The roles of mast cells and mast cell proteases during *Chlamydia* reproductive tract infection

Anne CHEVALIER

B.Sc. (*Bordeaux*); M.Sc. (*Montpellier*)

Discipline of Immunology and Microbiology
School of Biomedical Science and Pharmacy
Faculty of Health and Medicine
The University of Newcastle
Newcastle, NSW, Australia

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Immunology and Microbiology)

August 2019

Statement of originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision.

The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made.

I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Anne CHEVALIER

31 August 2019

Acknowledgements

I would like to express my gratitude to all of those who have made the completion of my PhD studies possible by their time, guidance and support.

Firstly, I would like to thank my primary supervisor, Professor Philip Hansbro, and my co-supervisors, Associate Professor Jay Horvat and Professor Darryl Knight, for offering me the opportunity to do this PhD. I would also like to thank them for their support, guidance and expertise throughout my PhD studies. I would also like to convey my special thanks to Professor Jay Horvat for his contribution, his help with the animal work, for the multitude advices he gave me, for his persistent encouragements and for spending time reviewing my thesis.

I would like to thank the University of Newcastle and the Hunter Medical Research Institute for providing me the opportunity to do my PhD and by giving me access to the resources necessary to conduct this research.

I would also like to thank the member of my confirmation panel, Professor Jonathan Hirst and Associate Professor Mark Baker, for their help and insightful advices.

I would also like to thank collaborators, Professor Richard Stevens and Associate Professor Michele Grimbaldeston, for their advice and for providing some of the mice and reagents used in my PhD studies.

I would also like to extend my acknowledgments to past and present members of the Horvat and Hansbro teams, as well as other colleagues from the Hunter Medical Research Institute, thank you for the daily help in the lab and for your support during hard times. I would specifically like to thank Kamal and Andi, for providing advices, expertise and help in the early stage of my PhD. I would like to thank the past and present member of the "*Chlamydia* infection study group", Huw, Daniel and Jemma, for their help with tissue collection as well as their advices and expertise. I would also like to thank Richard and Henry for their help and advices with experimental work.

I would like to thank my friends, who supported me during my PhD studies and helped me going through the hard time I have been facing. I would like to specifically thank Camille for

always being here for me, during both good and bad time. Thank you for taking the decision to come to Newcastle and for all the good memories we had in the last 3 years. I would also like to give special thanks to David, for his advices and for always finding ways to make me feel better when I was down. Finally, special thanks to Dee for all the laughs we had together and for giving me motivation throughout my PhD.

Last but not least, I would like to thanks my family for their support, encouragements and patience during my PhD. More specially, I would like to thank my mother, my father and my sister for coming to visit me at the opposite side of the world and for always being here for me.

Conference presentations arising from my PhD studies

First author oral presentations:

A. Chevalier, J.R. Mayall, H.W. McCarthy, R.L. Stevens, J.C. Horvat, P.M. Hansbro. *Mast cell deficiency is protective against Chlamydia-induced pathology of the female reproductive tract* - 6th Annual Australian Society for Medical Research (ASMR) Medical Research Week, Newcastle Satellite Scientific Meeting (June 2019).

A. Chevalier, J.R. Mayall, H.W. McCarthy, D.P. Hampsey, R.Y. Kim, W.M. Huston, R.L. Stevens, J.C. Horvat, P.M. Hansbro. *The role of mast cells and their proteases during Chlamydia infection*. International Union for Sexually Transmitted Infections (IUSTI) Asia-Pacific Congress, Auckland (November 2018).

A. Chevalier. *The role of mast cells and their proteases during Chlamydia reproductive tract infection*. Three-minute thesis, Faculty of Health and Medicine, University of Newcastle (July 2018).

A. Chevalier, D.P. Hampsey, J.R. Mayall, R.Y. Kim, D.A. Knight, W.M. Huston, R.L. Stevens, J.C. Horvat, P.M. Hansbro. *The role of mast cells and their proteases during Chlamydia female reproductive tract infection*. Australian *Chlamydia* Conference, Sydney (November 2017).

Presentations that I have contributed to during my candidature:

H.W. McCarthy, J.R. Mayall, J.C. Horvat, **A. Chevalier**, S.L. Masters, M.A. Cooper, A.B. Robertson, K. Schroder, R.Y. Kim, P.M. Hansbro. *The NLRP3 inflammasome induces natural killer cell responses in the uterus during Chlamydia FRT infections*. 6th Annual Australian Society for Medical Research (ASMR) Medical Research Week, Newcastle Satellite Scientific Meeting (June 2019).

J. Mayall, N. Mangan, **A. Chevalier**, D. Hampsey, A. Brown, M. Starkey, R. Kim, S. Maltby, P. Foster, P. Hertzog, J. Horvat, P. Hansbro. *The role of direct and indirect IFN-ε-mediated*

responses during Chlamydia infections. Australian *Chlamydia* Conference, Sydney (November 2017).

J. Mayall, N. Mangan, A. Brown, **A. Chevalier**, M. Starkey, R. Kim, P. Hertzog, P. Hansbro, J. Horvat. *IFN-ε regulated innate immune responses in the female reproductive tract during Chlamydia infection (P948).* American Association of Immunologists (AAI) Annual Meeting, Washington (May 2017).

J. Mayall, N. Mangan, **A. Chevalier**, B. Rae, M. Starkey, R. Kim, P. Hertzog, J. Horvat, P. Hansbro. *IFN-ε-regulated gene expression patterns in protection against female reproductive tract infection*. 16th International Congress of Immunology (ICI), Melbourne (August 2016).

J. Mayall, N. Mangan, **A. Chevalier**, M. Starkey, R. Kim, A. Brown, S. Maltby, P. Foster, P. Hertzog, J. Horvat, P. Hansbro. *IFN-ε-mediated local and systemic NK cell responses during Chlamydia infection*. ASI Annual Scientific Meeting, Canberra (December 2015).

Table of Contents

Statement of originality **2**

Acknowledgements **3**

Conference presentations arising from my PhD studies **5**

Table of Contents **7**

List of figures **16**

List of tables **21**

Abbreviations **22**

Synopsis **24**

Chapter one: Introduction **27**

1.1. *Chlamydia trachomatis* FRT infections **27**

1.1.1. Epidemiology of *Chlamydia trachomatis* infections 27

1.1.2. Diagnosis and treatments of *Chlamydia trachomatis* infections 28

1.1.3. Symptoms of *Chlamydia trachomatis* infections 28

1.2. Phylogeny and microbiology of *Chlamydia* **29**

1.2.1. The different species of *Chlamydia* 29

1.2.2. The biovars and serovars of *Chlamydia trachomatis* 30

1.2.3. The genome of *Chlamydia trachomatis* 32

1.2.4. The life cycle of *Chlamydia trachomatis* 33

1.3. Immune responses to *Chlamydia* FRT infections **36**

1.3.1. Animal models of *Chlamydia* FRT infections 36

1.3.2. Immunity to *Chlamydia* FRT infections 38

1.4. MCs and their proteases **45**

1.4.1. MC characteristics and activation 45

1.4.2. MC proteases 46

1.4.3. MC phenotypes 55

1.5. The roles of MCs and their proteases in mediating immune responses **56**

1.5.1. The roles of MCs in mediating immune responses 56

1.5.2. The roles of MC proteases in mediating immune responses 57

1.5.3. MCs in the FRT 59

1.6. Study rationale **63**

Chapter two: The role of mast cells and mast cell degranulation during *Chlamydia* female reproductive tract infection **65**

2.1. Abstract **65**

2.2. Introduction **67**

2.3. Materials and methods **71**

2.3.1. Ethics statement 71

2.3.2. Mouse models used to investigate the roles of MC and MC degranulation during *Chlamydia* FRT infection 71

2.3.3. Mouse model of *in vivo* inhibition of MC degranulation during *Chlamydia* FRT infection 75

2.3.4. Assessment of MC number and degranulation in the uterus 77

2.3.5. RNA extraction from FRT tissues 78

2.3.6. Reverse transcription of RNA from FRT tissues 78

2.3.7. DNA extraction from vaginal lavage 79

2.3.8. Real-time quantitative polymerase chain reaction 79

2.3.9. Evaluation of *Chlamydia*-induced pathology 81

2.3.10. Characterisation of immune cell numbers in the uterus and bone marrow during *Chlamydia* FRT infection using flow cytometry 81

2.3.11. Statistical analyses 85

2.4. Results 86

2.4.1. MCs are widespread in the FRT where they form an heterogeneous population 86

2.4.3. Progesterone and oestrogen treatments alter the number of uterine MCs but not their degranulation, in the absence of infection 89

2.4.4. *Chlamydia muridarum* infection does not alter the number and degranulation of uterine MCs early, but slightly decreases their number later 91

2.4.5. MC-deficient mice are protected against *Chlamydia*-induced pathology 93

2.4.6. MC-deficient mice exhibit a decline in the neutrophils, eosinophils, inflammatory monocytes and macrophages in their uterus during *Chlamydia* FRT infection 95

2.4.7. MC-deficient mice exhibit a decline in the eosinophils, γδ T cells and B cells but an increase in inflammatory monocytes and macrophages in their bone marrow during *Chlamydia* FRT infection 99

2.4.8. MC-deficient mice did not have changes in the expression of Th cell polarising transcription factors during *Chlamydia* FRT infection 105

2.4.9. The expression of the pro-inflammatory chemokine CXCL15 is reduced in the *Chlamydia*-infected uterus of MC-deficient mice 107

2.4.10. Inhibition of MC degranulation potently protects against the early stages of *Chlamydia* FRT infection but is largely detrimental to infection during the later stages 109

2.4.11. Inhibition of MC degranulation specifically during the early stages of *Chlamydia* FRT infection protects against *Chlamydia*-induced pathology .. 112

2.5. Discussion 115

2.6. Conclusion 126

Chapter three: The role of mast cell proteases during *Chlamydia* female reproductive tract infection 128

3.1. Abstract 128

3.2. Introduction 130

3.3. Material and methods 135

3.3.1. Ethics statement 135

3.3.2. Mouse strains used to investigate the roles of MC proteases during *Chlamydia* infection 135

3.3.3. RNA extractions from FRT tissues 139

3.3.4. Reverse transcription of RNA from reproductive tract tissues 139

3.3.5. DNA extraction from vaginal lavage 139

3.3.6. Real-time quantitative polymerase chain reaction 139

3.3.7. Evaluation of *Chlamydia*-associated pathology 141

3.3.8. Characterisation of the immune cell numbers in the uterus during *Chlamydia* FRT infection using flow cytometry 141

3.3.9. Statistical analyses 142

3.4. Results **143**

3.4.1. Mice treated with oestrogen have increased expression of MC proteases mMCP4, mMCP5, mMCP6 and Cpa3 in their FRT, in the absence of infection 143

3.4.2. Mice infected by *Chlamydia muridarum* have reduced expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3 in the FRT at late stages of infection 145

3.4.3. MC-deficient mice have strongly reduced expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3 in their uterus during *Chlamydia* FRT infection 147

3.4.4. The expression of Ndst2 is not affected by female sex hormones, *Chlamydia* FRT infection nor MC deficiency 149

3.4.5. Ndst2-deficient mice have increased *Chlamydia* burden in their uterus while being protected against *Chlamydia*-associated pathology 151

3.4.6. Ndst2-deficient mice exhibit reduced immune cell mobilisation in their uterus during *Chlamydia* FRT infection 153

3.4.7. Ndst2-deficient mice have reduced expression of the transcription factors associated with Th1 and Th17 responses during the early stages of *Chlamydia* FRT infection 157

3.4.8. Ndst2-deficient mice have altered expression of several immune mediators in their uterus during *Chlamydia* FRT infection 159

3.4.9. mMCP5-deficient mice are protected against *Chlamydia* FRT infection at early stages, but not at later stages 162

3.4.10. mMCP6-deficient/mMCP7-sufficient mice are partially protected against *Chlamydia* associated pathology 164

3.5. Discussion **168**

3.6. Conclusion **179**

Chapter four: The role of Prss31 during *Chlamydia* female reproductive tract infection **180**

4.1. Abstract **180**

4.2. Introduction **182**

4.3. Material and methods **185**

4.3.1. Ethics statement 185

4.3.2. Mouse strains used to investigate the roles of Prss31 during *Chlamydia* FRT infection 185

4.3.3. Intravaginal treatments with recombinant Prss31 during *Chlamydia* FRT infection 189

4.3.4. RNA extractions from FRT tissues 190

4.3.5. Reverse transcription of RNA from reproductive tract tissues 190

4.3.6. DNA extraction from vaginal lavage 190

4.3.7. Real-time quantitative polymerase chain reaction 190

4.3.8. Evaluation of *Chlamydia*-associated pathology 191

4.3.9. Characterisation of the immune cell numbers in the uterus during *Chlamydia* FRT infection using flow cytometry 192

4.3.10. Investigation of the cellular source of Prss31 in the FRT by immunohistochemistry 193

4.3.11. Statistical analyses 194

4.4. Results **195**

4.4.1. The expression of Prss31 is not altered by female sex hormones nor by *Chlamydia* FRT infection 195

4.4.2. Prss31 might not be specifically expressed by MCs 197

4.4.3. Prss31-deficient mice have increased susceptibility to *Chlamydia* FRT infection 200

4.4.4. Early intravaginal treatments with recombinant Prss31 improve clearance of *Chlamydia* FRT infection early and protect against *Chlamydia*-associated pathology at later stages..... 202

4.4.5. Prss31-deficient mice exhibit decreased immune cell recruitment to the uterus following *Chlamydia* FRT infection..... 204

4.4.6. Prss31-deficient mice have increased expression of IL-13 and IL-13rα during the early stages of *Chlamydia* FRT infection 208

4.4.7. Prss31-deficient mice have increased expression of pro-inflammatory mediators in the uterus during *Chlamydia* FRT infection 210

4.5. Discussion 213

4.6. Conclusion 219

Chapter five: General discussion and conclusions..... 221

5.1. Significance of the research 221

5.2. Female sex hormones regulate the number and phenotype of MCs in the FRT.....222

5.3. Effect of *Chlamydia* FRT infection on MC numbers and phenotype in the FRT.....223

5.4. MCs mediate immune responses to *Chlamydia* FRT infection.....223

5.4.1. MCs contribute to the development of oviduct pathology during *Chlamydia* FRT infection 223

5.4.2. Inhibition of MC degranulation during the early stages of *Chlamydia* FRT infection promotes clearance of infection early and reduces associated pathology later 224

5.4.3. MC factors stored in secretory granules through binding with heparin contribute to *Chlamydia* FRT infection and the development of *Chlamydia*-induced oviduct pathology 225

5.4.4. Different MC proteases stored in heparin-protease complexes differentially affect the pathogenesis of *Chlamydia* FRT infection 226

5.5. Potential role of eosinophils during *Chlamydia* FRT infection 228

5.6. Ramifications of my research for other STIs and STI-associated *sequelae* 229

5.7. Limitations of my studies 230

5.7.1. Lack of sham-infected controls 230

5.7.2. Use of a mouse model of *Chlamydia* FRT infection 231

5.7.3. Use of genetically modified mice 232

5.7.4. Differences between human and mouse MC proteases 232

5.8. Future directions 233

5.8.1. Further characterisation of the role of MCs during *Chlamydia* FRT infection 233

5.8.2. Further characterisation of the roles of MC proteases during *Chlamydia* FRT infection 234

5.9. Concluding remarks 237

Appendix **238**

6.1. Nucleotide sequences of the primers used for analyses of gene expression **238**

6.2. Antibody cocktail and gating strategy used for determining the percentages and numbers of immune cells in the uterus of WT, MC-deficient, Ndst2-deficient and Prss31-deficient mice **241**

6.3. Antibody cocktail and gating strategy used for determining the percentages and numbers of myeloid cells in the bone marrow of WT and MC-deficient mice **243**

6.4. Antibody cocktail and gating strategy used for determining the percentages and numbers of lymphoid cells in the bone marrow of WT and MC-deficient mice **245**

6.5. Problem encountered in evaluating the number of MCs in the uterus by flow cytometry **247**

References **249**

List of figures

Figure 1.1 – Schematic representation of *Chlamydia trachomatis* infectious life cycle..... 35

Figure 1.2 - Nomenclature of the reaction site between a serine protease and a substrate 47

Figure 1.3 – Schematic representation of the S1-pocket of the chymotrypsin-like, trypsin-like and elastase-like serine proteases..... 48

Figure 1.4 – Schematic representation of the MC serglycin proteoglycans stored in the secretory granules of mast cells (MCs)..... 53

Figure 2.1 - Mouse model of hormonal treatment and *Chlamydia muridarum* female reproductive tract (FRT) infection..... 74

Figure 2.2 - *In vivo* inhibition of mast cell (MC) degranulation during a mouse model of *Chlamydia muridarum* female reproductive tract (FRT) infection 76

Figure 2.3 - Mast cells (MCs) are widespread in mouse female reproductive tract (FRT) tissues. 88

Figure 2.4 – Mice treated with oestrogen have a reduced numbers of uterine mast cells (MCs) but a similar percentage of degranulated MCs compared to progesterone-treated mice 90

Figure 2.5 – Infection with *Chlamydia muridarum* does not significantly affect the number nor the degranulation of uterine mast cells (MCs) early, but slightly decrease MC number later..... 92

Figure 2.6 - **M**ast cell (MC)-deficient mice have a slightly lower *Chlamydia* burden and are protected against *Chlamydia*-induced pathology at 14 days post infection (dpi)... 94

Figure 2.7 - Mast cell (MC)-deficient mice have a reduced number of neutrophils, eosinophils, inflammatory monocytes and macrophages present in their uterus during *Chlamydia* infection..... 97

Figure 2.8 - Mast cell (MC)-deficient mice have a decline in the percentage of neutrophils, eosinophils, inflammatory monocytes and macrophages present in their uterus during *Chlamydia* infection..... 98

Figure 2.9 - Mast cell (MC)-deficient mice have a lower number of eosinophils, γδ T cells and B cells, and a higher number of inflammatory monocytes and macrophage,s in their bone marrow (BM) during *Chlamydia* infection. 102

Figure 2.10 - Mast cell (MC)-deficient mice have a significant higher percentage of immune cells and inflammatory monocytes and macrophages, but a significant lower percentage of eosinophils in their bone marrow (BM) during *Chlamydia* infection 104

Figure 2.11 – The expression of the transcription factors Tbet, GATA Binding Protein (GATA)3, RAR-related orphan receptor (ROR)γt and forkhead box P3 (FOXP3) is not altered in the uterus of *Chlamydia*-infected mast cell (MC)-deficient mice compared to WT controls 106

Figure 2.12 - The expression of the pro-inflammatory chemokine (C-X-C motif) ligand (CXCL)15 is reduced in the *Chlamydia*-infected uterus of mast cell (MC)-deficient mice compared to WT controls 108

Figure 2.13 – Inhibition of mast cell (MC) degranulation by cromolyn treatments throughout infection protects against the early stages of *Chlamydia* FRT infection but is largely detrimental at later stages 111

Figure 2.14 - Inhibition of mast cell (MC) degranulation by cromolyn treatments during the early stages of *Chlamydia* FRT infection protects against *Chlamydia*-induced pathology but not alter levels of infection in the vagina and uterus 114

Figure 3.1 - Mouse model of hormonal treatment and *Chlamydia muridarum* female reproductive tract (FRT) infections 138

Figure 3.2 – Mice treated with oestrogen have increased expression of the mast cell (MC) proteases mouse mast cell proteases (mMCP)4, mMCP5 and carboxypeptidase A3 (Cpa3) in their ovary, oviduct and uterus compared to mice treated with progesterone 144

Figure 3.3 – Mice infected with *Chlamydia muridarum* have reduced expression of the mast cell (MC) proteases mouse mast cell proteases 4 (mMCP4), mMCP5 and carboxypeptidase A3 (Cpa3) in the uterus at 14 days post infection (dpi)..... 146

Figure 3.4 - Mast cell (MC)-deficient mice have a reduced expression of the mast cell proteases mouse mast cell proteases (mMCP)4, mMCP5, mMCP6 and carboxypeptidase A3 (Cpa3) in the uterus 148

Figure 3.5 – The expression of *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2) in the female reproductive tract (FRT) is not altered by progesterone or oestrogen treatments, *Chlamydia*-infection nor mast cell (MC) deficiency..... 150

Figure 3.6 – *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice are more susceptible to *Chlamydia* infection while being protective against *Chlamydia*-associated pathology at 14 days post infection (dpi) 152

Figure 3.7 – *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice have a reduced number of immune cells, neutrophils, eosinophils myeloid dendritic cells, inflammatory monocytes and macrophages, $CD4^+$ and $CD8^+$ T cells in their uterus during *Chlamydia* infection 155

Figure 3.8 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice have a reduced percentage of immune cells, eosinophils, myeloid dendritic cells, inflammatory monocytes and macrophages and $CD4^+$ T cells; while having an increase in plasmacytoid dendritic cells in their uterus during *Chlamydia* infection..... 156

Figure 3.9 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice have reduced expression of the transcription factors Tbet and RAR-related orphan receptor (ROR)γt in their *Chlamydia*-infected uterus at 3 days post infection (dpi)..... 158

Figure 3.10 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice display enlarged expression of interleukin (IL)-1β and chemokine (C-X-C motif) ligand (CXCL)15 in their *Chlamydia*-infected uterus at 3 days post infection (dpi) 160

Figure 3.11 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice display enlarged expression of IL-4rα and chemokine (C-X-C motif) ligand (CXCL)1 in their *Chlamydia*-infected uterus at 14 days post infection (dpi)..... 161

Figure 3.12 – Mouse mast cell protease 5 (mMCP5)-deficient mice have reduced *Chlamydia* burden at early stages of *Chlamydia* infection, but not at later stages. 163

Figure 3.13 – Mouse mast cell protease 6 (mMCP6)-deficient mice have unaltered *Chlamydia* infection and associated pathology while mMCP6-deficient/mMCP7-sufficient mice are partially protected against *Chlamydia*-associated pathology 166

Figure 4.1 - Mouse model of hormonal treatment and *Chlamydia muridarum* female reproductive tract (FRT) infections 188

Figure 4.2 - *In vivo* treatments with recombinant protease serine member S31 (Prss31) during a murine model of *Chlamydia muridarum* reproductive tract infection 189

Figure 4.3 - The expression of protease serine member S31 (Prss31) in the female reproductive tract (FRT) is not significantly altered by progesterone or oestrogen treatments nor by *Chlamydia*-infection 196

Figure 4.4 - The expression of protease serine member S31 (Prss31) in the uterus is not altered in mast cell (MC)-deficient mice compared to WT controls 197

Figure 4.5 – Staining for protease serine member S31 (Prss31) in uterine cells and in the epithelium of the FRT of wild-type (WT) and Prss31-deficient mice 199

Figure 4.6 – Protease serine member S31 (Prss31)-deficient mice are more susceptible to *Chlamydia* infection at early stages 201

Figure 4.7 – Treatments with recombinant protease serine member S31 (rPrss31) are protective against *Chlamydia* infection at early stages and against *Chlamydia*-associated pathology at later stages 203

Figure 4.8 - Protease serine member S31 (Prss31)-deficient mice have a reduced number of viable cells, immune cells, eosinophils, myeloid dendritic cells, plasmacytoid dendritic cells, monocytes and macrophages, $CD4^+$ T cells and $CD8^+$ T cells in their uterus during *Chlamydia* infection 206

Figure 4.9 - Protease serine member S31 (Prss31)-deficient mice have a higher proportion of neutrophils in their uterus compared to wild type (WT) controls during *Chlamydia* infection 207

Figure 4.10 - Protease serine member S31 (Prss31)-deficient mice have enhanced expression of interleukin (IL)-13 and IL-13rα in their *Chlamydia*-infected uterus at 3 days post infection (dpi) 209

Figure 4.11 – Protease serine member S31 (Prss31)-deficient mice have enhanced expression of the signal transducer and activator of transcription (STAT)1, interleukin (IL)-10, chemokine (C-X-C motif) ligand (CXCL)1 and CXCL15 in their *Chlamydia*-infected uterus at 3 days post infection (dpi) 211

Figure 4.12 - Protease serine member S31 (Prss31) deficient mice have enhanced expression of the interleukin (IL)-1β and toll-like receptor in their *Chlamydia*-infected uterus at 14 days post infection (dpi) 212

Figure 6.1 - Gating strategy for characterising the immune cells in the uterus of wild type (WT), Mast cell (MC)-deficient mice, *N*-deacetylase/*N*-sulphotransferase-2- (Ndst2-)deficient mice and protease serine member S31- (Prss31-) deficient mice 241

Figure 6.2 - Gating strategy for characterising the myeloid immune cells in the bone marrow of wild type (WT) and Mast cell (MC)-deficient mice 243

Figure 6.3 – Gating strategy for characterising the lymphoid immune cells in the bone marrow of wild type (WT) and mast cell- (MC-) deficient mice 245

Figure 6.4 – Representative plots showing the absence of staining for mast cell (MC) in the uterus of wild type (WT) and MC-deficient mice 247

List of tables

Table 1.1 – The different biovars, strains and serovars of *Chlamydia trachomatis*, associated with their tissue tropism and main associated disease 31

Table 2.1 - Description of the mice used to investigate the roles of mast cell during *Chlamydia* infection 72

Table 2.2 – Surface markers used for identifying immune cells in the uterine tissue and bone marrow of *Chlamydia*-infected mice 84

Table 3.1 - Description of the wild type and transgenic mouse strains used to investigate the roles of mast cell proteases during *Chlamydia* infection. 136

Table 3.2 – Surface markers used for identifying immune cells in the uterine tissue of *Chlamydia*-infected Ndst2-deficient mice and WT controls 142

Table 4.1 - Description of the wild type and transgenic mouse strains used to investigate the roles of Prss31 during *Chlamydia* infection 186

Table 4.2 – Surface markers used for identifying immune cells in the uterine tissue of *Chlamydia*-infected Prss31-deficient mice and WT controls 193

Table 6.1 - Oligonucleotide sequences of the primer pairs used for quantitative polymerase chain reaction analyses of gene expression 240

Table 6.2 – Panel of the antibodies used for extracellular staining of uterine cells of wild type, mast cell-deficient, *N*-deacetylase/*N*-sulphotransferase-2-deficient and protease serine member S31-deficient mice 242

Table 6.3 – Myeloid panel, with the antibodies and their associated fluorochromes used for extracellular staining of bone marrow cells of wild type and mast cell deficient mice 244

Table 6.4 – Mature lymphoid panel, with the antibodies and their associated fluorochromes, used for extracellular staining of bone marrow cells of wild type and mast cell deficient mice 246

Abbreviations

ABR	Australian BioResources
Ala	Alanine
ANOVA	Analyses of variance
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine triphosphate
BMMC	Bone marrow mast cell
CAH	Central Animal House
cDNA	Complementary DNA
CMA1	Chymase 1
Cmu	*Chlamydia muridarum*
CPA3	Carboxypeptidase A3
CPAF	Chlamydial protease-like activating factor
CTMC	Connective tissue mast cell
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) rececptor
DAMP	Damage-associated molecular pattern
dbi	Days before infection
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dpi	Day post infection
DPPI	Dipeptidyl peptidase I
DTT	DL-dithiothreitol
EB	Elementary body
EDTA	Ethylenediaminetetraacetic acid
ER	Oestrogen receptor
FACS	Flow cytometry and cell sorting
FcεR	High affinity immunoglobulin E receptor
FCS	Fetal Calf Serum
FOXP3	Forkhead box P3
FRT	Female reproductive tract
GAG	Glycosaminoglycan
GATA3	GATA Binding Protein 3
Gly	Glycine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid
His	Histidine
HIV	Human immunodeficiency virus
HMRI	Hunter Medical Research I nstitute
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPV	Human papillomavirus
IFN	Interferon
ifu	Inclusion forming unit
Ig	Immunoglobulin
IL	Interleukin
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
Lys	Lysine
MC	Mast cell
MC_C	Mast cell chymase$^+$ tryptase$^-$
MC_T	Mast cell chymase$^-$ tryptase$^+$
MC_{TC}	Mast cell chymase$^+$ tryptase$^+$
Mcl	Myeloid cell leukemia
mDC	Myeloid dendritic cell
Met	Methionine
MHC	Major histocompatibility complex
MITF	*mi* transcription factor
MMC	Mucosal mast cell
mMCP	Mouse mast cell protease

MMP	Matrix metalloproteinase
MOMP	Major outer membrane protein
mT5	Mouse tryptase 5
NCR	Natural cytotoxicity triggering receptor
Ndst	*N*-deacetylase/*N*-sulfotransferase
NET	Neutrophil extracellular trap
NK cell	Natural killer cell
NLRP	Nucleotide-binding oligomerization domain-like receptor, pyrin domain-containing
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain-like
Omp	Outer membrane protein
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor
PB	Persistent body
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.05% Tween-20
pDC	Plasmacytoid dendritic cell
PDCA	Plasmacytoid dendritic cell antigen
PFA	Paraformaldehyde
Pgp	Plasmid glycoprotein
Phe	Phenylalanine
PR	Progesterone receptor
Prss	Protease serine member S
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RB	Reticulate body
RIP	Receptor-interacting protein
RLR	etinoic acid-inducible gene 1 like receptor
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor
ROS	Reactive oxygen species
rPrss31	Recombinant protease serine member S31
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of the mean
Ser	Serine
siRNA	Small interfering ribonucleic acid
SPF	Specific pathogen free
SPG	Sucrose-phosphate-glutamate
STI	Sexually transmitted infection
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th	T helper
Thr	Threonine
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPSAB	α/β-tryptase
TPSB	β-tryptase
TPSD	δ-tryptase
TPSG	γ-tryptase
Treg	Regulatory T cell
Trp	Tryptophan
Tyr	Tyrosine
UniSA	University of South Australia
Val	Valine
WT	Wild type

Synopsis

Chlamydia trachomatis is the most common bacterial sexually transmitted infection (STI), with approximately 130 million cases of infection occurring annually worldwide. Although *Chlamydia* infections are relatively simple to diagnose and treat, the majority of infected women do not develop any symptoms, hence they often go undiagnosed and untreated. Over time, untreated infections may ascend from the vagina into the upper female reproductive tract (FRT) and cause severe complications including pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility. The host immune responses to *Chlamydia* infections are very complex and a greater understanding of the host responses, including immune cells and factors, that contribute to clearance of infection *versus* those that underpin infection-associated pathology is required.

Mast cells (MCs) are large, tissue-resident, immune cells of haematopoietic origin that are present in the FRT. They are characterised by their numerous intracellular secretory granules that hold a wide variety of preformed inflammatory mediators, including histamine, serglycin proteoglycans and MC proteases. Upon MC activation, these preformed mediators are released into the extracellular matrix through a mechanism called degranulation. Although MCs are well recognised for their detrimental role in allergy, they are also key mediators of immune responses to an extensive number of pathogens. However, the role that MCs play during STIs remains largely unknown.

To address this, my PhD studies aimed to investigate the role(s) of MCs and MC proteases during *Chlamydia* FRT infections, using a suite of genetically modified mice that are deficient in MCs or in specific MC proteases and a murine model of *Chlamydia* FRT infection.

I show that the number of uterine MCs and their expression of the MC proteases, mouse MC protease (mMCP)4, mMCP5, mMCP6 and carboxypeptidase (Cpa)3 are regulated by female sex hormones and/or stage of oestrous cycle in the absence of *Chlamydia* FRT infection. Whilst the number, phenotype and degranulation of MCs is not changed 3 days post infection (dpi) with *Chlamydia*, the number of MCs and their expression of mMC4, mMCP5, mMCP6 and CPA3 are

slightly reduced at 14dpi. In contrast, I show that the expression of protease serine member S31 (Prss31), a unique MC protease that possesses a membrane anchor that binds it to the plasma membrane of MCs upon degranulation, is independent of female sex hormones and *Chlamydia* FRT infection. Together, these data reveal that female sex hormones and *Chlamydia* FRT infection can affect the number and phenotype of MCs in the FRT.

Moreover, my studies reveal a novel role for MCs in mediating *Chlamydia* FRT infection. I show that MC-deficient mice are protected against *Chlamydia*-induced pathology and have slightly reduced eosinophils, neutrophils, monocytes and macrophages in their uterus, suggesting a role for MCs in contributing to the recruitment of innate immune cells, associated with development of *Chlamydia*-induced pathology, in the upper FRT.

By using intravaginal treatments with the MC stabiliser cromolyn, I show evidence that MC degranulation is detrimental, especially during the early stages of *Chlamydia* FRT infection. Mice that received cromolyn throughout the early stages of *Chlamydia* infection have reduced infection at 3dpi. However, this protective effect is not maintained at later stages of infection. Importantly, mice that receive cromolyn treatment during the early stages of infection are protected against infection-induced pathology during the later stages. These observations show that the inhibition of MC degranulation does not recapitulate the effects observed in MC-deficient mice, suggesting that some of the factors released through degranulation might have differential effects to other factors released by MCs through other pathways.

I next sought to identify the role(s) of some of the key factors, specifically factors that are stored in the secretory granules of MCs that are released during MC degranulation, in the pathogenesis of *Chlamydia* FRT infection. In my first series of experiments, *N*-Deacetylase/*N*-Sulfotransferase 2 (Ndst2)-deficient mice were subjected to *Chlamydia* FRT infection. These mice lack the important enzyme for *N*-deacetylation and *N*-sulfation of heparan sulfate in MCs, which causes abnormal storage of the MC mediators that are normally bound to heparin in the secretory granules of MCs, including histamine and the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3. Interestingly, I show that Ndst2-deficient mice are more susceptible to

infection, while being protected against *Chlamydia*–induced pathology. This increase in susceptibility to infection and protection against pathology is associated with a decrease in the number of innate and adaptive cells present in the uterus, suggesting that the factors that are affected by Ndst2 deficiency, play important role in the induction of the recruitment of immune cell associated with clearance of infection (E.g. $CD4^+$ T cells) and with development of *Chlamydia*–associated pathology (E.g. neutrophils).

I next sought to assess the individual roles played by specific MC proteases in the pathogenesis of *Chlamydia* FRT infection. To do this, mMCP5-, mMCP6-, Prss31-deficient and mMCP6-deficient/mMCP7-sufficient mice were infected with *Chlamydia* FRT. I show that whilst mMCP6-deficient mice have similar course of infection as wild type (WT) control mice, mMCP5-deficient mice are protected against infection at 3dpi. Interestingly, the presence of mMCP7 (which is naturally deficient in the WT control mice used) in mMCP6-deficient mice slightly protected against the early stages of infection as well as infection-induced pathology during the later stages of infection compared to WT controls. I also show that Prss31-deficient mice are more susceptible to infection early, but have no change in infection-induced pathology at later stages of *Chlamydia* infection. My studies also show that absence of Prss31 results in a decrease in immune cell recruitment to the uterus during infection. Importantly, daily intravaginal treatment with recombinant Pss31 protects against infection and infection-induced pathology.

Together, my studies show important role(s) for MCs and MC degranulation in the pathogenesis of *Chlamydia* FRT infection. My studies also show that different MC proteases may play different roles in infection and infection-induced disease. Importantly, whilst the mechanisms involved remain to be elucidated, my studies highlight that MC-mediated responses may be therapeutically manipulated in order to treat/prevent *Chlamydia* FRT infection and/or infection-induced FRT pathology.

Chapter one: Introduction

1.1. *Chlamydia trachomatis* FRT infections

1.1.1. Epidemiology of *Chlamydia trachomatis* infections

Worldwide, STIs are a major health burden with approximately 1 million new infections, caused by over 30 micro-organisms, acquired every day (1, 2). The four most prevalent STIs are estimated to account for around 376.4 million new infections annually, in women and men aged between 15 to 49 years old. In more details, in 2016, the parasite *Trichomonas vaginalis* was estimated to be responsible for 156 million infections (corresponding to incidence rates of 40 cases per 1000 women and 42 cases per 1000 men), the bacteria *Chlamydia trachomatis* to be responsible for 127.2 million infections (corresponding to incidence rates of 34 cases per 1000 women and 35 cases per 1000 men), *Neisseria gonorrhoeae* to be responsible for 86.9 million infections (corresponding to incidence rates of 20 cases per 1000 women and 26 cases per 1000 men) and *Treponema pallidum* to be responsible for 6.3 million infections (corresponding to incidence rates of 1.7 cases per 1000 women and 1.6 cases per 1000 men) (1, 3). Other common STIs include viral infections caused by hepatitis B virus, herpes simplex virus and human immunodeficiency virus (HIV), with 257, 417 and 36.9 million people living with these viral infections respectively (4-6).

In Australia, *Chlamydia trachomatis* is the most prevalent STI, with 100,775 cases reported in 2017. Infections are as common in women as in men, however adolescents and young adults are most likely to be infected with people aged between 15 to 29 years old accounting for 73% of the infections reported (7). Aboriginal and Torres Strait Islander populations have higher rates of STIs in general, and for *Chlamydia trachomatis* in particular, with notification rates approximately three times higher than the rest of the population (7-9). In women, the use of hormonal oral contraceptive have been associated with increasing susceptibility to *Chlamydia* infections (10).

1.1.2. Diagnosis and treatments of *Chlamydia trachomatis* infections

The diagnosis of *Chlamydia trachomatis* infections is completed by the detection of *Chlamydia* nucleic acid in first pass urine or in vaginal and/or endocervical swabs (11, 12). In remote areas and low-income countries, point-of-care testing has been developed to allow wide access to rapid low-cost testing (11-13). However, the sensitivity and specificity of these tests remain inconsistent, hence they are not implemented as a general screening method (12, 13).

After diagnosis, antibiotics are given immediately, with either a single-dose of 1g azithromycin orally or a 7-day course of two daily doses of 100mg doxycycline orally (14, 15). Because of the high rate of re-infections, testing 3 months after a positive diagnosis is recommended (15).

Globally, the rapid development of antibiotic-resistant bacteria represents a major threat, with *Neisseria gonorrhoeae* being resistant to multiple antibiotics and antimicrobial drugs, including widespread resistance to quinolones, decrease in susceptibility to sulphonamides and ciprofloxacin, as well as emerging resistance to azithromycin and third generation cephalosporins (1, 16, 17). Although the swine pathogen *Chlamydia suis* possesses a stable tetracycline resistance gene (18, 19), antibiotics remain largely effective for the treatment of *Chlamydia trachomatis* infection in women (18, 19), even if some cases of treatment failure with tetracyclines and macrolides have been reported (14, 20).

1.1.3. Symptoms of *Chlamydia trachomatis* infections

The majority of *Chlamydia trachomatis* infections are asymptomatic, with up to 90% of women and 50% of men not developing any *Chlamydia*-associated symptoms (11, 21). Therefore, infections often go undiagnosed and untreated, which can, at term, lead to severe sequelae (11, 15, 21). Indeed, men can develop dysuria, urethral discharge, urethritis, proctitis and epididymitis, while women can develop dysuria, vaginal discharge, urethritis, vaginitis, cervicitis, endometritis, salpingitis, pelvic inflammatory disease and tubal factor infertility (14, 22-25). Re-infections often lead to greater damage to the reproductive tract tissues and more severe complications (25). Globally, two thirds of the cases of infertility in women are associated with *Chlamydia*

trachomatis infection, which represents 5% of all women (26). Moreover, *Chlamydia* infections are responsible of about a third of all ectopic pregnancies and can cause miscarriage and premature birth (26-29). Perinatal transmission of *Chlamydia trachomatis* to the new born can cause conjunctivitis and pneumonia in babies (28, 30, 31). In rare instance, *Chlamydia trachomatis* reproductive tract infections can disseminate to the joint, leading to the development of sexually acquired reactive arthritis (32, 33).

Chlamydia trachomatis can also increase the susceptibility to other STIs. Indeed, infection of endo-cervical epithelial cells by *Chlamydia trachomatis* have been associated with increased crossing of the mucosal barrier by HIV, allowing replication in underlying target cells, hence potentially favouring the establishment of a reservoir for HIV in the endo-cervix (34). Moreover, reproductive tract co-infection with human papillomavirus (HPV) has been associated with increased oncogenicity of HPV and higher risk of cervical cancer (35-37). In addition, a recent study identified a doubling in ovarian cancer risk when patients had serological evidence of a prior or current *Chlamydia trachomatis* infection (38).

The annual cost related to the treatment of *Chlamydia trachomatis* infections and associated pathology is estimated to be approximately 51.4 million dollars in Canada (39) and between 90 to 160 million dollars in Australia (24). Therefore, the development of a vaccine and/or of alternative therapeutic strategies that prevent the development of *sequelae* is needed to help reduce the number of infections worldwide (22, 40-43).

1.2. Phylogeny and microbiology of *Chlamydia*

1.2.1. The different species of *Chlamydia*

Chlamydiae were discovered in 1907 by Stanislaus von Prowazek and Ludwig Halberstaedter after detecting unusual intracytoplasmic inclusions containing microorganisms in Giemsa-stained conjunctival scrapings (44). In 1910, similar inclusions were observed in scrapings from urethritis, cervicitis and conjuctivitis patients by Linder *et al.*, and from lymphogranuloma venereum (LGV) patients by Durand *et al.*, in 1913; establishing, for the first time, a unique

pathogen for those diseases (44). *Chlamydiae* were first classified as protozoa or viruses because of their intracellular location, their incapacity to be grown on culture media, their dependence on host biosynthetic machinery and their capacity to pass through bacterial filters. Only in 1966, the use of electron microscopy allowed to identify the presence of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), ribosomes and a cell wall, revealing that *Chlamydia* are Gram negative bacteria (45).

Through time, the phylogeny of *Chlamydia* has greatly evolved with the discovery of new species. The improvement of molecular techniques, such as the analyses of 16S ribosomal RNA or the comparisons of whole genome (46), resulted in the classification of *Chlamydia* in a single genus in the order of *Chlamydiales* and the family of *Chlamydiaceae* (44, 47). There are eleven different species, with *Chlamydia trachomatis*, *Chlamydia psittaci, Chlamydia muridarum*, *Chlamydia pneumoniae*, *Chlamydia pecorum*, *Chlamydia suis*, *Chlamydia abortus*, *Chlamydia felis*, *Chlamydia caviae*, *Chlamydia avium* and *Chlamydia gallinacean*, all responsible for a range of diseases, in a variety of hosts, with different tissue tropisms (21, 44, 46, 48). *Chlamydia trachomatis* and *Chlamydia pneumoniae* are the two main species that infect humans, while other species such as *Chlamydia psittaci* and *Chlamydia abortus,* and in rarer occasions *Chlamydia felis*, *Chlamydia suis* and *Chlamydia caviae,* have been associated with human zoonoses (44, 48).

1.2.2. The biovars and serovars of *Chlamydia trachomatis*

Chlamydia trachomatis species can be divided into the trachoma biovar and the LGV biovar (**Table 1.1**) (49-51). These two biovars are further divided into 19 serovars, according to the serological specificity of their major outer membrane protein (MOMP) (52, 53).

The trachoma biovar, composed of the serovars A-K, is further divided between the strains responsible for either trachoma or oculo-genitals infections (49, 51, 54). Indeed, the serovars A, B, Ba and C are responsible for endemic trachoma, a chronic ocular infection that represent the leading cause of preventable blindness in the world (54-56). Similarly to the serovars A-C, the serovars D-K can produce ocular infections, but no severe complication have been reported. They also cause reproductive tract infections that can breach the cervical barrier and ascend to the

fragile upper reproductive tract tissues inducing the development of severe pathology as described previously (**Section 1.1.3**) and detailed in **Table 1.1** (24, 54, 57).

The LGV biovar is composed of the highly invasive serovars L1, L2 and L3 that are also sexually transmitted but, contrary to the serovars D-K that infect mostly the mucosal epithelial cells of the reproductive tract, the LGV serovars also infect tissue resident monocytes and macrophages, hence migrating to the draining lymph nodes and causing the bubonic disease of LGV, a disease mainly limited to lower-income countries in East/West Africa, Southeast Asia and Caribbean, where it can be considered as endemic (58, 59).

Species	Biovars	Strains	Serovars	Tissue tropism	Main diseases
Chlamydia trachomatis	Trachoma	Trachoma strains	A, B, Ba and C	Ocular	Trachoma and subsequent blindness
		Oculo-genital strains	D, Da, E, F, G, H, I, Ia, J and K	Ocular	Self-limited form of acute conjunctivitis
				Genital	Men: urethritis, proctitis, and epididymitis Women: salpingitis, pelvic inflammatory disease, tubal factor infertility and ectopic pregnancy New born: neonatal conjunctivitis and pneumonia
	LGV	LGV	L1, L2 and L3	Genital, disseminated to lymph nodes	Genital ulceration and scaring, chronic proctitis and bubonic disease

Table 1.1 – The different biovars, strains and serovars of *Chlamydia trachomatis*, associated with their tissue tropism and main associated disease.

1.2.3. The genome of *Chlamydia trachomatis*

Chlamydia trachomatis possess a relatively small genome of approximately 1,042,519 base pairs, containing 894 protein-coding genes (60). In addition, *Chlamydia trachomatis*, similarly to *Chlamydia muridarum*, holds multiple copies of a highly conserved, non-conjugative and non-integrative, cryptic plasmid of 7,493 base pairs (44, 60). This plasmid, constituted of non-coding RNA and of 8 open reading frames coding for the plasmid glycoprotein (Pgp)1-8, acts as a virulence factor despite not containing antibiotic-resistance gene (61, 62). Only rare clinical cases of plasmid-deficient *Chlamydia* have been reported, suggesting evolutionary selective pressure (63, 64). Indeed, murine models have shown that infections with plasmid-deficient *Chlamydia trachomatis* or *Chlamydia muridarum* reduce *Chlamydia* burden, induce weaker immune responses and have reduced pathology compared to plasmid-proficient strains (65-71). The plasmid is highly conserved in the different species of *Chlamydia* with most of the variability restricted to the *Pgp3* gene. The Pgp3 protein is associated with the development of *Chlamydia*-associated pathology in murine models (72, 73). The exact role of Pgp3 has not yet been completely understood, but it was shown to be secreted into the host cytoplasm (74) and to inactivate the host antimicrobial peptide cathelicidin LL-37 (75). Vaccination trials targeting this protein showed partial protection against infection and immunopathology (76, 77).

Chlamydia trachomatis possesses a type III secretion system that also acts as a virulence factor by permitting the translocation of proteins between *Chlamydia* and the host cells, allowing *Chlamydia* to capture host nutrients, but also to affect host cytoskeletal rearrangements and mediate host immune responses (21, 78).

As reviewed by Nunes *et al*., the main dissimilarities in the genomes of the different species of *Chlamydiae* are located in a hypervariable region of the genome, called the plasticity zone. As an example, *Chlamydia trachomatis* possesses a subset of a tryptophan operon, whilst, at the contrary, *Chlamydia muridarum* lacks tryptophan biosynthesis genes (44, 51).

The lipopolysaccharide (LPS) of *Chlamydia trachomatis* has been shown to induce 100 times less activation of immune cells compared to LPS from *Salmonella minnesota* or *Neisseiria*

gonorrhoeae, suggesting that *Chlamydia trachomatis* infections induce relatively weak inflammatory responses in hosts, which may contribute to the high levels of asymptomatic infections associated with *Chlamydia* infections (79).

1.2.4. The life cycle of *Chlamydia trachomatis*

Chlamydia trachomatis is a Gram negative obligate intracellular bacteria that infects epithelial cells at the mucosal surfaces. Because *Chlamydia* cannot produce their own adenosine triphosphate (ATP) or perform *de novo* synthesis of nucleotides, they rely on parasitizing eukaryotic cells to hijack their ATP, nucleotides and nutrients for energy production and protein synthesis (21, 80).

Chlamydia can differentiate into three different forms, each morphologically and metabolically different. The elementary bodies (EBs) are the small extracellular, metabolically inactive, infectious form. They contain RNA polymerase and ribosomes, and have a spherical shape directed by a rigid cell wall, allowing survival in the extracellular environment. The reticulate bodies (RBs) are the intracellular, non-infectious yet replicative form. They are larger and have a more flexible structure. The persistent bodies (PBs) are a viable, but latent, form of the bacteria, which are important for allowing *Chlamydia* persistence and survival in detrimental environments (21, 80).

The unique infectious life cycle of *Chlamydia,* characterised for the first time in 1932 by Bedson and Bland, is constituted of a biphasic cycle lasting between 36 to 44 hours depending on the species (**Figure 1.1**) (49). The life cycle begins with the infectious extracellular EBs attaching to host mucosal epithelial cells. The host receptor involved has not yet been clearly identified. Indeed, while some evidence showed that heparan sulfate proteoglycans on the surface of epithelial cells could bind to *Chlamydia* protein, such as the outer membrane protein (Omp) B (81-83), others showed differences between species and serovars, with the serovars D, E and L2 of *Chlamydia trachomatis* not requiring heparan sulfate proteoglycans, contrary to the L1 serovar (84-86). It is possible that other host receptors, such as the protein disulfide isomerase, could be involved (87). After attachment, the EBs are endocytosed and a membrane-bound vacuole called

an inclusion is formed, protecting the EBs from the host immune response while allowing them to access host cytoplasmic nutrients (21, 80). The inclusion avoids fusion with endosomes and lysosomes, and migrate to the peri-nuclear region. After cytoskeletal rearrangements, EBs differentiate into RBs, marking the beginning of gene transcription. RBs replicate by binary fission and, as the inclusion fills, RBs differentiate back into EBs, before being released through exocytosis or extrusion into the extracellular environment where the EBs can start a new infectious cycle in surrounding epithelial cells (21, 80).

In the presence of environmental conditions that are detrimental to *Chlamydia* replication, such as those caused by the reduced availability in iron or tryptophan or by the presence of antibiotics or interferon (IFN)γ; *Chlamydia* differentiate into PBs, which allows its survival and the development of a latent infection. When the environmental conditions become more favourable, the PBs can differentiate back into RBS, thus allowing the reactivation of infection (88, 89).

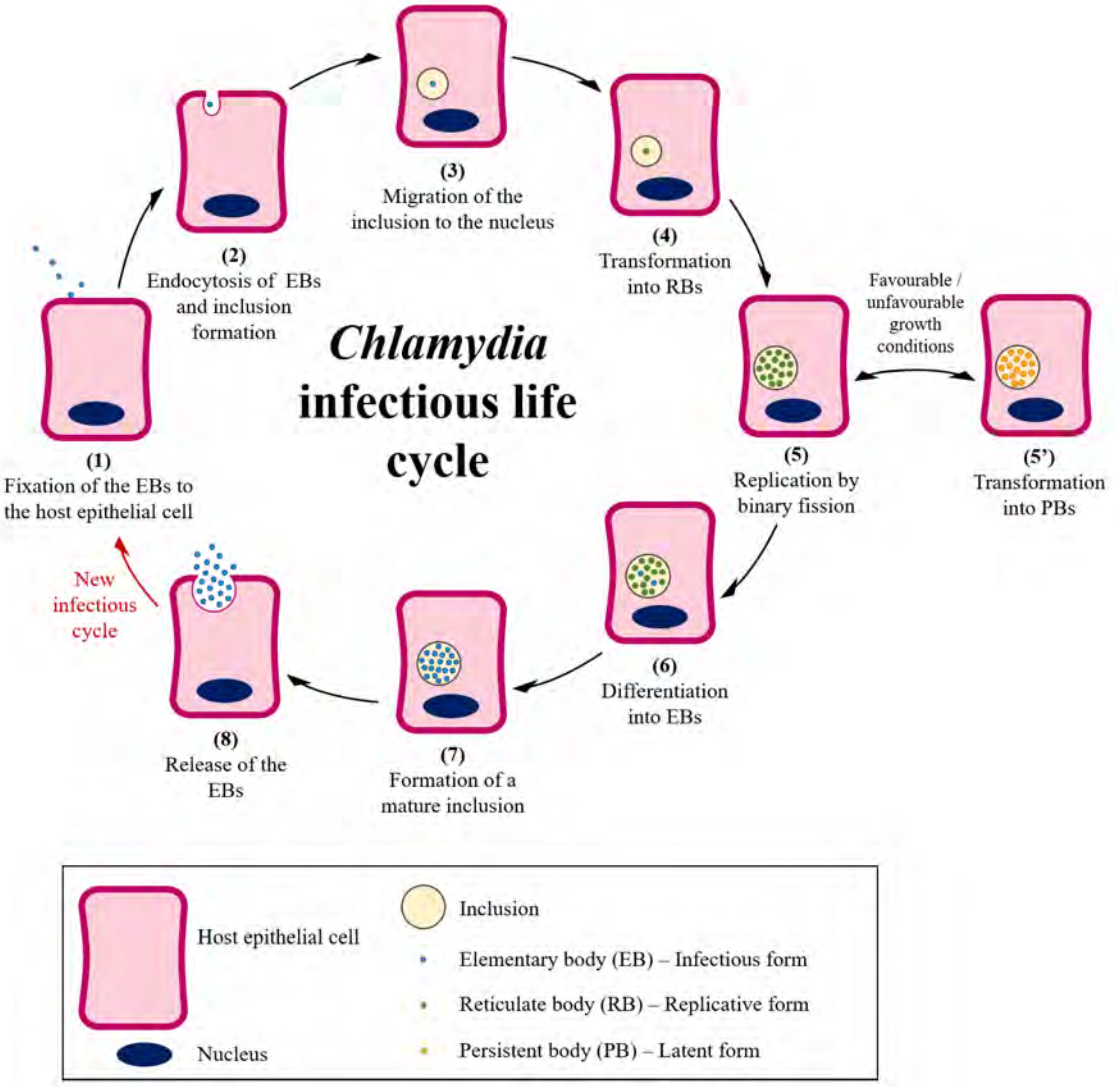


Figure 1.1 – Schematic representation of *Chlamydia trachomatis* infectious life cycle. (1) The infectious elementary bodies (EBs) binds to host epithelial cells in the reproductive tract. **(2)** They are endocytosed, forming an inclusion that protect EBs from the host immune system. Follows **(3)** migration of the inclusion to the perinuclear region and **(4)** differentiation of the EBs into the replicative reticulate bodies (RBs). **(5)** RBs replicate by binary fission until sufficient number of RBs are in the inclusion. **(6)** RBs differentiate back into EBs and **(7)** form a mature inclusion. **(8)** The EBs are then released in the reproductive tract where they can start new infectious cycles. **(5')** Under detrimental conditions, RBs can differentiate into persistent bodies (PBs), enabling latent infection and protection of the bacteria until the environmental conditions become favourable. The PBs can then transform back into RBs, hence triggering the reactivation of *Chlamydia* infectious cycle.

1.3. Immune responses to *Chlamydia* FRT infections

1.3.1. Animal models of *Chlamydia* FRT infections

A wide variety of environmental, bacterial and hosts factors can affect the course of a *Chlamydia* infection and its clinical presentation. Such factors include the bacterial load during inoculation, the serovar, co-infection with other pathogens and the differences between primary and secondary infections. Host variability, including age, hormone levels, immunological responses and genetic differences, also plays an important role in determining the outcome of infection. Low availability and difficulty in sourcing appropriate human tissues, especially from young pre-menopausal women, has meant that most of the research on *Chlamydia* infections, associated host immune responses and pathology has been generated from observations made in animal models. Results of these studies can then be used, in combination to complementary clinical studies, to gain insights into the immunobiology and pathophysiology of *Chlamydia* infection and disease (90).

Various host animals have been used to study *Chlamydia* FRT infection, including mice, rats, guinea pigs, pigs, macaques and marmosets. Mouse models are the most commonly used as the availability of inbred mice reduces the complications of genetic variability, the immunology of the mouse is well characterised and genetically modified mouse strains allow for examination of the mechanistic roles of different immune factors for clearance/susceptibility of *Chlamydia* infection and/or development of associated pathologies (90). Even though human and mouse are both mammals and have approximately 85% identity in the coding regions of their genomes (91), there are notable differences in the FRT of the two species, with human menstrual cycle lasting 28 days compared to 4 days of oestrous cycle in mice and the existence of a midline simplex uterus in human compared to a duplex uterus in mice, characterised by the presence of two uterine horns (92).

A number of mouse models of *Chlamydia* FRT infection exist in which the oestrous cycle of the mice need to be synchronised with progesterone before intravaginal or intrauterine infection with *Chlamydia*. Different strains of mice can display different levels of susceptibility to

Chlamydia FRT infection due to genetic and immunologic differences, with Balb/c and C57BL/6J mice susceptible to *Chlamydia*-associated pathology, and DBA/2J and A/J mice resistant (93). Some mouse models use intravaginal infections with the human pathogen *Chlamydia trachomatis*, but those infections are often cleared quickly and don't ascend to the upper FRT tissues (90, 94, 95). At the contrary, direct inoculation of *Chlamydia trachomatis* into the uterus or the oviduct of mice can cause the development of *sequelae* (90, 96). Other models use intravaginal infections with the natural mouse pathogen, *Chlamydia muridarum*, which result in infections that ascend to the upper FRT and induce tubal inflammation and occlusion similar to observed in women (90, 97, 98).

There are conflicting opinions on which model recapitulates the best the course of an infection in women. Indeed, *Chlamydia muridarum* is not a natural human pathogen, and, even if the genomes of the human infectious agent *Chlamydia trachomatis* and of the murine strain *Chlamydia muridarum*, are relatively similar, some differences exist with, for instance, the lack of tryptophan operon in the *Chlamydia muridarum* genome (44, 51). Moreover, the life cycle of *Chlamydia muridarum* appears to more rapid than the one of *Chlamydia trachomatis*, which could account for the higher virulence of the murine strain (99). The differences in the genomes and in the life cycles of *Chlamydia trachomatis* and *Chlamydia muridarum* can affect the immune responses that are triggered during infection, hence affecting the course of infection (44, 90). *Chlamydia muridarum* has been shown to trigger strong immune responses, which often results in quick development of pathology, while most women display weak immune responses and, in most cases, don't develop symptoms (99, 100). However, as noted previously, intrauterine inoculation with *Chlamydia trachomatis* is required to produce upper FRT infections in mice, which is not the natural route of infection in women. On the contrary, intravaginal *Chlamydia muridarum* infections are characterised with both replication at the initial site of infection, as well as with ascending infection that replicate in the upper FRT tissues leading to the development of pathology. Therefore, *Chlamydia muridarum* infections recapitulate the natural course of symptomatic *Chlamydia* FRT infection in women. As such, *Chlamydia muridarum* infections in mice allow the examination of the complexes interactions that occur between infection and

immune responses at different levels of the FRT *in vivo* and, as such, enable to characterise the immune pathway mediating clearance/susceptibility to *Chlamydia* infection and/or development of associated pathology (90, 101).

1.3.2. Immunity to *Chlamydia* FRT infections

The FRT is made up of a series of organs that undergo major morphologic and immunologic changes under the influence of sex hormones during the menstrual cycle and in pregnancy (102, 103). Compared to other mucosal tissues, immunity in the FRT is unique and complex, with the special need to balance protection against pathogens with tolerance to allogeneic sperm and to the implantation and development of a foetus (104).

1.3.2.1. Innate immunity to *Chlamydia* FRT infections

1.3.2.1.1. Mucosal immunity to *Chlamydia* FRT infections

The first line of defence against *Chlamydia* infection is the epithelium, which creates a physical barrier between the external lumen and the underlying tissues. In the lower FRT, a multi-layered squamous epithelium is present, while the upper FRT tissues are bordered with a single columnar epithelium (102, 103). Underneath the epithelial layer, stromal cells composed of fibroblasts and other structural cells contribute to tissue support, but can also secrete immune mediators that participate in both immune and reproductive functions. Resident leukocytes such as macrophages, dendritic cells (DCs), natural killer (NK) cells, neutrophils, MCs and T cells are also widely distributed in the different regions of the FRT (102, 103). In addition, the lower FRT is colonised by a variety of commensal bacteria that can mediate immune responses and participate in protecting the FRT against pathogens (105).

In complement to their roles in physical protection, epithelial cells directly participate in a variety of immune processes. Indeed, they possess a variety of surface and intracellular receptors known as pattern recognition receptors (PRRs), which include toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) and RIG-1 like receptors (RLRs). These receptors can directly identify infection through the recognition of evolutionarily conserved pathogen-associated molecular patterns (PAMPs) such as LPS, flagelin and single- or

double-stranded RNA (106). They can also detect indirect markers of infection such as damage-associated molecular patterns (DAMPs), which are often produced and/or released following tissue damage and/or cellular stress induced by infections (106, 107).

After detection of an infection through PRR signalling, epithelial cells can secrete mucus thereby trapping *Chlamydia*, initiate tissue repair processes and mediate the transport of immunoglobulins (Ig) to the lumen (106, 107). They can also release various anti-microbial compounds such as elafins, protegrins, cathelicidins and defensins (102, 108-110). However, as mentioned previously, studies have shown that the Chlamydial virulence factor, Pgp3, encoded by the plasmid of *Chlamydia*, can protect against cathelicidin LL-37 by forming stable complexes with it, thereby inhibiting the development of pro-inflammatory responses (75, 111).

The detection of *Chlamydia* infection by epithelial cells, structural cells as well as resident immune cells is key for coordinating the recruitment of innate and adaptive immune cells to the infected FRT through their secretion of a wide variety of cytokines and chemokines, including interleukin- (IL-)1α, IL-6, IL-8, IL-18, tumor necrosis factor (TNF)α, IFNγ and granulocyte-macrophage colony-stimulating factor (GM-CSF) (107, 112, 113). Indeed, TLR2 has been shown to be important for the detection of a *Chlamydia* infection and signal transduction through the production of cytokines such as IL-6, TNFα and IFNγ (114, 115). TLR3 signalling has been linked with protection against *Chlamydia* infection by inducing the production of IFNβ by epithelial cells and by maintaining the integrity of the epithelial layer to limit the spreading of the infection (116-118). In addition, the nucleotide-binding oligomerization domain-like receptor (NOD)1 has been shown to activate the production of the neutrophil chemo-attractant IL-8 by signalling through the receptor-interacting protein (RIP)2 (119). However, whilst pro-inflammatory mediators are required for clearance of infection, they are also associated with development of pathology. For example the release of IL-1 from epithelial cells has been shown to be a major contributor to the induction of pathology in the upper FRT during *Chlamydia* infection (120). Importantly, the differential production of cytokines and chemokines upon *Chlamydia* infection has been associated with the risk of developing *sequelae*, including infertility. For example cervical cells from fertile women produce mainly IFNγ and IL-12 upon exposure to *Chlamydia*,

while cervical cells from women suffering from fertility disorders secrete more IL-4, IL-6, IL-8 and IL-10 (121).

1.3.2.1.2. NK cells and *Chlamydia* FRT infections

Residential and infiltrating NK cells have been shown to contribute to protection against *Chlamydia* during the early stages of infection (122). Indeed, they can recognise and destroy cells that are infected by *Chlamydia* (123). Moreover, NK cells produce cytokines such as TNFα and IFNγ which are associated with protection against infection (122, 124). Crosstalk between immune cells have been shown to be necessary for optimal production of IFNγ by NK cells, with secretion of IL-12 and IL-18 by epithelial cells and DCs necessary for induction of optimal secretion of IFNγ by NK cells (125, 126).

1.3.2.1.3. Neutrophils and *Chlamydia* FRT infections

Neutrophils are short-lived phagocytes that play a major role in the acute immune response to *Chlamydia* infections (127). Following detection of an infection, neutrophils are rapidly recruited to the infection sites by the secretion of various cytokines and chemokines including IL-8, GM-CSF or TNFα (128-130). Neutrophils produce nuclear extracellular traps (NETs) that ensnare bacteria and prevent spreading (131). As a protective mechanism, *Chlamydia* releases chlamydial protease-like activating factor (CPAF), which interferes with the formyl peptide receptor 2 on the surface of neutrophils hence preventing their activation and favouring its escape from host responses (131).

The definitive function of neutrophils in mediating clearance of *Chlamydia* infection remain unclear. Neutrophils have been shown to contribute to the clearance of *Chlamydia muridarum* infection through phagocytose and killing of *Chlamydia* EBs after antibody-mediated opsonisation (128, 132). However, neutrophils do not appear to mediate protection against transcervical *Chlamydia trachomatis* infection, as neutrophil-depleted mice (*via* anti-Gr1 or anti-Ly6G treatments) appear to have similar *Chlamydia* burden as control mice (127)

Despite a role in mediating clearance of *Chlamydia* infection unclear, neutrophils have been largely recognised as playing a detrimental role in the development of *Chlamydia*-associated

pathology. Excessive neutrophil infiltration to the infected FRT has been associated with increased tissue damage, fibrosis and pathology of the upper FRT (127, 133-135). Possible mechanisms include the production, activation and secretion of detrimental compounds such as the metalloproteinase (MMP)9 (134). Indeed, MMP9, along with other MMPs, is associated with destruction and remodelling of the oviducts, tissue damage and pathology. The increased expression of MMP9 in *Chlamydia*-infected fallopian tubes in women is associated with increased evidence for tubal scarring and MMP9-deficient mice display reduced levels of oviduct pathology (135-137). In addition, the defensins produced by neutrophils during *Chlamydia* infection have been associated with development of endometritis (138).

1.3.2.1.4. Eosinophils and *Chlamydia* FRT infections

The roles of eosinophils during *Chlamydia* FRT infection has not been greatly studied as most research has focused on the protective role of T helper (Th) type 1 responses, with eosinophils mostly associated with Th2 responses (139). However, a study revealed that IL-4 released by eosinophils could mediate tissue repair, stromal cell proliferation and prevent endometrial damage during *Chlamydia* FRT infection (140).

1.3.2.1.5. Macrophages and dendritic cells and *Chlamydia* FRT infections

Resident and infiltrating macrophages are important sentinel cells during *Chlamydia* infection. Indeed, they can produce and release nitric oxide (NO) to favour clearance of *Chlamydia* infection (141). They also contribute to inflammation by releasing a variety of cytokines such as TNFα, IL-1β, IL-6, IL-10 or IL12 (142). In addition, they can recognise and phagocytose *Chlamydia*, which results in the bacteria being directed to lysosomes where the bacteria is killed (141, 143, 144). However, *Chlamydia* can evade killing and persist in some macrophages through the formation of extrusions, which suggests that macrophages may play a role in persistence and dissemination of *Chlamydia* infection under certain circumstances (145-148). Macrophages might also contribute to development of FRT damage, with the caspase-1-mediated secretion of IL-1β by macrophages, as well as by neutrophils, associated with development of *Chlamydia*-associated pathology (149, 150).

After detection of an infection, DCs can produce cytokines such as IL-12 and chemokine (C-X-C motif) ligand (CXCL)10 that can mediate recruitment of T cells and induce Th1 polarization (151). They also produce IL-18, which, as noted earlier, mediates the production of IFNγ by NK cells (125). Both myeloid DCs and plasmacytoid DCs are recruited to the FRT during *Chlamydia* infection, where they have been shown to play different roles (152). While myeloid DCs appear to contribute to protection through the induction of *Chlamydia*-specific T cell responses and Th1 polarisation; plasmacytoid DCs are associated with detrimental T cell responses including the induction of $CD4^+$ regulatory T cell (Treg) and $CD8^+$ T cell responses. Plasmacytoid DCs are associated with higher risks of cervicitis in women, and depletion of plasmacytoid DCs has been shown to result in a decrease in *Chlamydia*-associated pathology, marked by less dilatation and collagen deposition in the oviducts, which suggests a role for these cells in the pathogenesis of *Chlamydia*-induced disease (152-154).

Both DCs and macrophages are antigen-presenting cells that can expose *Chlamydia*-specific antigens to their cell surface by the intermediary of their major histocompatibility (MHC) complexes (107, 155, 156). They can also migrate to the draining lymph nodes, activate naïve $CD4^+$ T cells and influence Th differentiation, thereby playing an important role in orchestrating the nature and magnitude of specific adaptive immune responses (156, 157).

1.3.2.2. Adaptive immunity to *Chlamydia* FRT infections

1.3.2.2.1. T cells and *Chlamydia* FRT infections

T cells are key lymphoid immune cells that can recognise microbial peptides presented by MHC class I and II molecules on the surface of antigen-presenting cells *via* highly variable T cell receptors (TCR). The two main types of T cells are the helper $CD4^+$ T cells, which express CD4 on their surface and recognise exogenous peptides presented by MHC class II complexes on the surface of phagocytes, and the cytotoxic $CD8^+$ T cells, which express CD8 on their surface and are activated by endogenous peptides presented by MHC class I complexes on the surface of nucleated cells (158).

1.3.2.2.2. $CD4^+$ T cells and *Chlamydia* FRT infections

$CD4^+$ T cells are recognised as the major protective immune cells during *Chlamydia* infection by mediating clearance of infection (96, 159-163). Mice deficient in MHC class II complex fail to clear *Chlamydia* infection, which highlights the importance of $CD4^+$ T cells (164). Moreover, $CD4^+$ T cells have been shown to be important in protecting against reinfection in mouse models, and in women, through their potent production of IFNγ (162, 165, 166).

The polarisation of $CD4^+$ T cells towards the Th1, Th2, Th17 or Treg phenotypes are heterogeneous following *Chlamydia* FRT infection. Th1 responses are the main phenotype induced by this intracellular pathogens and are associated with protection against infection (95, 96, 160, 165, 167, 168). Indeed, the strong production of the Th1 cytokines IL-12, TNFα and IFNγ is associated with increased efficiency of clearance of *Chlamydia muridarum* in mice, while IFNγ deficiency is associated with delayed clearance and increased dissemination of the infection to other organs (163, 168-171). IFNγ is known to be one of the most important cytokines for the clearance of infection. For instance, IFNγ stimulates macrophage activity, prompts inducible NO synthase expression and subsequent production of the bactericide NO, limits the availability of tryptophan through the activation of indoleamine 2,3-dioxygense and enhances a number of other pro-inflammatory and antibody-mediated responses (172-174). IFNγ depletion shifts the $CD4^+$ T cell phenotype from a Th1- to a Th17-dominant immune phenotype which results in the amplification of FRT pathology in mice (95). Indeed, Th17-dominant responses are associated with increased pathology of the FRT, but do not extensively affect clearance of infection (95, 175). Development of Th2 responses have been observed in women infected by *Chlamydia trachomatis*, however, murine models are not generally characterised by the induction of potent Th2 responses (95, 176). The use of genetically modified animals has highlighted a detrimental role of increased IL-13 in the clearance of infection (177), while IL-4 and IL-4rα have been shown to be protective against tissue damage (140). Interestingly, mice deficient in Myd88, an important signalling adaptor for PRRs-induced responses, were shown to develop increased Th2 responses and be more susceptible to ascending infection and pathology (178). Johnson et *al.*, revealed a new protective subtype of $CD4^+$ T cells in the reproductive tract called CD4γ13 T cells that

produce both IFNγ and IL-13 and appear to prevent oviduct pathology without affecting clearance (179). Tregs have been shown to mediate Th17 responses, thereby favouring the development of *Chlamydia*-associated pathology (180) and IL-10 appear to diminish the antigen presentation by DCs by modulating the assembly of the nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing (NRLP)3 inflammasome, thereby preventing optimal clearance of *Chlamydia* infections (181).

1.3.2.2.3. $CD8^+$ T cells and *Chlamydia* FRT infections

$CD8^+$ T cells appear to only have minor positive role for clearance of primary or secondary infection with *Chlamydia* despite their capacity to secrete IFNγ and to activate cytolysis of infected cells *via* perforin production (162, 182-184). On the other hand, $CD8^+$ T cell appear to mediate immunopathology in the oviduct, through contributing to the induction of neutrophil recruitment and the secretion of TNFα, a known activator of pro-inflammatory cytokines associated with pathology, such as IL-1β or IL-17 (182, 183).

1.3.2.2.4. B cells and *Chlamydia* FRT infections

The roles of B cells in mediating immune responses to *Chlamydia* infection has been relatively under-appreciated (185). Indeed, B cell deficiency has been shown to lead to increased susceptibility to *Chlamydia* secondary infection (162). Moreover, B cells appear to prevent *Chlamydia* dissemination and mediate $CD4^+$ T cells activity (160). Globally, B cells are involved in various immune processes, including inducing the secretion of pathogen-specific antibody by plasma cells that can neutralise the bacteria and activate complement-mediated anti-bacterial immune responses (185). During *Chlamydia* infection, the main immunoglobulins in the vaginal and cervical mucosa are IgA and IgG (186, 187). B cells can also act as antigen-presenting cells, regulating and activating macrophages and the clonal expansion of $CD4^+$ T cells (160, 179, 185).

1.4. MCs and their proteases

1.4.1. MC characteristics and activation

MCs were first identified as metachromatic granulated cells by Paul Ehrlich in 1878 (188, 189). They are large tissue-resident immune cells that are widespread in the body, especially in vascularised tissues at the interface with the external environment, such as the skin or the respiratory, intestinal and genital mucosa. They are often found at higher concentrations in close proximity to blood vessels, nerves, smooth muscle and epithelial cells (190, 191).

MCs originate from the bone marrow and are released into the blood stream as immature committed progenitors. They differentiate in peripheral tissues under the influence of local stimuli, leading to the establishment of a wide heterogeneity of phenotype of tissue MCs (192, 193). They are relatively long-lived cells that can proliferate upon specific stimulation (194, 195). They are evolutionary conserved, suggesting primordial roles of these cells beyond their commonly associated detrimental roles in the development of several pathophysiological diseases including allergic hypersensitivity disorders, asthma, anaphylaxis cardiovascular disease and mastocytosis (189, 196). Indeed, MCs have numerous functions in various physiological processes including tissue homeostasis, angiogenesis, tissue repair and mediation of immune responses (189).

MCs are characterised by their numerous secretory granules that contain a large variety of very potent preformed mediators including proteoglycans, the biogenic amines, histamine and serotonin, cytokines as well as various MC proteases (189). MCs can be activated by a wide variety of stimuli and pathways, with the most well recognised being the crosslinking of antigen-specific IgE antibodies bound to the high affinity immunoglobulin E receptor (FcεRI). MCs can also be activated by recognition of PAMPS by the various TLRs and NODs that they express, recognition of exogenous and endogenous peptides such as bacterial toxins or the complement components C3a and C5a (197). Shortly after activation, MCs degranulate and release their preformed mediators into the extracellular environment. They also synthesise and release lipid mediators such as prostaglandins and leukotrienes. At later stages following immediate

degranulation, activated MCs may also produce and release a number of cytokines and chemokines including IL-1β, IL-4, IL-6, IL-8, IL-13, TNFα and GM-CSF, *via de novo* synthesis, allowing a more specific response to the activating stimulus (189, 198).

1.4.2. MC proteases

MC proteases constitute approximately 50% of all the proteins in the secretory granules of MCs (199). In general, the term MC proteases refers to MC-specific proteases, which include a number of chymases, tryptases and carboxypeptidases. However, MCs also contain many other non-MC-specific proteases, including MMP9, cathepsins and granzymes (200, 201). MC proteases are first synthesized as inactive precursors referred to as a pre-pro-enzymes. The pre-peptide component of the pre-pro-enzyme serves as a signal peptide that guides the enzyme through to the required secretory pathway in the endoplasmic reticulum. After removal of the pre-peptide, a pro-peptide is revealed, acting as an activation peptide and maintaining the enzyme in a latent state. In this inactive state, the protease is called a zymogen. This inactive state allows the regulation of the protease's activity. The pro-peptide is cleaved by specific proteolytic mechanisms that can be auto-catalytic or mediated by other granule enzymes, such as the dipeptidyl peptidase I (DPPI) or cathepsins. The removal of the pro-peptide reveals the protease's active site and confers enzymatic activity to the protease. At this stage the protease is considered mature and biologically active (190, 202-204).

Proteases are characterised by their capacity to catalytically cleave peptide bonds in specific regions of molecules, which initiates conformational changes that alter the function of the target molecules. Such functional changes may result in activation or de-activation of the target molecule or may change how the molecule behaves biologically. Importantly, proteases may have numerous targets that are dependent on the specific interactions that occur between protease enzymatic sites and substrate cleavage sites that are present in target proteins. Therefore, proteases play a major, yet diverse, role in mediating biological responses through their ability to modify the activity and function of numerous proteins (205).

1.4.2.1. Serine proteases

Serine protease are broadly distributed in most tissues where they have a variety of activities in different physiological processes such as fertilisation, embryonic development, blood coagulation, digestion, innate and adaptive immunity (205, 206). The terminology of the serine protease:substrate interaction is established around the enzymatic active site and the substrate cleavage site (207). On the substrate, the amino acids upstream of the cleavage point are referred to as P1, P2, P3 and P4 while the ones downstream are P'1, P'2, P'3 and P'4. Equivalently, the amino acids downstream and upstream of the protease active site are labelled respectively S1, S2, S3 and S4; and S'1, S'2, S'3 and S'4 (**Figure 1.2**) (207).

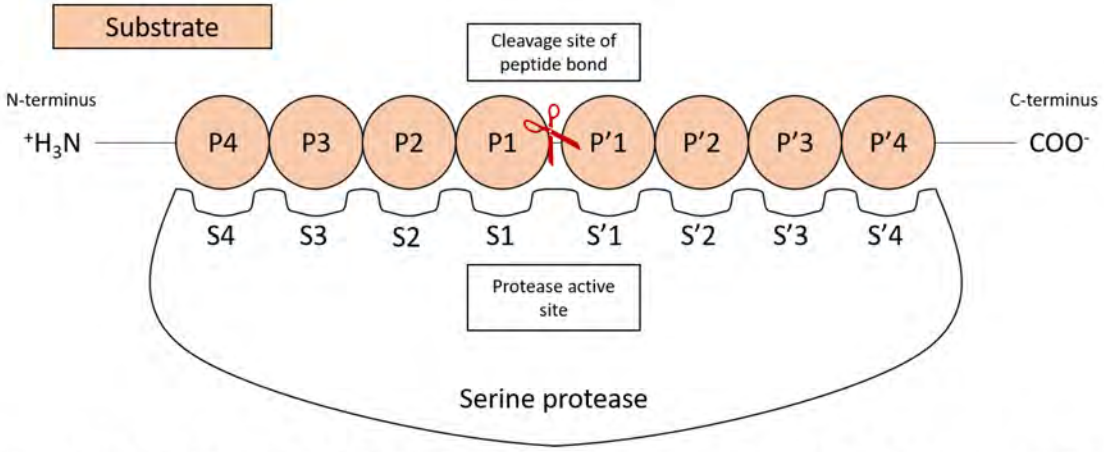


Figure 1.2 - Nomenclature of the reaction site between a serine protease and a substrate. The amino acids located upstream (N-terminal) of the substrate cleavage site are called P1, P2, P3 and P4 while the one downstream (C-terminal) are P'1, P'2, P'3 and P'4. Correspondingly, on the serine protease, the amino acids upstream of the active site are referred to as S1, S2, S3 and S4 while those downstream are S'2, S'2, S'3 and S'4. Figure adapted from (207).

Serine proteases are endopeptidases characterized by the presence of a highly reactive serine amino acid, in close association with two other amino acids forming a catalytic triad that is part of an extensive hydrogen-bonding network crucial for the enzymatic reaction (208). According to the catalytic triad, serine proteases are classified into different groups, including the chymotrypsin family, which can be further divided into chymotrypsin-like, trypsin-like and elastase-like proteases depending on their primary specificity (206, 208). The primary specificity

is dependent on the capacity of the serine protease to recognise the amino acid on P1 of the substrate and is largely dependent on the enzymatic S1-pocket, a structure forming a pocket and two loops at the active site of the enzyme and containing three amino acids called the specificity-conferring triplet (206, 208). As described in **Figure 1.3**, chymotrypsin-like proteases have a wide S1-pocket allowing cleavage after large and aromatic hydrophobic residues, trypsin-like proteases possess a negatively charged aspartic acid residue in its cleavage site allowing them to attract and cleave preferentially after positively charged amino acids. Elastase-like proteases hold two large amino acids in their specificity-conferring triplet, therefore, the S1-pocket is smaller. This results in elastase-like proteases only cleaving proteins after the presence of small uncharged amino acids (206, 208-210).

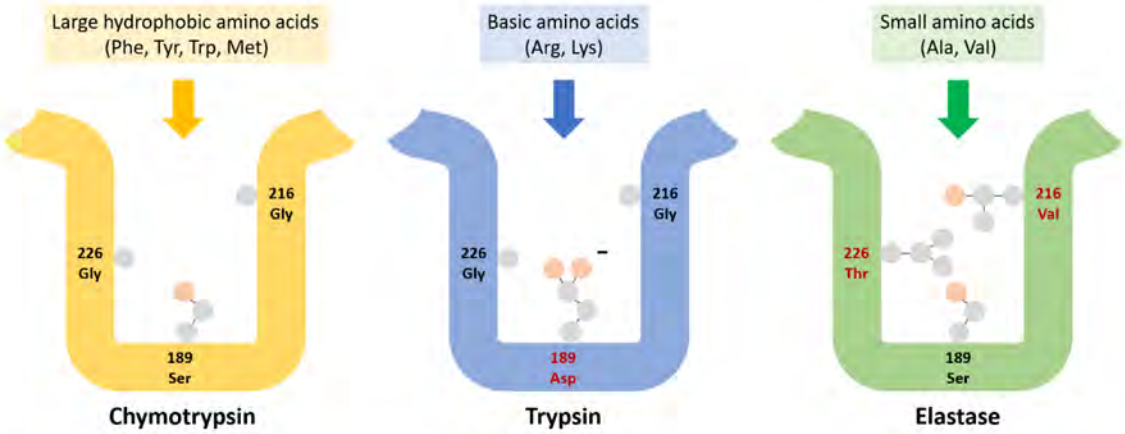


Figure 1.3 – Schematic representation of the S1-pocket of the chymotrypsin-like, trypsin-like and elastase-like serine proteases. The amino acids at the position 189, 216 and 226 confess different substrate specificity with chymotrypsin-like proteases cleaving large hydrophobic amino acids, trypsin-like proteases cleaving positively charged amino acids and elastase-like proteases cleaving small amino acids. *Phe: phenylalanine, Tyr: tyrosine, Trp: tryptophan, Met: methionine, Arg: arginine, Lys: Lysine, Ala: alanine, Val: valine, Gly: glycine, Ser: serine, Asp: aspartic acid and Thr: threonine. Grey circle: carbon atoms and orange circle: oxygen atoms*. Figure adapted from (208).

1.4.2.1.1. MC tryptases

Tryptases are trypsin-like serine proteases holding a substrate-binding cleft offering restricted substrate specificity compared to trypsin (201). In humans, the tryptase locus is present on the chromosome 16p13.3 and contain the genes *TBSAB1* (encoding for the allelic variants αI- and βI-tryptases), *TPSB2* (encoding for the allelic variants βII- and βIII-tryptases), *TPSD1* (encoding for δ-tryptase), *TPSG1* (encoding for γ-tryptase/Prss31) and Prss22 (encoding for ε-tryptase) (202, 211, 212). In mice, the corresponding tryptase locus is located on the chromosome 17A3.3 and encodes for 13 functional tryptases including the MC proteases, mMCP6, mMCP7 and Prss31 (200, 202, 212).

β-tryptases are the most abundant tryptases stored in, and secreted from, the secretory granules of MCs and are also the most enzymatically active (213). The different allelic forms of the β-tryptase are relatively similar and display only slight differences in substrate specificity, because of variances in affinity with the amino acids in P2, P3 and P4 position of the substrate (214). β-tryptases are stored in the secretory granules of MCs as active tetramers, tightly bound to serglycin proteoglycans, with the active site of each β-tryptase protected in the central pore of the tetramer. This tetrameric arrangement physically restricts the substrate specificity of the enzyme but also provides further resistance to inhibitors hence allowing prolonged enzymatic activity of the β-tryptases in the extracellular environment (215, 216). Even if α-tryptases possess more than 90% identity in their amino acid sequences with β-tryptases, they appear to possess no/low catalytic activity because of a mutation on their pro-peptides preventing its removal, and of a single mutation on their substrate-binding cleft restricting their substrate specificity (217). Another difference is the fact that, unlike most MC proteases, the α-tryptases are not stored in the secretory granules of MCs but rather constitutively secreted as inactive monomers upon production (213). Because α- and β-tryptases are allelic variants, 29% of the human population is estimated to be deficient in α-tryptase, with major differences between ethnicities (218). δ-tryptases are transcribed at relatively low levels and appear to be enzymatically inactive because of a premature stop codon, making the δ-tryptase shorter than mature α- and β-tryptases by

approximately 40 amino acids. To date, no biological role has been established for δ-tryptases (219, 220).

The mouse ortholog of β-tryptase, mMCP6, also spontaneously forms tetramers bound to serglycin proteoglycans with their active sites facing the central pore (221). While mMCP7 displays similarity with the fifth exon of δ-tryptases, mMCP7 is enzymatically active (219, 222). Both mMCP6 and mMCP7 can form homo- and hetero-tetramers bound to serglycin proteoglycans in the secretory granules of MCs but, while mMCP6 remain bound to serglycin proteoglycans upon MC degranulation and are released as proteoglycan-tetramer complexes in the extracellular matrix, mMCP7 dissociate from serglycin proteoglycans upon degranulation allowing them to reach the circulation (219, 221-223).

The human γ-tryptase and the murine Prss31 are the only membrane-anchored tryptases in MCs, with other MC proteases being released upon MC degranulation (202, 212). Similarly to all serine proteases, these membrane-anchored tryptases possess a signal pre-peptide, followed by an activation pro-peptide, but, subsequently, they also hold a hydrophobic peptide membrane anchor in their C-terminal region (212, 224, 225). The γ-tryptases/Prss31 are stored in the secretory granules of MCs and, upon degranulation, become bound to the plasma membrane of the degranulated MC. Therefore, they are only able to cleave extracellular substrates present in the immediate local environment of the degranulated MC. Serpin-class inhibitors can inactivate γ-tryptases but are not efficient against β-tryptases, probably because of their ability to form tetramer-proteoglycan complexes (202, 226).

In mice, MC tryptases are expressed in a strain-dependent manner. Indeed, while mMCP6 appears to be expressed by all strains of mouse, a mutation on the first nucleotide at the exon-2/intron-2 splice site of the *Mcpt7* gene causes the activation of a cryptic splice site and the apparition of a premature stop codon in the *Mcpt7* gene in C57BL/6, but not in BALB/c mice (227). This renders C57BL/6 mice as naturally mMCP7-deficient. In contrast, Prss31 is expressed in C57BL/6 mice, but not in BALB/c mice, most likely as a result of a cytokine-dependent post-transcriptional mechanism (212).

1.4.2.1.2. MC chymases

Chymases are part of the chymotrypsin-like sub-family of serine proteases and harbour a relatively broad substrate specificity (208, 228). The MC chymase locus is located on the human chromosome 14 and encodes a single chymase, the α-chymase CMA1. Even if the MC chymase locus is also located on the chromosome 14 in mouse, major differences exist, with the mouse locus being approximately three times bigger and containing a total of 14 genes, coding for, among others, the α-chymase mMCP5, and the β-chymases mMCP1, mMCP2, mMPC4, mMCP8 and mMCP9 (229-231). mMCP1, mMCP2, mMPC4, mMCP8 and mMCP9 have only been identified in rodent MCs (230, 231). In the secretory granules of MCs, most chymases assemble into monomers ionically bound to serglycin proteoglycans and they remain tightly bound with serglycin proteoglycans upon MC degranulation (228). While mMCP5 is the most closely related mouse chymase to human chymase in terms of the amino acid sequence, a mutation in the active site of mMCP5 changes its enzymatic activity from chymotrypsin-like to elastase-like activity, characterised by a substrate specificity for small amino acids rather than large hydrophobic amino acids (209, 210). mMCP4 is often considered as the functional homolog to CMA1, as it displays similar substrate specificity and tissue expression as the human chymase (200, 232-234).

1.4.2.2. Carboxypeptidase

CPA3 is a zinc-dependent metalloprotease characterised by the presence of a zinc ion in its active site, which is involved in the hydrolysis of the substrate peptide bond and in the stabilisation of the tetrahedral-intermediate that precedes cleavage (235, 236). Contrary to serine proteases that are endopeptidases, CPA3 is an exopeptidase that only cleaves peptide bonds at the C-terminal end of its substrates, with a specificity for large hydrophobic amino acids (235). The *CPA3* gene is located on the chromosomes 3q24 in human and 3A3 in the mouse (200, 235). CPA3 is specific to MCs and basophils (237). Similarly to serine proteases, CPA3 is first generated as an inactive pre-pro-protein that is activated by proteolytic cleavage by cathepsins to form a mature protease, that is stored as monomer bound to serglycin proteoglycans in the secretory granules of MCs (200, 238). The storage of Cpa3 and mMCP5 appear to be largely co-dependent, with mice

genetically deficient for one of these genes, also displaying secondary loss of the other (233, 239, 240), suggesting co-dependence for correct storage and, potentially, involvement in similar enzymatic cascades (235).

1.4.2.3. Serglycin proteoglycans

In the secretory granules of MCs, proteoglycans are composed of a small core protein, called serglycin, which consists of multiple repetitions of serine and glycine residues linked to numerous unbranched glycosaminoglycan (GAG) side chains (**Figure 1.4**) (241). In MCs, serglycin proteoglycans are composed of GAGs formed by some of the most negatively charged molecules in the body, heparin or chondroitin sulfate (200). Their highly carboxylated and sulfated disaccharide units, conferring high anionic charges, allow serglycin proteoglycans to form tight electrostatic interactions with positively charged molecules, such as MC proteases (242).

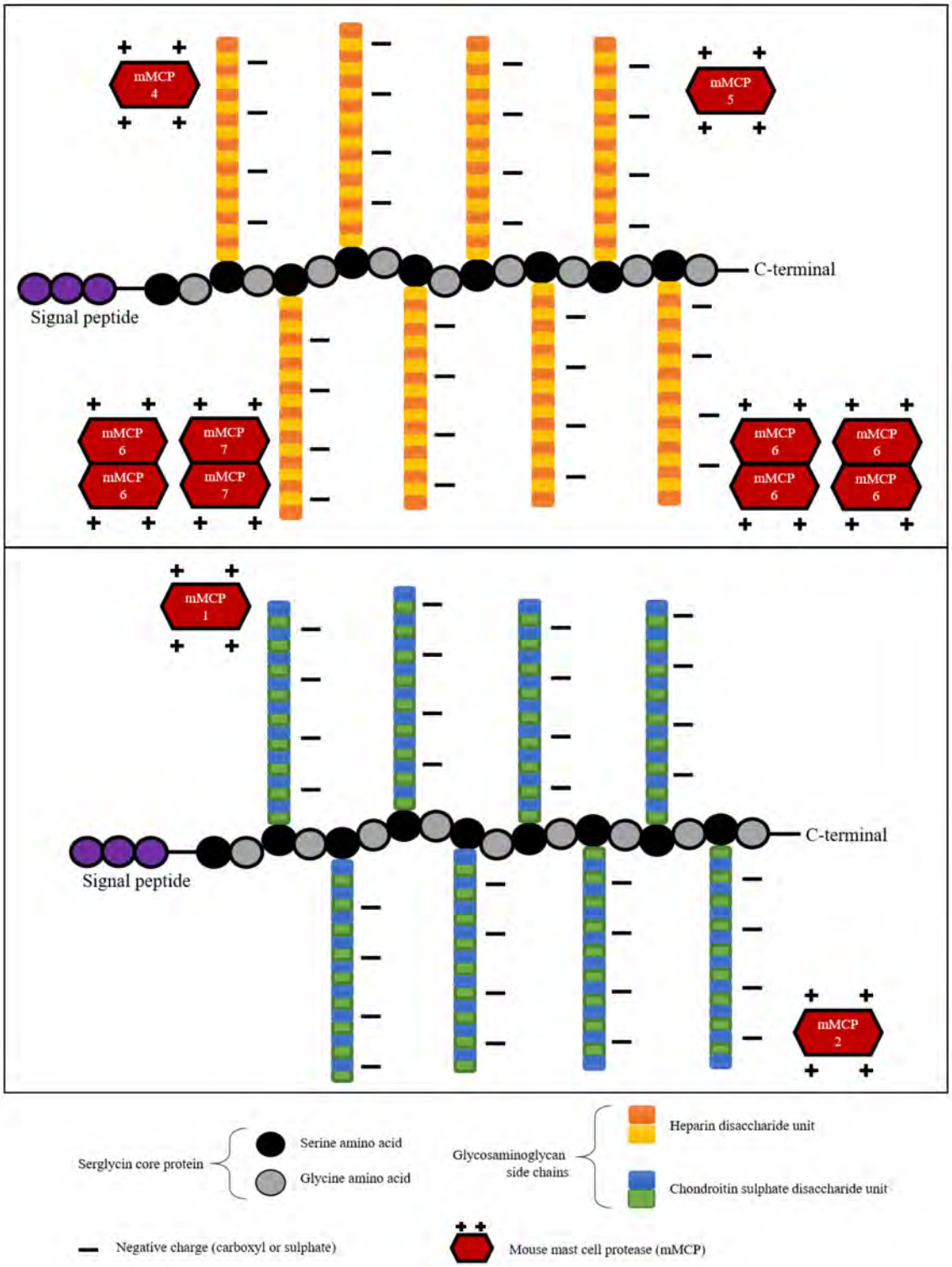


Figure 1.4 – Schematic representation of the MC serglycin proteoglycans stored in the secretory granules of mast cells (MCs). Serglycin proteoglycans are composed of a serglycin core protein characterised by the presence of repeated serine and glycine residues and of multiple glycosaminoglycan side chains. Those glycosaminoglycan side chains are constituted of repeated disaccharide units of either heparin or chondroitin sulfate, that are highly negatively charged, allowing them to create tight bounds with some MC factors, including MC proteases. *mMCP: mouse mast cell protease.*

The main role of serglycin proteoglycans in MCs is to offer a storage matrix for MC proteases and to protect them upon release into the extracellular environment (242, 243). While mMCP1 and mMCP2 are thought to mainly bind to chondroitin sulfate, mMCP4, mMCP5, mMCP6 and Cpa3 bind to heparin (**Figure 1.4**) (202, 244, 245). Because not all the proteases have the same level of positive charge, they do not all bind proteoglycans to the same degree. For instance, mMCP7 becomes independent of proteoglycans upon degranulation, while mMCP4, mMCP5, mMCP6 and Cpa3 remain tightly bound to heparin proteoglycans (202, 221).

The biosynthesis of heparan sulfate and heparin is complex and involves a large number of enzymes. Among them, the Ndst enzymes are responsible for initiating the sulfation and designing the sulfation patterns of heparan sulfate and heparin (246). Four Ndst enzymes have been identified, Ndst1-4. Ndst1 and Ndst2 are ubiquitously expressed in most cells and tissues of the body, whereas Ndst3 and Ndst4 are restricted, with expression localised to part of the brain such as the hippocampus and cerebellum, as well as the epithelium of the colon (247-250). Overall, Ndst1 and Ndst2 appear to be the main enzymes for the biosynthesis of heparin, with Ndst2 being the main enzyme involved in the biosynthesis of the heparin residues that are attached to the serglycin proteoglycans enclosed in the secretory granules of MCs. On the other hand, Ndst1 is proposed to be the major enzyme involved in the biosynthesis of heparin that is attached to syndecan, glypican, or perlecan proteoglycans in other cell types (247). Ndst2-deficient mice are viable and fertile and, even if they do not appear to have major differences in the composition of the heparan sulfate in most cell types, they display dramatic changes in their MCs, with reduced levels of histamine and of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3, stored in their secretory granules (244, 245). Although the genes coding for those proteases are still actively transcribed in MCs, the proteins are not expressed in MCs, confirming the major role of heparin for the storage of those MC proteases (244-246).

1.4.3. MC phenotypes

In humans, MCs are often characterised based on their protease content, with MC_T MCs containing only tryptases, MCc MCs containing only chymases and CPA3 and MC_{TC} MCs containing both tryptases, chymases and CPA3 (200). In mice, MCs have been characterised according to their tissue location. Connective tissue MCs (CTMCs) are located in the skin, tongue, peritoneal cavity and express the MC proteases mMCP4, mMCP5, mMCP6, mMCP7 and Cpa3 bound to heparin proteoglycans. Mucosal MCs (MMC) are located within the mucosal tissues of the intestine and predominantly express the β-chymases mMCP1 and mMCP2 along with chondroitin sulfate proteoglycans (200). MMCs have been shown to be recruited to the mucosal region of the jejunum during helminth infection under the influence of T cell responses, while CTMCs are thought to be constitutively present in the tissues that they reside (251). However, MC distinctions that are based on MC protease content or tissue location are not representative of the complexity of MCs. It is now recognised that MCs are much more heterogeneous than previously described with the phenotype of MCs and the proteases that are stored in their granules heavily dependent on the nature of the immune/inflammatory micro-environment, tissue location as well as host genetics (252).

1.5. The roles of MCs and their proteases in mediating immune responses

1.5.1. The roles of MCs in mediating immune responses

While MCs have long been recognised for their detrimental roles in allergic inflammatory conditions, they are important effectors of a wide variety of physiological processes including angiogenesis and tissue homeostasis and repair, as well as in the initiation and development of various pathological conditions, including mastocytosis, cardiovascular disease and cancer (189, 191). Moreover, MCs have been shown to mediate immune responses to pathogens, in a range of tissues (253-261). For instance, during *Chlamydia* respiratory tract infections, MCs appear to play a detrimental role by mediating excessive immune cell recruitment to the airway contributing to pathogenesis (262).

Given their location within tissues and their ability to rapidly release inflammatory mediators, MCs play a key sentinel role in host defence. Upon activation, MCs release a large amount of preformed and neo-formed inflammatory mediators as well as cytokines and chemokines, which allow them to induce a wide range of anti-pathogenic and pro-inflammatory responses as well as directly target pathogens (263).

MC mediators have been shown to stimulate mucus production of goblet cells, which helps immobilise pathogens. They can also act on smooth muscle to facilitate parasitic expulsion. Moreover, the coupled action of MC mediators, that increase vascular permeability, and their release of pro-inflammatory cytokines and chemokines, such as eotaxin, IL-6, IL-8, and TNFα, stimulate the recruitment of innate immune cells (264-271). Indeed, the release of mMCP6, leukotrienes, IL-6 or TNFα from MCs have been shown to mediate the recruitment of neutrophils and eosinophils during bacterial and parasitic infections (253, 272-276). Interestingly, some of the MC mediators also display anti-inflammatory properties, with heparin preventing the infiltration of eosinophils during formation of an oedema and nasal allergen challenge (277, 278).

MCs can also participate directly and indirectly in the induction of adaptive immune responses. MCs possess MHC complexes that can directly present antigens to T cells (279, 280).

MCs also mediate the infiltration and maturation of DCs during infection, as well as enhance their migration to draining lymph nodes, allowing the presentation of antigens to naïve T cells and priming of specific T cell responses (254, 260, 279, 281, 282).

MCs have been mostly associated with Th2 responses (283-286). DCs have receptors for histamine and, if they mature in the presence of histamine, which is released by MCs, DCs induce the polarisation of $CD4^+$ T cells into a Th2 phenotype (283). Similarly, MC degranulation appears to reduce the capacity of DCs to prime Th1 T cells polarisation *in vivo*, resulting in T cells producing larger amount of IL-4 while secreting less IFNγ (284). However, MCs and some of their mediators have also been associated with induction of Th1 and Th17 responses (260, 287, 288). For instance, MCs were shown to mediate DC maturation during *Leishmania* infection, inducing the polarisation of $CD4^+$ T cells that release higher levels of IFNγ and IL-17 (260, 289). MCs have also been associated with induction of humoral immune responses. Indeed, MCs were shown to stimulate the proliferation and activation of B cells, as well as their differentiation in plasma cells, through direct contact and secretion of MC-derived cytokines (289, 290).

MCs also display antimicrobial properties as they can secrete compounds such as cathelicidins that have been shown to protect against group A *Streptococcus* infections (291). Moreover, they can directly kill bacteria through phagocytosis and reactive oxygen species (ROS) production (292, 293). Furthermore, similarly to neutrophils, MCs can secrete extracellular nets constituted of DNA, histones, granules mediators and cathelicidins that can trap bacteria and reduce their growth and dissemination, without phagocytosis (294).

Therefore, MCs and their mediators harbour a range of immunomodulatory properties that can mediate host innate and adaptive immune responses to pathogens.

1.5.2. The roles of MC proteases in mediating immune responses

The physiological functions of most MC proteases is not clearly defined, but they appear to have various roles in mediating immune responses. Indeed, during Japanese encephalitis virus infection, chymases have been shown to be detrimental, through enhancing the cleavage of tight junction proteins and the breakdown of the blood-brain barrier (295). Similarly, following injury,

the chymases mMCP4 and mMCP5 have been shown to disrupt epidermal tight junctions (296, 297). During infections, those tight junctions, whose primary functions are to mediate paracellular permeability and to participate in signalling for cellular proliferation and differentiation, can be targeted by pathogens and used as receptors for attachment or as gateway for tissue dissemination. Disruption of tight junction, by pathogens or by host factors, has been shown to elicit inflammatory responses (298, 299). The chymase mMCP1 has been shown to protect against gastrointestinal infection with the parasite *Trichinella spiralis*, by inducing inflammatory responses, NO production and TNFα secretion (300, 301). Similarly, the tryptase mMCP6 can mediate pro-inflammatory responses, eosinophil migration and protects against chronic *Trichinella spiralis* infection (275). Moreover, mMPC6 mediates neutrophil migration to the lung and helps protects against *Klebsiella pneumoniae* infection (274). However, whilst the mechanisms involved are not fully elucidated, mMCP5 appears to be detrimental during *Streptococcus pneumonia* and *Pseudomonas aeruginosa* infections, as mMCP5-deficient mice were shown to have enhanced clearance of infections, altered number of macrophages or neutrophils, as well as modified cytokine and chemokine profiles in their lungs, compared to WT controls (302).

While the mechanisms involved are not yet fully understood, most MC proteases, including mMCP4, mMCP5, mMCP6 and mMCP7 can cleave fibronectin, which suggest roles in tissue remodelling and coagulation, but also in activating pro-inflammatory responses and immune cell recruitment (222, 233, 239, 303-306). Unpublished data from Miyasaki *et al.*, suggest that mMCP7-mediated fibronectin cleavage can induce IL-6-mediated neutrophil and eosinophil infiltration into the conjunctiva (202). Furthermore, mMCP4-mediated cleavage of fibronectin was show to protect against group B *Streptococcus* by preventing attachment of the bacteria to mucosal tissues (305).

In addition to fibronectin, MC proteases can cleave, degrade and activate a variety of cytokines and chemokines, including IL-1β, IL-6, IL-13, IL-33 and TNFα (226, 307-312). In addition, MC proteases can activate other pro-inflammatory proteases, with mMCP4, mMCP5 and mMCP6 able to activate MMP2, MMP3, MMP9 and MMP13 during arthritis or skin

blistering (239, 313-315). Moreover, chymases and Cpa3 have also been shown to protect against venoms by directly cleaving toxic peptides (316, 317).

1.5.3. MCs in the FRT

1.5.3.1. Female sex hormones and MCs in the FRT

MCs are present in relatively high numbers in FRT tissues, especially in close proximity with blood vessels, glands and nerves, in most species including humans (318-322), cows (323), horses (324), goats (325, 326), hamsters (327), rats (328) and mice (328-331). In the human uterus, all three types of MCs have been identified, with higher proportions of MC_T and MC_{TC} observed compared to MC_C (318). In mice, uterine MCs have been shown to display heterogeneous phenotypes with 5% to 20% of uterine MCs expressing mMCP5 (329).

Conflicting reports exist with regards to the presence of female sex hormone receptors on MCs. Indeed, the oestrogen receptor (ER)α and progesterone receptor (PR) have been identified as being expressed by the human mast cell line HMC-1 and in mouse bone marrow-derived mast cells (BMMCs) (332, 333). However, uterine MCs from human biopsies, have been reported to only express ERβ (318) or no female sex hormone receptors at all (334).

Progesterone and oestrogen have been shown to induce the up-regulation of the chemokines CCR3, CCR4 and CCR5 on MC surface, suggesting that the fluctuations in female sex hormones that occur during the menstrual/reproductive cycle and in pregnancy may affect MC recruitment (332). Studies show that the number, location, maturation and/or activation of MCs in the FRT might be dependent on the menstrual cycle supporting an important role of hormones in regulating MCs number and function. Briefly, in hamsters, a minimum number of uterine MCs were reported the day before ovulation (327), while in mice and rats the number of uterine MCs is maximal during the receptive phase of the oestrous cycle (329, 335). In cows, the number of MCs in the oviduct correlate with the levels of progesterone in plasma (323). A positive correlation between the levels of oestrogen and the number of MCs in the cervix was identified in horses, with no changes in the uterus or vagina (324). In women, a decrease in the number of MCs was reported after 6 months of usage of the intrauterine device Mirena®, which delivers the progestogen

levonorgestrel in the uterus (334). An increase in uterine MC activation was reported during the secretory phase of the menstrual cycle (318), while an increase in MC activation was reported just prior and during menstruation (336). Similarly, in biopsies, an increase in MC activation was discovered during the late secretory and early menstrual phases (337, 338). After menopause, which is characterized by minimal hormonal levels, the number of MCs appear to be reduced (319, 320), while pregnancy has been shown to increase the number of MCs in the FRT, especially in tissues close to implantation sites, and to shift the phenotypes of MCs from mostly MC_{TC} to mostly MC_T (329, 339, 340). Even if the mechanisms involved are not fully understood, fluctuations in the levels of female sex hormones have often been suggested to regulate those changes in number and/or phenotype of MCs in the FRT.

1.5.3.2. The roles of mast cells in the FRT physiology and pathophysiology

In the FRT, MCs appear to contribute to various physiological and pathophysiological processes. Indeed, they appear to mediate the follicular development of the ovaries of neonatal mice (341, 342), which could be mediated by their secretion of IL-8, a cytokine important for ovulation and luteinisation (343). In addition, some studies reveal an enhancement of MC activity just before menstruation, suggesting a potential role of MCs in extracellular matrix breakdown, uterine menstrual contractions, shedding of the endometrium or tissue remodelling (313, 319, 336-339, 344, 345).

During pregnancy, MCs appear to facilitate implantation, with MC-deficient mice having a delay in the development of their implantation sites. Moreover, systemic reconstitution of MCs in MC-deficient mice, by intravenous injection of BMMCs, as well as local reconstitution of MCs, by injection of BMMCs in the uterine horns, were shown to increase the number of implantation sites (329). In addition, MCs have been shown to migrate towards the trophoblast (332) and histamine release is increased during implantation (346). MCs also appear to contribute to angiogenesis, with inhibition of MC degranulation, using the MC stabiliser cromolyn, resulting in a decrease in the expression of VEGF and vessel formation, which could have detrimental effects on cervical ripening and parturition (347, 348). MC-deficient mice exhibit reduced blood

flow, leading to restricted growth of the foetus, while mice deficient in both uterine MCs and uterine NK cells have further abnormalities in spiral arteries and foetal development, suggesting that uterine NK cells and uterine MCs display overlapping roles in assuring implantation, appropriate vascularisation and placental development (331, 349). Adoptive transfer of Tregs in abortion-prone mice have been shown to cause an increase in uterine MCs and subsequent improvement in remodelling of spiral arteries and placentation (330). However, other studies have not shown any differences in placentation and foetal development between MC-deficient mice and WT controls (340). MCs have also been shown to be potentially important in the induction of labour, with serotonin, histamine and MC degranulation shown to induce contraction of the cervix and myometrium (350, 351). However, a reduction in the number of MCs in the decidua have been associated with pre-term birth, contradicting the hypothesis that MCs could induce labour (352).

MCs are also involved in pathophysiology of the FRT as they have been associated with hypersensitivity reactions and chronic pain characteristic of endometriosis (353). An increase in the number and activity of MCs have been identified in biopsies from women suffering from endometriosis, with MCs being located in close proximity to nerves (354-357). Chronic cervicitis, polypoidal endocervicitis and vestibulitis have also been associated with an increase in MCs (321, 358) and treatments targeting MCs with anti-histamines or the MC stabiliser, cromolyn, being protective against dyspareunia, vaginitis and dysfunctional uterine bleeding (359).

1.5.3.3. The roles of mast cells in the mediating immune responses in the FRT

The roles for MCs in FRT infections are relatively understudied. In women infected with HIV, an increase in the number of MCs has been observed in the cervical epithelium and some suggest that MCs might act as a reservoir for the virus (360-362). Moreover, studies suggest that MCs might mediate immune responses to the parasite *Trichomonas vaginalis*, with infection of epithelial cells inducing the recruitment of MCs, as well as their activation, subsequent release of histamine, TNFα and IL-8, and neutrophil migration (363-366).

MCs appear to be protective against group B *Streptococcus* infections. Indeed, MCs were shown to degranulate after infection and to release pro-inflammatory mediators able to decrease the bacterial burden (367). MC-deficient mice were shown to have increased bacterial burden and decreased immune cell infiltration (mainly neutrophils) following a systemic intraperitoneal infection. They also displayed an enhancement in bacterial persistence in the vagina and the uterus during vaginal infection (367). In addition, the β-chymase mMCP4 is protective against group B *Streptococcus* infection, by preventing the adherence of the bacteria to host surfaces by proteolytically cleaving fibronectin (305).

Recently, MCs have been shown to be detrimental during *Chlamydia* FRT infection, with MC-deficient mice protected against *Chlamydia*-associated pathology, while displaying similar levels of clearance. Although the mechanisms involved were not fully resolved, *Chlamydia trachomatis* and *Chlamydia muridarum* have been shown to induce MC activation and the release of cytokines and chemokines *in vitro*. In addition, MC-deficient mice have been shown to have a reduced number of DCs in their FRT-draining lymph nodes following *Chlamydia* infection, suggesting that MCs might mediate activation and migration of DCs. Moreover, splenocytes isolated from *Chlamydia*-infected MC-deficient mice released less IFNγ, IL-10, IL-13 and IL-17 upon stimulation with inactivated *Chlamydia*, suggesting a role of MCs in adaptive T cell responses. All together, these results highlight a role of MCs in mediating immune responses to *Chlamydia* FRT infection (368).

1.6. Study rationale

Chlamydia trachomatis is a major health problem worldwide. Whilst the detection and treatment of infection with antibiotics is relatively straight forward and effective, most infections are asymptomatic and, therefore, go undiagnosed and untreated, and can result in a number of serious FRT diseases, including pelvic inflammatory disease and tubal factor infertility. The complexity of the immune response to *Chlamydia* infection has hindered the development of preventions and/or treatments that therapeutically target the progression of infection-induced pathology. Therefore, research is needed to fully understand the factors that mediate clearance of infection *versus* those that induce pathology in the FRT. Such studies may inform novel targets for the development of novel therapies.

MCs have been shown to play roles in the pathogenesis of infections though mechanisms involving direct killing of pathogens, the quick release of preformed and neo-formed mediators, and the *de novo* synthesis of cytokines and chemokines, which modulate immune responses and cell recruitment to the infection site. Moreover, MCs are widespread in the FRT, where evidence suggests that they mediate physiological processes such as menstruation, implantation and labour. They have also been shown to protect against group B *Streptococcus* infection in the FRT by mediating immune responses and preventing bacterial attachment. A recently available Master thesis also reveals that MCs mediate immune responses to *Chlamydia* FRT infections and suggest a detrimental role in the development of *Chlamydia*-induced pathology.

The objective of my PhD studies is to characterise the roles of MCs, MC degranulation and MC proteases during *Chlamydia* FRT infection and on associated *Chlamydia*-induced pathology.

In **Chapter 2**, the effects of female sex hormones and *Chlamydia* infection on the number and degranulation of uterine MCs will be investigated. Using MC-deficient mice (*Cpa3-Cre; Mcl-1*$^{fl/fl}$), the roles of MCs in mediating clearance/susceptibility to *Chlamydia* infection and development of *Chlamydia*-associated pathology will be examined. The number of innate and adaptive immune cells, as well as the gene expression of key mediators of *Chlamydia* immune responses, will be evaluated to determine the involvement of MCs in the induction of immune

cell infiltration and in the development of inflammatory responses during *Chlamydia* infection. Moreover, by using intravaginal treatments with the MC stabiliser, cromolyn, the effects of MC degranulation on clearance/susceptibility to *Chlamydia* infection and on the development of *Chlamydia*-associated pathology will be examined.

In **Chapter 3**, the effects of female sex hormones and *Chlamydia* infection on the expression of the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, in the FRT will be investigated. Moreover, the roles of the MC mediators that are normally bound to heparin in the secretory granules of MCs, including histamine and the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, in mediating clearance of *Chlamydia* infection and development of associated pathology will be assessed by using Ndst2-deficient mice. The number of innate and adaptive immune cells, as well as the gene expression of key mediators of *Chlamydia* immune responses, will be evaluated to determine the involvement of MCs in the induction of immune cell infiltration and in the development of inflammatory responses during *Chlamydia* infection. Furthermore, the individual roles of the MC proteases, mMCP5/Cpa3, mMP6 and mMCP7 in mediating clearance of *Chlamydia* infection and development of associated pathology will be assessed by using mMCP5-deficient mice, mMCP6-deficient mice and mMCP6-deficient/mMCP7-sufficient mice.

In **Chapter 4**, the effects of female sex hormones and *Chlamydia* infection on the expression of the membrane-anchored tryptase Prss31 in the FRT will be investigated and an attempt at identifying its cellular source will be made. The roles of Prss31 in mediating clearance of *Chlamydia* infection and development of associated pathology will be assessed by using Prss31-deficient mice and intravaginal treatments with the recombinant Prss31 protein. The number of innate and adaptive immune cells, as well as the gene expression of key mediators of *Chlamydia* immune responses, will be evaluated to determine the involvement of MCs in the induction of immune cell infiltration and in the development of inflammatory responses during *Chlamydia* infection.

Chapter two: The role of mast cells and mast cell degranulation during *Chlamydia* female reproductive tract infection

2.1. Abstract

Chlamydia trachomatis is the most common bacterial STI worldwide. Infections can result in a range of severe pathologies such as pelvic inflammatory disease and tubal factor infertility. To date, no vaccines have become available and no therapeutic strategies exist for the treatment and/or prevention of pathology in those with infection-induced *sequelae*. A greater understanding of the complex mechanisms that underpin the clearance of *Chlamydia* infection *versus* those that result in the development of infection-associated pathology are required to inform such therapies. MCs are tissue resident immune cells characterised by their granules that contain a wide variety of potent physiological response mediators. Many of these mediators play important roles in orchestrating immune responses to bacterial infections, but their role in STIs in the FRT remain largely unknown. In this chapter, I will outline my studies investigating the general roles of MCs during *Chlamydia* FRT infection.

I show that MCs are widespread in the FRT of WT C57BL/6 mice and their number and expression of MC proteases appears to be regulated by female sex hormones. I show that MC deficiency is protective against *Chlamydia*-induced pathology in the oviducts. Using flow cytometry, I show that MC-deficient mice have reduced inflammatory monocytes and macrophages, neutrophils and eosinophils in their uterine tissues during *Chlamydia* infection. In addition, MC-deficient mice have reduced B cells, γδ T cells and eosinophils and an increase in inflammatory monocytes and macrophages in their bone marrow compared to WT mice during infection. Whilst the expression of many pro-inflammatory factors remains unchanged, the expression of the neutrophil chemoattractant, CXCL15, is decreased in MC-deficient mice. Therefore, my data suggest that MCs might participate in the induction of pro-inflammatory

responses following *Chlamydia* infection leading to excessive infiltration of innate immune cells that cause immunopathology.

Daily intravaginal treatments with the MC stabiliser cromolyn was protective against the early stages, but not the later stages, of *Chlamydia* infection. Moreover, cromolyn treatment in the early stages of infection offered partial protection against pathology at later stages. Together, my findings suggest that MC degranulation during the early stages of *Chlamydia* infection may be detrimental to infection and infection-induced pathology.

Taken together, these data suggest that MCs and MC degranulation play important roles during *Chlamydia* infection and in the progression of infection-induced pathology. Importantly, the fact that cromolyn treatment did not mimic results obtained with MC-deficient mice indicates a possible differential role for MC degranulation *versus de novo* synthesis of MC cytokines and chemokines and/or differential effects of different MC proteases following degranulation, thus highlighting the importance of investigating the roles of individual MC mediators, including MC proteases, during *Chlamydia* infection and infection-induced pathology.

2.2. Introduction

Chlamydia trachomatis, a Gram negative obligate intracellular bacteria, is the most common bacterial sexually transmitted pathogen, with estimated 130 million new infections occurring every year (51, 369). The detection and the treatment of *Chlamydia* FRT infections with antibiotics are relatively straight forward and effective (11, 14). However, a large proportion of women, estimated to be up to 90%, develop asymptomatic infections. This means that a high number of women go undiagnosed and, therefore, untreated. In these cases, infections can persist in, and cause immunopathological damages to, the fragile tissues of the upper FRT (11, 21). In some cases, the infection, as well as the immune responses it triggers, cause severe and irreversible *sequelae*, including pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility (23, 42). In the FRT mucosa, immune responses are highly complex owing to the fact that they need to simultaneously balance between protecting against infection and tolerating the presence of genetically dissimilar sperm and embryos (370). A better understanding of the immune responses that mediate the clearance of *Chlamydia* infection *versus* those that underpin pathology are necessary in order to develop new strategies to prevent/treat *Chlamydia* FRT infection and/or infection-induced pathology.

Different animal models that replicate the course of an infection in women have been employed to help understand the immunobiology and pathophysiology of infection. The mouse models of *Chlamydia* FRT infection rely on inoculation of the mouse pathogen *Chlamydia muridarum* or the human pathogen *Chlamydia trachomatis* (140, 162, 371, 372). *Chlamydia muridarum* readily ascends into the upper murine FRT tissues after a single intravaginal inoculation and causes scarring and swelling of the oviducts, whereas *Chlamydia trachomatis* is poorly infectious in mice and does not readily ascend the FRT following intravaginal inoculation. Therefore, *Chlamydia muridarum* is often considered as the most representative pathogen to use in mice to model *Chlamydia* infections in women in order to help to understand the roles of specific immune mediators in both clearance/susceptibility to *Chlamydia* infection and development of upper FRT pathology (101).

Type 1 immune responses have been shown to play an important role in the clearance of infection, with $CD4^+$ Th1 cells and NK cells having a predominant roles through their production of IFNγ (122, 162, 163, 173). Although macrophages and neutrophils have sometime been associated with protection against infection, excessive infiltration of these cells have been shown to contribute to the development of *Chlamydia*-associated pathology in the oviducts, through their production of detrimental immune mediators including MMP9 and IL-1β (132-135, 149). These examples demonstrate the complexity of nature of the host-pathogen relationship that occurs during *Chlamydia* infection with some of the protective immune responses involved in clearance of infection also being associated with development of pathology. Therefore, it is crucial to improve the comprehension of the immune responses involved during *Chlamydia* infection and, importantly, to discover the roles of novel mediators of immune responses *Chlamydia* infection that could be targeted to prevent/treat infection and/or infection-induced pathology (42).

MCs are large, tissue-resident immune cells of hematopoietic origin. Once in their target tissues, MCs finish their maturation under the direction of local stimuli that, depending on the tissue type and the organ, gives rise to a large diversity of MC phenotypes (193). MCs are widely distributed around the body, especially in high-vascularised tissues and at sites directly exposed to the external environment (191). They are characterised by their numerous secretory granules that contain a wide variety of preformed mediators including proteoglycans, histamine, serotonin, cytokines and MC proteases (190). In tissues, MCs can be activated by a large variety of mechanisms including the crosslinking of antigen-specific IgE antibodies bound to the FcεRI receptor, the recognition of PAMPS by PRRs, including TLRs and NODs, as well as the recognition of exogenous peptides, such as bacterial toxins, and endogenous peptides, such as the complement components C3a and C5a (191, 373). Within a matter of seconds following activation, MCs degranulate, releasing all the preformed mediators contained within their granules. They also release newly synthesized lipid mediators such as prostaglandin D_2 and leukotriene C_4 (373). At later stages, MCs are capable of acting via a more specific response through the *de novo* synthesis and release of specific cytokines and chemokines including IL-1α, IL-1β, IL-4, IL-5 IL-6, IL-13, TNFα, GM-CSF and VEGF (374). The mediators released by MCs

following activation have potent and diverse effects in physiology and pathophysiology. MCs are evolutionary conserved, suggesting primordial roles for these cells beyond their commonly associated detrimental roles in hypersensitivity disorders such as anaphylaxis, allergy or asthma (375). Indeed, MCs have more recently been associated with inducing protective immune responses during bacterial infections, including during *Klebsiella pneumoniae*, group A and group B *Streptococcus*, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Mycoplasma pneumoniae* infections (253, 255, 256, 273, 276, 291, 367, 376-379). MCs can directly kill bacteria through secretion of a variety of antimicrobial mediators, phagocytosis, ROS-mediated oxidative burst as well as generation of extracellular nets (291-294). Moreover, MCs are able to indirectly promote clearance of infection through their capacity to induce the recruitment of neutrophils and other immune cells (253, 255, 256, 273, 276, 376-379). Interestingly, MCs have been shown to play a detrimental role during *Chlamydia pneumoniae* lung infection, during which MC-deficient mice and mice treated with the MC stabiliser cromolyn have been shown to have reduced bacterial burden, mortality and immune cell infiltration in their airways (262).

In the FRT, MCs are present in relatively high numbers in many species, including humans (318, 322, 338) and mice (329, 367). The female sex hormones, progesterone and oestrogen, have been shown to alter MC migration, activation and degranulation, which is consistent with a role for MCs in mediating physiological responses in the FRT (329, 332, 333, 335, 339, 380, 381). MCs mediate the secretion and activation of MMPs as well as the induction of ischaemic spasm of spiral arteries associated with the shedding of the endometrium and subsequent tissue remodelling during menstruation (344, 380, 382). Moreover, MCs are considered to play various roles during pregnancy, specifically in mediating implantation, placentation, foetal growth, cervical ripening and the induction of labour (329, 330, 332, 339, 347, 350, 351, 383-385). MCs have also been associated with FRT pathology, with increased numbers of MCs associated with ovarian, uterine and cervical cancers (386), vulvar vestibulitis (358) and endometriosis (356). Therapeutically targeting MCs with drugs such as cromolyn or antihistamines has been shown to be effective for the treatment of clinical cases of recalcitrant idiopathic vulvar vestibulitis, chronic

dyspareunia, vaginitis and dysfunctional uterine bleeding (359, 387). In the FRT, the roles of MCs in mediating immune responses to infection remain poorly understood. An increase in the number of MCs in the cervical epithelium of women infected with HIV has been shown (360). Furthermore, gut MCs have been postulated to participate in HIV dissemination through their ability to present virions to $CD4^+$ T cells (257). *In vitro* studies reveal that MCs may be important mediators of the immune responses required for the control of *Trichomonas vaginalis* infection. Indeed, *Trichomonas* and *Trichomonas*-infected epithelial cells appear to induce the recruitment and activation of MCs, their release of histamine, TNFα and IL-8 and induction neutrophils recruitment (363, 364). Moreover, MCs have been shown to have a protective role during group B *Streptococcus* infections, with MC-deficient mice displaying increased bacterial burden and decreased immune cell infiltration following a systemic intraperitoneal infection and augmented bacterial persistence in their vagina and uterus following vaginal infection (367). A recent study suggests that MCs may play a role in the development of pathology in the upper FRT following *Chlamydia* infection, but the mechanisms involved remain unclear (368).

Given that MCs have been shown to mediate immune response to bacteria, but that their role during *Chlamydia* FRT infections remain to be fully characterised, this chapter will investigate the role(s) of MCs and MC degranulation during *Chlamydia* FRT infections. For this, the number of uterine MCs will be evaluated according to female sex hormones and *Chlamydia* infection. MC-deficient mice will be subjected to a mouse model of *Chlamydia muridarum* and the *Chlamydia* burden in the vagina and uterus, the *Chlamydia*-induced pathology in the oviducts and the *Chlamydia*-induced immune responses in the uterus will be characterised. Moreover, the effects of MC degranulation on clearance of infection and associated pathology will be assessed by using intravaginal treatments with the MC stabiliser cromolyn during *Chlamydia muridarum* infection.

2.3. Materials and methods

2.3.1. Ethics statement

All the animals and procedures used in this study were approved by the Animal Care and Ethics Committee at the University of Newcastle (Callaghan, NSW, Australia).

2.3.2. Mouse models used to investigate the roles of MC and MC degranulation during *Chlamydia* FRT infection

WT C57BL/6 female mice were obtained from the Australia BioResources (ABR) facility (Moss Vale, NSW, Australia) in order to conduct studies to evaluate the effects of progesterone and oestrogen treatments and the effects of *Chlamydia muridarum* FRT infection on MC number and phenotype (**Table 2.1**).

MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) female mice and their appropriate controls (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) were obtained from the University of South Australia (UniSA) animal facility (Adelaide, SA, Australia), thanks to collaborator Associate Professor Michele Grimbaldeston at the Centre for Cancer Biology, to assess the effect of MC deficiency on *Chlamydia muridarum* FRT infection (**Table 2.1**) (237). These mice were produced by targeted mutation of the intracellular anti-apoptotic factor myeloid cell leukemia sequence-1 (Mcl-1) that resulted in MC deficiency ranging from 92% to 100% (237).

WT C57BL/6 female mice were obtained from the Central Animal House (CAH) facility of The University of Newcastle (Callaghan, NSW, Australia) in order to conduct studies using the MC stabiliser cromolyn to assess the effect of MC degranulation on *Chlamydia muridarum* FRT infection (**Table 2.1**) (262).

Upon delivery, mice were housed in specific pathogen free (SPF) conditions in the Bioresources facility at the Hunter Medical Research Institute (HMRI; New Lambton Heights, NSW, Australia), with *ad libitum* access to food and water under a 12 hours light-dark cycle.

Transgenic mouse line	Mouse background	Facility of origin	Description of the genetic modification
WT	C57BL/6	ABR	-
WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$)	C57BL/6	UniSA	-
Cpa3-Cre;Mcl-1$^{fl/fl}$	C57BL/6	UniSA	Deficiency in the intracellular anti-apoptotic factor myeloid cell leukemia-1 (Mcl-1) required for MC survival resulting in MC deficiency
WT	C57BL/6	CAH	-

Table 2.1 - Description of the mice used to investigate the roles of mast cell during *Chlamydia* infection. *WT: wild type, MC: mast cell, ABR: Australia BioResources, UniSA: University of South Australia, Cpa3: carboxypeptidase A3, CAH: Central Animal House.*

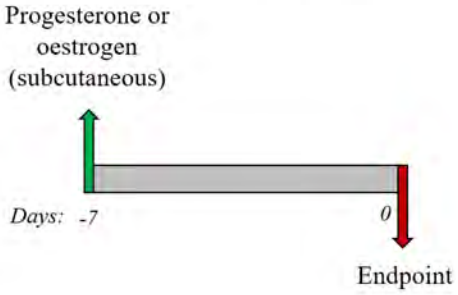
In order to assess the effects of female sex hormones on MC number and degranulation in the FRT, WT mice (10-11 weeks old) were subcutaneously administrated with either medroxyprogesterone acetate (Depo-Provera; Pfizer, NY, USA; 2.5mg per mouse in 200µL saline) or 17β-oestradiol (Sigma-Aldrich, Castle Hill, NSW, Australia; 0.5mg per mouse in 200µL corn oil) under isofluorane gas anaesthesia to synchronise their oestrus cycle and induce diestrus or oestrus, respectively (332, 388). Seven days later, mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb; Virbac, Milperra, NSW, Australia) and FRT tissues were collected for analyses (**Figure 2.1 A**).

In order to assess the effects of *Chlamydia* infection on MC number and degranulation in the FRT (**Figure 2.1 B**) and to characterise the role of MCs during *Chlamydia* infection (**Figure 2.1 C**), WT C57BL/6 mice, MC-deficient mice and their WT controls (9-18 weeks old) were subcutaneously administrated with medroxyprogesterone acetate (Depo-Provera; 2.5mg per mouse in 200µL saline) under isoflurane gas anaesthesia in order to synchronise their oestrous cycles and make them susceptible to *Chlamydia* FRT infection. Seven days later, mice were infected intravaginally with $5x10^4$ inclusion forming units (ifu) of *Chlamydia muridarum* (Cmu; ATCC VR-123) in 10µl sucrose-phosphate-glutamate buffer (SPG; filter sterilised solution of 10mM sodium phosphate, 250mM sucrose and 5mM L-glutamic acid, [pH 7.2]) or sham-infected with 10µl SPG alone under ketamine:xylazine anaesthesia (Ilium Ketamil® and Ilium Xylazil-20®; Troy Laboratories, Glendenning, NSW, Australia; 80mg/kg:5mg/kg per mouse

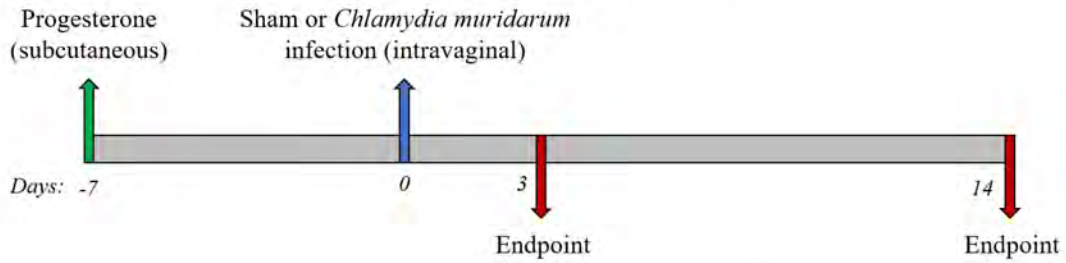
administrated intraperitoneally) (177, 389). For infections in the MC-deficient mice and their associated WT controls, ketamine:xylazine anaesthesia was replaced by ketamine:medetomidine anaesthesia (with atipamezole reversal; Ilium Ketamil®, Ilium Medetomidine® and Ilium Atipamezole®; 75mg/kg:1mg/kg per mouse administrated intraperitoneally, followed by subcutaneous injection of 1m/kg of atipamezole as reversal agent) in order to conform with best practices*. At 3dpi and/or 14dpi, mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb) and FRT tissues and/or bone marrow were collected for analyses.

* All the other infections were approved and/or conducted prior to an update in the Animal Care and Ethics Committee guidelines for best practice for anaesthesia.

(A) Effect of female sex hormones on MC numbers and degranulation (WT mice)



(B) Effect of *Chlamydia* infection on MC numbers and degranulation (WT mice)



(C) Role of MCs during *Chlamydia* infection (*Cpa3-Cre;Mcl-1$^{WT/WT}$* and *Cpa3-Cre;Mcl-1$^{fl/fl}$* mice)

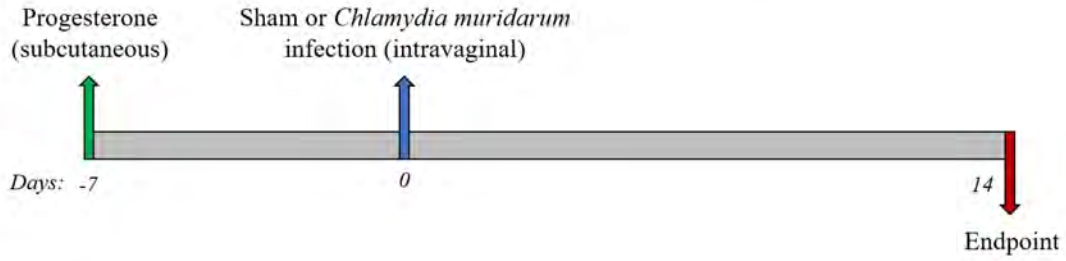
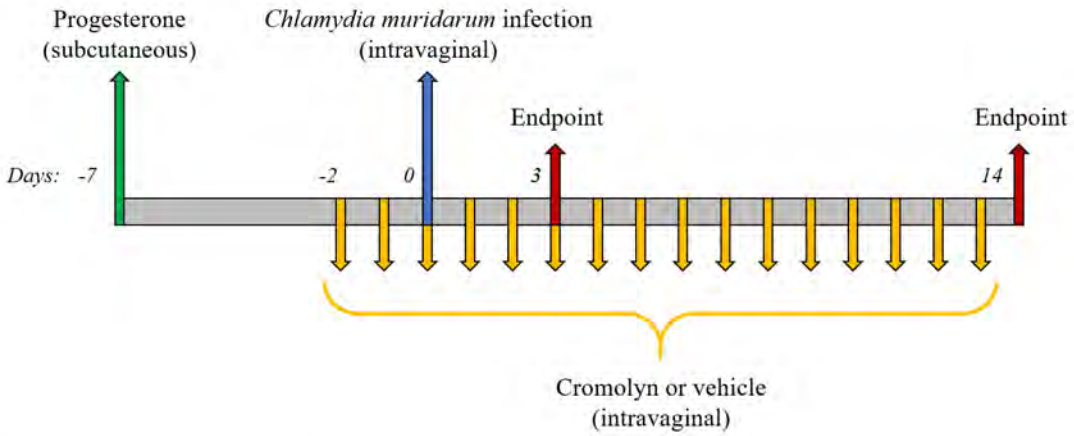


Figure 2.1 - Mouse model of hormonal treatment and *Chlamydia muridarum* female reproductive tract (FRT) infection. (A) Progesterone or oestradiol was administrated subcutaneously to wild type (WT) female C57BL/6 mice. Seven days later, FRT tissues were collected for subsequent analyses of mast cell (MC) number and degranulation. **(B)** WT C57BL/6 female mice were administrated progesterone subcutaneously seven days prior to intravaginal infection with $5x10^4$ inclusion forming units (ifu) of *Chlamydia muridarum* or sham-infection. Tissues were collected at 3 and 14 days post infection (dpi) for subsequent the assessment of MC number and degranulation. **(C)** MC-deficient (*Cpa3-Cre;Mcl-1$^{fl/fl}$*) female mice and their associated WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were administrated progesterone subcutaneously seven days prior to intravaginal infection with $5x10^4$ ifu of *Chlamydia muridarum*. FRT tissues and bone marrow were collected at 14dpi for subsequent assessment of the roles of MCs during *Chlamydia* infection.

2.3.3. Mouse model of *in vivo* inhibition of MC degranulation during *Chlamydia* FRT infection

To characterise the effects of MC degranulation during *Chlamydia* FRT infection, WT C57BL/6 female mice (6-9 weeks old) were subjected to the previously described murine model of *Chlamydia* FRT infection and, in addition, treated daily intravaginally with 5mg/kg of the MC stabilizer cromolyn (Sigma-Aldrich), in 20μL sterile saline/phosphate-buffered saline (PBS) or with sterile saline/PBS vehicle alone, under isoflurane gas anaesthesia. Two regimen of treatments were performed. Firstly, throughout treatments, starting 2 days before infection (dbi) and performed daily until 3dpi and 14dpi to analyse the effects of MC degranulation throughout *Chlamydia* infection (**Figure 2.2 A**). Then, early treatments were performed, starting 2dbi and performed daily until 3dpi, with the endpoint at 14dpi to analyse the effects of MC degranulation specifically during the early stages of *Chlamydia* infection (**Figure 2.2 B**). At all endpoints, mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb) and FRT tissues were collected for analyses.

(A) Role of MC degranulation during *Chlamydia* infection (WT mice)



(B) Role of MC degranulation during the early stages of *Chlamydia* infection (WT mice)

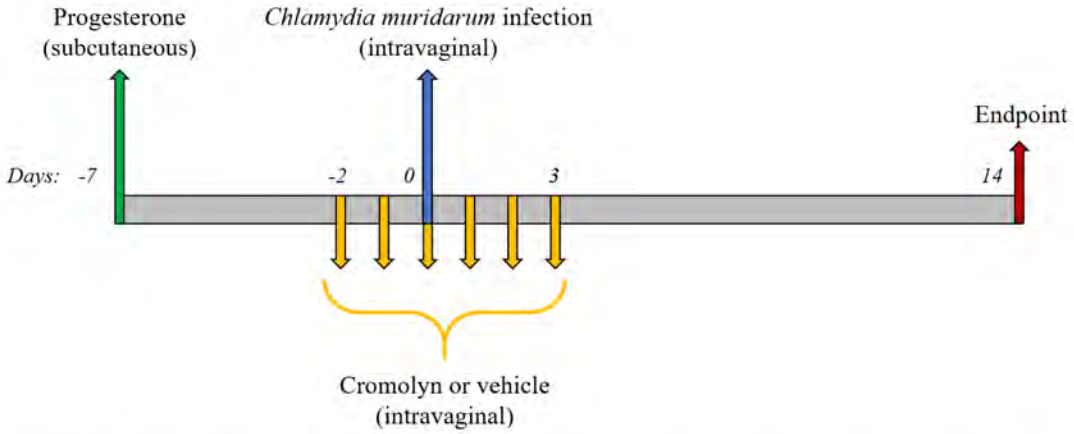


Figure 2.2 - *In vivo* inhibition of mast cell (MC) degranulation during a mouse model of *Chlamydia muridarum* female reproductive tract (FRT) infection. Wild type (WT) female C57BL/6 mice were administrated progesterone subcutaneously seven days prior intravaginal infection with $5x10^4$ inclusion forming units of *Chlamydia muridarum*. Mice were administered with 5mg/kg cromolyn or vehicle control intravaginally, **(A)** daily from 2 days before infection (dbi), with FRT tissues collected at 3 and 14 days post infection (dpi) for subsequent assessment of the effects of MC degranulation throughout *Chlamydia* infection; or **(B)** daily from 2dbi to 3dpi with FRT tissues collected at 14dpi for subsequent assessment of the effects of MC degranulation specifically during the early stages of *Chlamydia* infection.

2.3.4. Assessment of MC number and degranulation in the uterus

Mice were sacrificed as described above, and the right side of the FRT (right ovary, right oviduct, right uterine horn) as well as cervix and vagina were isolated and fixed in formalin for histological analyses to identify MCs in the different FRT tissues and investigate the changes in the number of uterine MCs according to the hormonal and infection status of the mice. Samples were sent to the John Curtin School of Medical Research (Australian National University, ACT, Australia) or to the HMRI Core Histology Facility (New Lambton Heights, NSW, Australia) for processing. Briefly, tissues were embedded in paraffin and cut in longitudinal sections of 5µm thickness. Sections were deparaffinised using xylene, rehydrated using absolute alcohol gradients and stained with either toluidine blue or alcian blue/safranin to visualise MCs. Briefly, for toluidine blue staining, sections of the FRT were incubated for 5 minutes in a 0.25% toluidine blue solution, washed and stabilised using 0.1% acetic acid. For alcian blue/safranin, sections of the FRT were incubated 10 minutes with alcian blue, then 10 minutes with 0.7N hydrochloric acid before counterstaining with safranin O for 2 minutes.

Slides were visualised using a Zeiss Axio Imager M2 microscope (Carl Zeiss Microscopy, North Ryde, NSW Australia). The alcian blue/safranin stain was used to evaluate the heterogeneity of MC phenotype with red MCs being heparin positive, blue MCs being chondroitin sulfate positive and violet MCs representing an intermediate population (390). The toluidine blue stain, which reveal MC granules as purple, was used to enumerate MCs and observe degranulation. Briefly, the numbers of MCs were evaluated at 20X magnification on at least 15 random fields of view, taken over the full length of uterine tissues. The myometrium region was selected as a representation of the uterus because of the large number of MCs in this region (while most samples did not contain MCs in the endometrium). On each field of view, the size of the myometrium was evaluated using the ZEN 2.3 software program (Blue edition, Carl Zeiss Microscopy), to obtain the total number of MCs per mm^2 of tissue. In addition, the degranulation status of each MC was evaluated by identification of purple granule staining surrounding the MC, to obtain a percentage of degranulated MCs in the uterus.

2.3.5. RNA extraction from FRT tissues

Mice were sacrificed as described above and the left uterine horns were harvested, snap frozen and stored at -80°C. Total RNA extractions were performed using the TRIzol® method according to the manufacturer's instructions. Briefly, tissues were homogenised in TRIzol® (Sigma-Aldrich) using a Tissue-Tearor (BioSpec Products, Bartlesville, OK, USA). Samples were incubated for 10 minutes at room temperature in chloroform (Sigma-Aldrich) before centrifugation. The top aqueous phase containing the nucleic acid was transferred to fresh tubes and supplemented with isopropyl alcohol (Sigma-Aldrich) in order to precipitate the RNAs. After ethanol washes, RNAs were reconstituted in nuclease-free distilled water (Invitrogen by Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) and stored at -80°C.

2.3.6. Reverse transcription of RNA from FRT tissues

The purity, quality and concentration of the RNA samples were measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The RNA samples were then diluted to 1µg RNA per sample. Treatments with deoxyribonuclease (DNase) I (Sigma-Aldrich) were performed for 15 minutes at room temperature to remove DNA contaminants. The DNase I was then chemically inactivated by incubation with DNase I STOP solution (Sigma-Aldrich) for 10 minutes at 65°C in a T100™ Thermal Cycler (Bio-Rad, Gladesville, NSW, Australia).

The RNA samples were then reversed transcribed into complementary DNA (cDNA) using M-MLV reverse transcriptase enzyme (Life Technologies, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, RNA samples were incubated with 1µL of 50ng/µL random hexamer primers (Bioline, Alexandria, NSW, Australia) and 1µL of 10mM deoxyribonucleotide triphosphates mix (dNTPs; Bioline) at 65°C for 5 minutes in a T100™ Thermal Cycler (Bio-Rad). Samples were then incubated with 4µL of 5X First-Strand reaction buffer (Life Technologies; [250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM MgCl]), 1µL of 100mM DL-dithiothreitol (DTT; Life Technologies) and 1µL of nuclease-free distilled water (Invitrogen) at 37°C for 2 minutes. 200 units of M-MLV reverse transcriptase were added to the reaction before incubation at 25°C for 10 minutes, 37°C for 50 minutes and 70°C for 15 minutes.

The newly transcribed cDNA was resuspended in nuclease-free distilled water (Invitrogen) to a volume of 100µL and stored at -20°C until real-time quantitative polymerase chain reaction (qPCR) analyses.

2.3.7. DNA extraction from vaginal lavage

In order to evaluate the *Chlamydia muridarum* burden in the lower reproductive tract, vaginal lavages were collected at all endpoints by washing the vagina twice with 60µL of sterile SPG. DNA was extracted from the lavage samples using the GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies Sdn. Bhd., Subang Jaya, Selangor Darul Ehsan, Malaysia) according to the manufacturer's instruction. Briefly, the thawed vaginal lavage fluids were centrifuged, resuspended in buffer R1 and treated with lysozyme for 20 minutes at 37°C. After centrifugation, the protein were denaturised for 20 minutes at 65°C in buffer R2 and proteinase K, before addition of buffer BG and further incubation at 65°C for 10 minutes. After addition of ethanol, the samples were transferred to columns and washed. The DNA was then collected in elution buffer and stored at -20°C until qPCR analyses.

2.3.8. Real-time quantitative polymerase chain reaction

2.3.8.1. RNA expression analyses

For RNA expression analyses, cDNA samples were supplemented with custom designed primers (**Appendix, Table 6.1**; IDT, Coralville, IA, USA and Sigma-Aldrich; 5µM), iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and nuclease-free distilled water (Invitrogen). They were put through cycling conditions of 50°C for 2 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds and 55/65°C (as determined by gradient testing for each primer pair) for 1 minute in a Mastercycler® ep Realplex2 Real-time PCR System (Eppendorf, North Ryde, NSW, Australia), a CFX96 or a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Dissociation analyses were performed to verify the specificity of each primer pairs.

The relative expression of each target gene was then determined by comparison to the mouse housekeeping reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) to verify

the efficiency and the integrity of the reverse transcription, normalize the data and control for variations in amount of RNA.

To estimate the *Chlamydia* burden in the left uterine horn of mice, primers targeting the *Chlamydia muridarum* 16S ribosomal RNA (rRNA; IDT; **Appendix, Table 6.1)** were used to evaluate the relative expression of *Chlamydia muridarum* 16S rRNA in each uterine horn. The 16S rRNA encodes for the component of the 30S subunit of the bacterial ribosome and is commonly used to identify bacteria and to study bacterial phylogeny because of its slow rate of evolution and of the presence of conserved and highly variable regions (46, 177, 389). This measurement will allow to measure the levels of ascending infection as, following intravaginal infection, *Chlamydia muridarum* is known to breach through the cervical barrier of mice to reach the upper FRT tissues where it can cause pathology.

The expression profile of various genes coding for cytokines, chemokines and immune mediators associated with susceptibility/clearance of *Chlamydia* infection and/or associated pathology and/or host immune responses (Tbet, GATA Binding Protein [GATA]3, RAR-related orphan receptor [ROR]γt and forkhead box P3 [FOXP3], IFNγ, TNFα, IL-1β, IL-4rα, IL-6, IL-10, IL-12p40, IL-13, IL-17, CXCL1, CXCL15, chemokine (C-X-C motif) receptor [CXCR]2, GM-CSF, MMP9, TLR3 and PAR2) were determined using specific primers (IDT; **Appendix, Table 6.1**) to investigate the changes in inflammation status in the *Chlamydia*-infected uterus of MC-deficient mice and WT controls.

The expression of the NK cell specific receptor, natural cytotoxicity triggering receptor (NCR)1, was measured using specific primers (IDT; **Appendix, Table 6.1**) to investigate if MCs mediate NK cell proliferation during *Chlamydia* infection (391).

2.3.8.2. DNA expression analyses

In order to quantify the bacterial burden in vaginal lavage fluid primers targeting the gene encoding for *Chlamydia* MOMP (IDT; **Appendix, Table 6.1**), a region of the *Chlamydia* genomic DNA, were used to estimate the *Chlamydia* burden in DNA extracted from each vaginal lavage. The presence of the *Chlamydia* MOMP gene in each sample was then compared to DNA standards

extracted from known concentrations of *Chlamydia muridarum* to evaluate the number of *Chlamydia* in each vaginal lavage fluid sample.

2.3.9. Evaluation of *Chlamydia*-induced pathology

At 14dpi, *Chlamydia*-infected mice have developed tubal pathology that can be observed in the form of hydrosalpinx. The levels of hydrosalpinx in mice were evaluated by measuring the size of their oviducts in two planes (up/down and left/right) using a digital calliper (Sontax, Canning Vale, WA, Australia). These two measurements were then multiplied to estimate the cross sectional area of each oviduct as a representation of the level of oviduct swelling caused by *Chlamydia*-induced hydrosalpinx.

2.3.10. Characterisation of immune cell numbers in the uterus and bone marrow during *Chlamydia* FRT infection using flow cytometry

Flow cytometry was used to characterise the immune cell numbers in the uterine tissues and bone marrow of *Chlamydia*-infected MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) mice compared to their WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls. Because of the difficulty in processing uterine tissues and the low number of cells available, uteri from several mice had to be pooled to gain sufficient numbers of cells for staining and subsequent analyses.

Briefly, mice were sacrificed as described above and uterine horns were isolated, cut in four pieces and dissociated in C tubes (Miltenyi Biotech, Macquarie Park, NSW, Australia) containing 5mL of 1X 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid (HEPES) buffer (10mM HEPES-NaOH [pH7.4], 150mM NaCl, 5mM KCI, 1mM $MgCl_2$ and 1.8mM $CaCl_2$) using a gentleMACS™ Dissociator (Miltenyi Biotech). After 30 minutes incubation with 40U/mL of DNase I and 2mg/mL of collagenase D (Roche, Dee Why, NSW, Australia) at 37°C and further dissociation in the gentleMACS™ Dissociator, samples were passed through a 70µm nylon cell strainer to remove debris and obtain a single cell suspension containing all uterine cells. The cells were then incubated in red blood cell lysis buffer (155mM NH_4Cl, 12mM $NaHCO_3$ and 0.1mM ethylenediaminetetraacetic acid [EDTA], [pH 7.35]) at 4°C for 5 minutes. The number of living

cells was enumerated using trypan blue exclusion and a Countess™ automated cell counter (Invitrogen).

Bone marrow was isolated from the left femur each mouse. After centrifugation, the bone marrow cells were incubated in red blood cell lysis buffer at 4°C for 5 minutes. The number of living cells was enumerated using trypan blue exclusion.

The single cell suspensions from uterine tissues and bone marrow were blocked at 4°C for 15 minutes with 10ng/mL of anti-mouse CD16/32 (InVivoMAb; BioXCell, Lebanon, NH, USA) in 100µL flow cytometry and cell sorting (FACS) buffer (2% foetal calf serum [FCS] and 2mM EDTA in PBS) to prevent nonspecific staining of IgG to the FcγIII (CD16) and FcγII (CD32) receptors and allow for antigen specific staining.

For staining, the single cell suspensions were incubated at 4°C for 20 minutes with a cocktail of antibodies specific for surface markers conjugated with either a fluorochrome or biotin (**Appendix, Tables 6.2, 6.3 and 6.4**). For single cell suspensions from uterine tissues, the single cell suspensions were incubated at 4°C for 20 minutes with a streptavidin antibody conjugated with a BV605 fluorochrome to detect the antibodies conjugated to biotin (**Appendix, Table 6.2**).

As a negative control, a portion of the single cell suspensions were handled as above without addition of antibodies to generate an unstained control, in order to set the voltages on the BD LSRFortessa™ X-20 cell analyzer (BD Biosciences) and gate the different cell populations in subsequent analyses. Moreover, OneComp eBeads™ compensation beads (Invitrogen) were used to make single fluorochrome controls for each antibody used in the panel, with 3 drops/mL of compensation beads stained with 10µL/mL of a single antibody, in order to set the voltages and the compensation on the BD LSRFortessa™ X-20 cell analyzer for removal of signal overlap between fluorochromes.

Finally, the stained cells were fixed overnight at 4°C in 4% paraformaldehyde (PFA). The fluorescence of each sample was measured using a BD LSRFortessa™ X-20 cell analyzer and analysed with the BD FACSDiva™ Software (BD Biosciences) to determine the percentages and total numbers of innate and adaptive immune cells in the uterine tissues of *Chlamydia*-infected

MC-deficient mice and their WT controls, with the gating strategy based on the specific forward scatter, side scatter and surface markers of each immune cell type (**Table 2.2** and **Appendix, Figure 6.1**). Similarly, the percentages and total numbers of myeloid and lymphoid immune cells in the bone marrow of *Chlamydia*-infected MC-deficient mice and their WT controls, with the gating strategy based on the specific forward scatter, side scatter and surface markers of each immune cell type (**Table 2.2** and **Appendix, Figures 6.2 and 6.3**).

Stains	Cell types	Surface marker expressions
Uterine stain	Immune cells	$CD45^+$
	Neutrophils	$CD45^+$ $CD11b^+$ $Ly6G^+$
	Eosinophils	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^+$
	Myeloid dendritic cells	$CD45^+$ $CD11b^+$ $CD11c^+$ $Ly6C^-$ $PDCA^-$
	Plasmacytoid dendritic cells	$CD45^+$, $CD11b^-$ $CD11c^+$ $Ly6C^+$, $PDCA^+$
	Resident monocytes/macrophages	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^-$ $F4/80^+$ $Ly6C^-$
	Infiltrating monocytes/macrophages	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^-$ $F4/80^+$ $Ly6C^+$
	T cells	$CD45^+$ $CD3^+$
	$CD4^+$ T cells	$CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$
	$CD8^+$ T cells	$CD45^+$ $CD3^+$ $CD4^-$ $CD8^+$
Bone marrow stain for myeloid cells	Immune cells	$CD45^+$
	Neutrophils	$CD45^+$ $CD11b^+$ $SiglecF^-$ $Ly6G^+$
	Eosinophils	$CD45^+$ $CD11b^+$ $SiglecF^+$
	Resident monocytes/macrophages	$CD45^+$ $CD11b^+$ $SiglecF^-$ $Ly6G^-$ $Ly6C^-$
	Infiltrating monocytes/macrophages	$CD45^+$ $CD11b^+$ $SiglecF^-$ $Ly6G^-$ $Ly6C^+$
Bone marrow stain for lymphoid cells	Immune cells	$CD45^+$
	NK cells	$CD45^+$ $CD3^-$ $NKp46^+$
	T cells	$CD45^+$ $CD3^+$
	$CD4^+$ T cells	$CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$
	$CD8^+$ T cells	$CD45^+$ $CD3^+$ $CD4^-$ $CD8^+$
	γδ T cells	$CD45^+$ $CD3^+$ γδTCR$^+$
	NKT cells	$CD45^+$ $CD3^+$ $NKp46^+$
	B cells	$CD45^+$ $CD3^-$ $NKp46^-$ $B220^+$

Table 2.2 –Surface markers used for identifying immune cells in the uterine tissue and bone marrow of *Chlamydia*-infected mice.

2.3.11. Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). All statistical tests were performed using the GraphPad Prism software (version 7.0; GraphPad Software, La Jolla, California). Grubbs outlier tests were realised followed by normality testing using the D'Agostino & Pearson normality test for analyses of 8 or more samples, or the KS normality test for analyses of less than 8 samples. Then, as appropriate, Student's t-tests or Mann-Whitney tests were performed, with a $p<0.05$ considered as statistically significant.

2.4. Results

2.4.1. MCs are widespread in the FRT where they form an heterogeneous population

In order to examine the tissue location and heterogeneity regrading proteoglycan content of MCs, FRT tissues were isolated from WT female mice, fixed in formalin and embedded in paraffin. Longitudinal sections of 5µm thickness were generated and stained with toluidine blue (**Figure 2.3 A-F**) or alcian blue/safranin (**Figure 2.3 G-M**).

Toluidine blue is a basic thiazine metachromatic dye with high affinity for negatively charged compounds, including serglycin proteoglycans (390). In all the FRT tissues, most MCs are observed to be filled with serglycin proteoglycan-containing granules as shown with strong violet staining in MCs (**Figure 2.3 E**). Moreover, some degranulated MCs are also present, as revealed by the presence of small granules stained violet in the extracellular tissue environment surrounding the MC (**Figure 2.3 F**).

In the ovary, MCs are mostly present in the middle section of the tissue (medulla), which is characterised by high vascularisation (**Figure 2.3 A-G**). MCs are also present in the oviduct, principally in the outer layers (*tunica muscularis* and *tunica serosa*) (**Figure 2.3 B-H**). In the uterus, MCs are present in highest concentration in the myometrium, the muscular portion of the uterus that is responsible for uterine contraction during labour. MCs can also be visible in the endometrium, but they are relatively rare, with fewto no MCs visualised in this part of the uterus (**Figure 2.3 C-I**). In the vagina, MCs are relatively numerous and are present in the stroma in close proximity to the vaginal epithelium (**Figure 2.3 D-J**).

Alcian blue/safranin staining also stains for the highly negative serglycin proteoglycans, but also allows for the discrimination between MCs containing chondroitin sulfate (positive for alcian blue staining), those containing heparin (positive for safranin staining) and those containing both chondroitin sulfate and heparin (double positive for alcian blue and safranin) (390). In the FRT, the three phenotypes of MC are present, which demonstrates that MCs form a heterogeneous population in the FRT (**Figure 2.3 K-M**). Globally, the more frequent MCs in the FRT are those

staining for alcian blue only and those that stain positive for both alcian blue and safranin, with MCs staining for safranin only being much rarer.

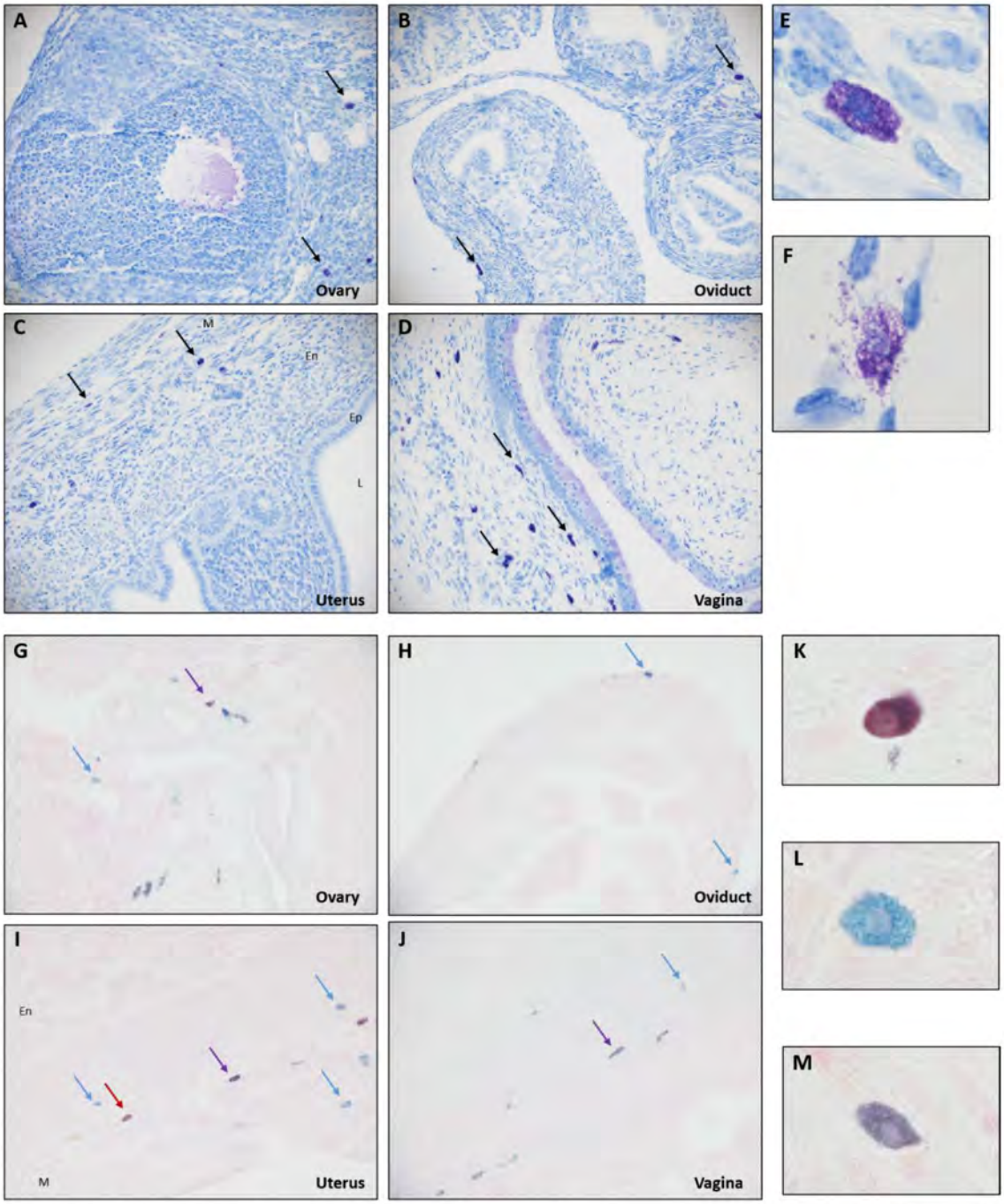


Figure 2.3 - Mast cells (MCs) are widespread in mouse female reproductive tract (FRT) tissues. The FRT tissues of C57BL/6 wild-type mice were fixed in formalin, embedded in paraffin and longitudinal sections of 5µm thickness were stained with **(A-F)** toluidine blue or **(G-M)** alcian blue/safranin. The sections were observed with a Zeiss Axio Imager M2 microscope (20X and 100X) and representative pictures of the **(A, G)** ovary, **(B, H)** oviduct, **(C, I)** uterus (*L:lumen, Ep: epithelium, En: endometrium, M: myometrium*) and **(D, V)** vagina were taken. Both **(E)** non-degranulated and **(F)** degranulated MCs are present. **(A-D)** Examples of MCs are indicated with black arrows. Three phenotypes are apparent with MCs **(K)** positive for safranin, **(L)** alcian blue and **(M)** double positive, with the alcian blue positive and the double positive MCs being the most common. **(G-J)** Examples of those different MC phenotypes in the FRT tissues are indicated with respectively red, blue and violet arrows.

2.4.3. Progesterone and oestrogen treatments alter the number of uterine MCs but not their degranulation, in the absence of infection

Several studies have investigated the variation in MC numbers and activation in different part of the FRT during the oestrous/menstrual cycle, however the conclusion of those studies were variable. In order to examine the effects of oestrous stage and female sex hormone treatments on MC numbers in the FRT, WT female mice were treated with progesterone (to induce diestrus) or oestrogen (to induce oestrus) (**Figure 2.1 A**). Seven days later, the FRT tissues were extracted, fixed in formalin, embedded in paraffin, sectioned and stained with toluidine blue. The number of MCs were counted in the myometrium region of at least 15 random fields of view pictures representative of the full length of the uterine tissues (20X magnification). The size of the myometrium was evaluated to obtain the total number of MC per mm^2 of tissue. Moreover, the degranulation status of each MC was evaluated to obtain a percentage of degranulated MCs in the uterus. The myometrium region was selected as a representation of the uterus because the large majority of uterine MCs are present in this region.

Oestrogen-treated mice have a reduced number of MCs in their myometrium compared to progesterone treated mice (**Figure 2.4 A-C**). However, the percentage of degranulated MCs was similar between mice treated with progesterone and those treated with oestrogen (**Figure 2.4 D**).Those results suggest that female sex hormones and/or oestrous cycle might affect the number of MCs in the FRT.

In future studies, alcian blue/safranin staining will be performed and the different types of MCs will be enumerated, in order to determine if female sex hormones and/or oestrous cycle alter the numbers and/or proportions of heparin-positive MCs and chondroitin sulfate-positive MCs.

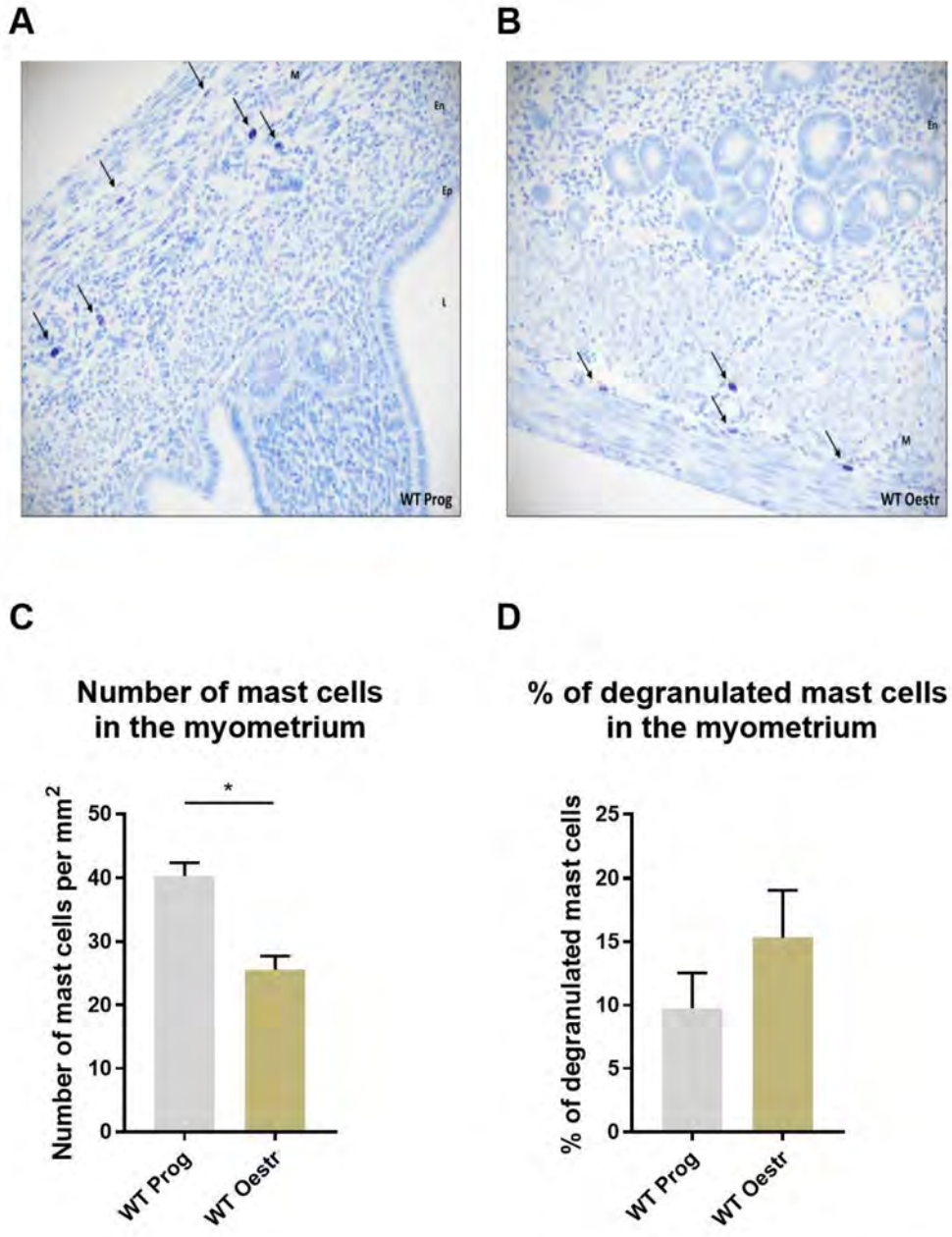


Figure 2.4 – Mice treated with oestrogen have a reduced numbers of uterine mast cells (MCs) but a similar percentage of degranulated MCs compared to progesterone-treated mice. C57BL/6 wild-type (WT) mice were treated with either progesterone (Prog) or oestrogen (Oestr) and sacrificed 7 days later. The reproductive tract tissues were fixed, embedded in paraffin and longitudinal sections of 5µm thickness were stained with toluidine blue. **(A, B)** Representative pictures of the uterus (*L:lumen, Ep: epithelium, En: endometrium, M: myometrium*) of **(A)** WT progesterone-treated and **(B)** WT oestrogen-treated mice, with MCs distinguishable by their purple colour and indicated with a black arrow. **(C)** The number of MCs was quantified in at least 15 field of views of myometrium tissue at a magnification of 20X using the Zeiss Axio Imager M2 microscope and the respective area of myometrium (mm^2) was quantified. Cell counts are expressed as number of cells per square millimetre (cells/mm^2). **(D)** The percentage of MC degranulation was obtained by enumerating the number of degranulated MCs and non-degranulated MCs in the myometrium. All data are presented as mean±SEM (n=4) and Mann-Whitney tests were performed, with * representing $p<0.05$.

2.4.4. *Chlamydia muridarum* infection does not alter the number and degranulation of uterine MCs early, but slightly decreases their number later

In order to examine if *Chlamydia* infections affect the number and degranulation of MCs in the FRT, WT female mice were treated with progesterone and infected intravaginally with *Chlamydia muridarum* or sham-infected (**Figure 2.1 B**). At 3dpi and 14dpi, the FRT tissues were extracted, fixed in formalin, embedded in paraffin, sectioned and stained with toluidine blue. Similarly to what explained in **Section 2.4.2**, the MCs were enumerated in the myometrium and their percentage of degranulation was enumerated.

At 3dpi, the number of MCs and their degranulation appear similar in the myometrium of *Chlamydia*-infected and control mice (**Figure 2.5 A-D**). At 14dpi, the number of MCs in the myometrium was slightly reduced in *Chlamydia*-infected mice compared to non-infected control mice (**Figure 2.5 E-G**, p=0.0571). No difference in the percentage of degranulation was observed in the uterus of *Chlamydia*-infected and control mice at 14dpi (**Figure 2.5 H**).

In future studies, alcian blue/safranin staining will be performed and the different types of MCs will be enumerated, in order to determine if *Chlamydia* infection alters the numbers and/or proportions of heparin-positive MCs and chondroitin sulfate-positive MCs. Furthermore, the number of mice will be increased, in order to validate if the decrease in MCs observed in *Chlamydia*-infected mice become statistical as, due to time restraints, it was not possible to perform those during my PhD study.

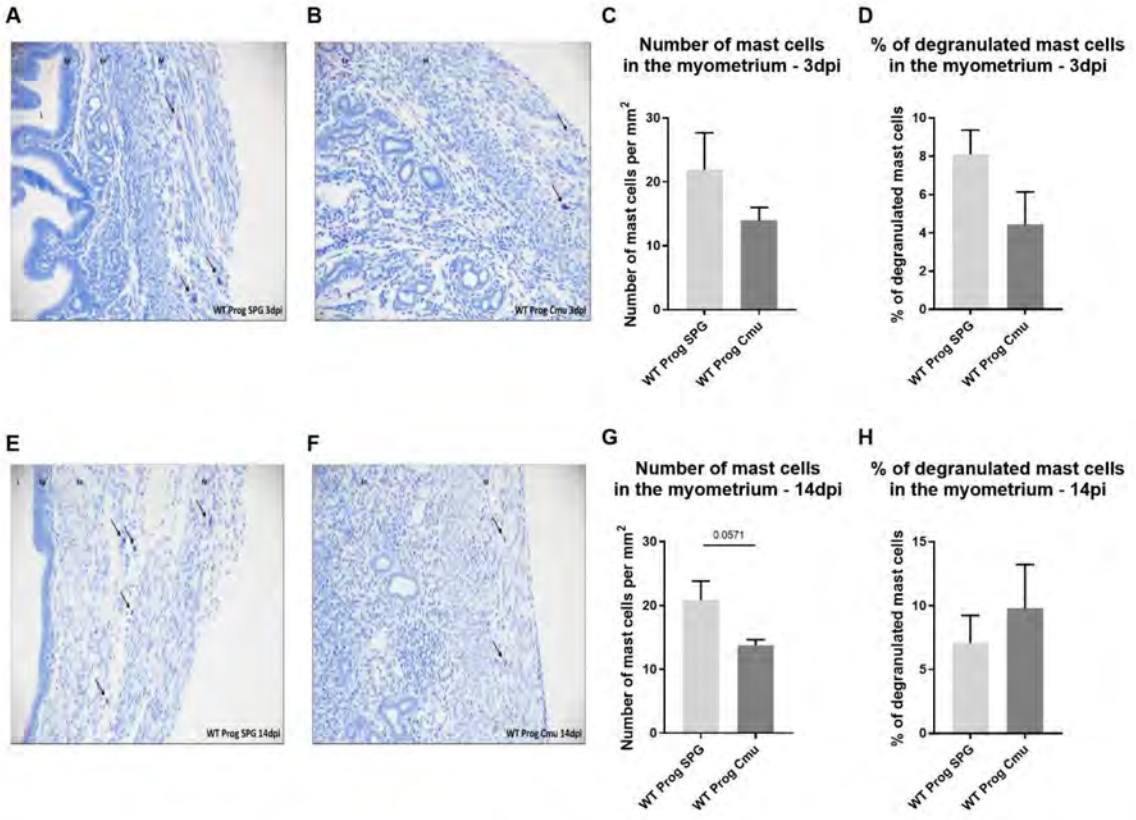


Figure 2.5 – Infection with *Chlamydia muridarum* does not significantly affect the number nor the degranulation of uterine mast cells (MCs) early, but slightly decrease MC number later. C57BL/6 wild-type (WT) mice were treated with progesterone (Prog), infected intravaginally with *Chlamydia muridarum* (Cmu) or sham-infected with sucrose-phosphate-glutamate buffer (SPG). Mice were sacrificed at **(A-D)** 3dpi and **(E-H)** 14dpi. The reproductive tract tissues were fixed in formalin, embedded in paraffin and longitudinal sections of 5µm thickness were stained with toluidine blue. **(A, B, E, F)** Representative pictures of the uterus (*L:lumen, Ep: epithelium, En: endometrium, M: myometrium*) of **(A, E)** non-infected control mice and **(B, F)** *Chlamydia*-infected mice, with MCs distinguishable by their purple colour and indicated with a black arrow. **(C, G)** The number of MCs was quantified in at least 15 field of views of myometrium tissue and the respective area of myometrium (mm^2) was quantified. Cell counts are expressed as number of cells per square millimetre (cells /mm^2). **(D, H)** The percentage of MC degranulation was obtained by enumerating the number of degranulated MCs and non-degranulated MCs in the myometrium. All data are presented as mean±SEM ($n \geq 3$) and Mann-Whitney tests were performed.

2.4.5. MC-deficient mice are protected against *Chlamydia*-induced pathology

MCs are present in the different tissues of the FRT and have been shown to play an important role in immune responses to various bacterial infections (260, 262, 274). The *Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice have a targeted deficiency in the intracellular anti-apoptotic factor, Mcl-1 in Cpa3-expressing cells, causing severe MC deficiency, ranging from 92% to 100% (237). In order to assess the roles of MCs during *Chlamydia* FRT infection, MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 2.1 C**). The levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

The number of *Chlamydia* per vaginal lavage (**Figure 2.6 A**; p=0.0722) and the *Chlamydia* burden in the uterus (**Figure 2.6 B**) were reduced in MC-deficient mice compared to WT controls, although the differences did not reach statistical significance. High variability is present, particularly in WT mice, which is often the case in mouse models of *Chlamydia* infection. Increasing the number of mice would allow to definitely conclude on the effect of MC deficiency in *Chlamydia* infection in the vagina and uterus, however, due to logistical restrictions associated with breeding of genetically modified mice, it was not possible to obtain more mice in the timeframe of my PhD.

The cross sectional area of the left and right oviducts of MC-deficient mice is decreased compared to that in WT control mice, demonstrating that MC-deficient mice are protected against *Chlamydia*-induced tubal blockage and subsequent hydrosalpinx (**Figure 2.6 C-D**).

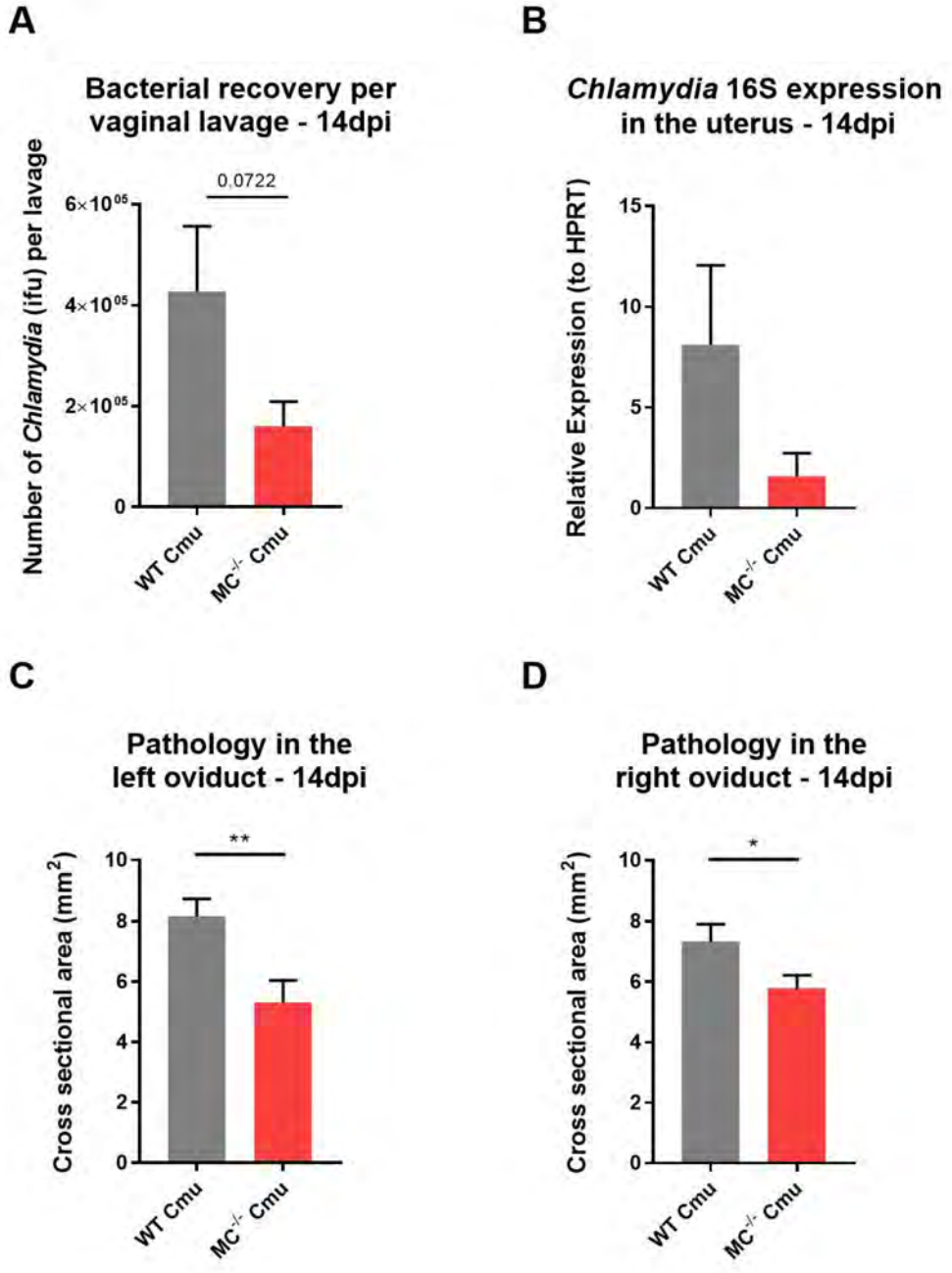


Figure 2.6 - Mast cell (MC)-deficient mice have a slightly lower *Chlamydia* burden and are protected against *Chlamydia*-induced pathology at 14 days post infection (dpi). MC-deficient (*MC$^{-/-}$*, *Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and their associated WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection (dpi). **(A)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per vaginal lavage. **(B)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross sectional area of the **(C)** left and **(D)** right oviducts were measured using a calliper to evaluate the levels of *Chlamydia*-associated pathology. All data are presented as mean±SEM ($n \geq 8$) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and ** representing $p<0.01$.

2.4.6. MC-deficient mice exhibit a decline in the neutrophils, eosinophils, inflammatory monocytes and macrophages in their uterus during *Chlamydia* FRT infection

My data demonstrate that MCs contribute to the development of *Chlamydia*-induced pathology in the FRT. Several studies show that MCs are associated with immune cell recruitment to the infectious site and that, during *Chlamydia* FRT, excessive immune cell infiltration into the FRT is linked with tissue damage (133, 183, 273, 274, 392). Therefore, in order to assess the effects of MCs on immune cell recruitment to the FRT during *Chlamydia* infection, MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 2.1 C**). The uteri were collected and digested into a single cell suspension and the total number of cells were enumerated. Single cell suspensions were then stained for extracellular markers and key immune cells were identified by flow cytometry. The number of cells in the uterine tissue was expressed both as the total number of each immune cell type and as a percentage of each immune cell type among all viable cells. To complement these data, the expression of the NK cell-specific marker, NRC1, was assessed by qPCR in the uterine tissue of MC-deficient mice and their WT controls.

Although the number of uterine cells (**Figure 2.7 A**) as well as the number and the percentage of immune cells in the uterus were similar between MC-deficient mice and their WT controls (**Figures 2.7 B and 2.8 A**), the number of some key innate immune cells appeared slightly reduced in the uterus of MC-deficient mice. For example, the total number and percentage of neutrophils (**Figures 2.7 C and 2.8 B**, p=0.1283 and p=0.0730) and eosinophils (**Figures 2.7 D and 2.8 C**, p= 0.1014 and p=0.1375) were slightly decreased in MC-deficient mice, although the changes were not statistically significant. It is possible that the limited number of MC-deficient mice available for this study did not permit to reach statistical significance given the relative variability of mouse model of *Chlamydia* infection. While the number and percentage of myeloid DCs (**Figures 2.7 F and 2.8 D**), plasmacytoid DCs (**Figures 2.7 G and 2.8 E**) and resident monocytes and macrophages (**Figures 2.7 H and 2.8 F**) remained similar between MC-deficient mice and

their WT controls, the number and percentage of inflammatory monocytes and macrophages were slightly decreased in MC-deficient mice (**Figures 2.7 I and 2.8 G**; p=0.1384 and p=0.0522). The expression of NRC1 was similar in MC-deficient mice and their WT controls, suggesting that similar number of NK cells are present (**Figures 2.7 E and 2.8 D**).

Furthermore, the number and percentage of T cells (**Figures 2.7 J and 2.8 H**), $CD4^+$ T cells (**Figures 2.7 K and 2.8 I**) and $CD8^+$ T cells (**Figures 2.7 L and 2.8 J**) were not different between MC-deficient mice or their WT controls during *Chlamydia* infection.

Therefore, MC-deficient mice do not appear to have any significant changes in the proliferation and/or recruitment of innate and adaptive immune cells in the uterus during *Chlamydia* infection that may explain the reduction in hydrosalpinx. However, the reductions in the percentages and numbers of neutrophils, eosinophils and inflammatory monocytes and macrophages need to be explored further by increasing the number of mice per group.

Unfortunately, due to restrictions in the breeding of genetically modified mice, the number of mice available was insufficient to include sham-infected control mice in this study. In future experiments, the numbers and percentages of each immune cell type in the uterus of sham-infected MC-deficient mice (and WT controls) will be determined in order to establish if the differences observed in this study are caused by MC deficiency alone or if they are mediated by *Chlamydia* infection.

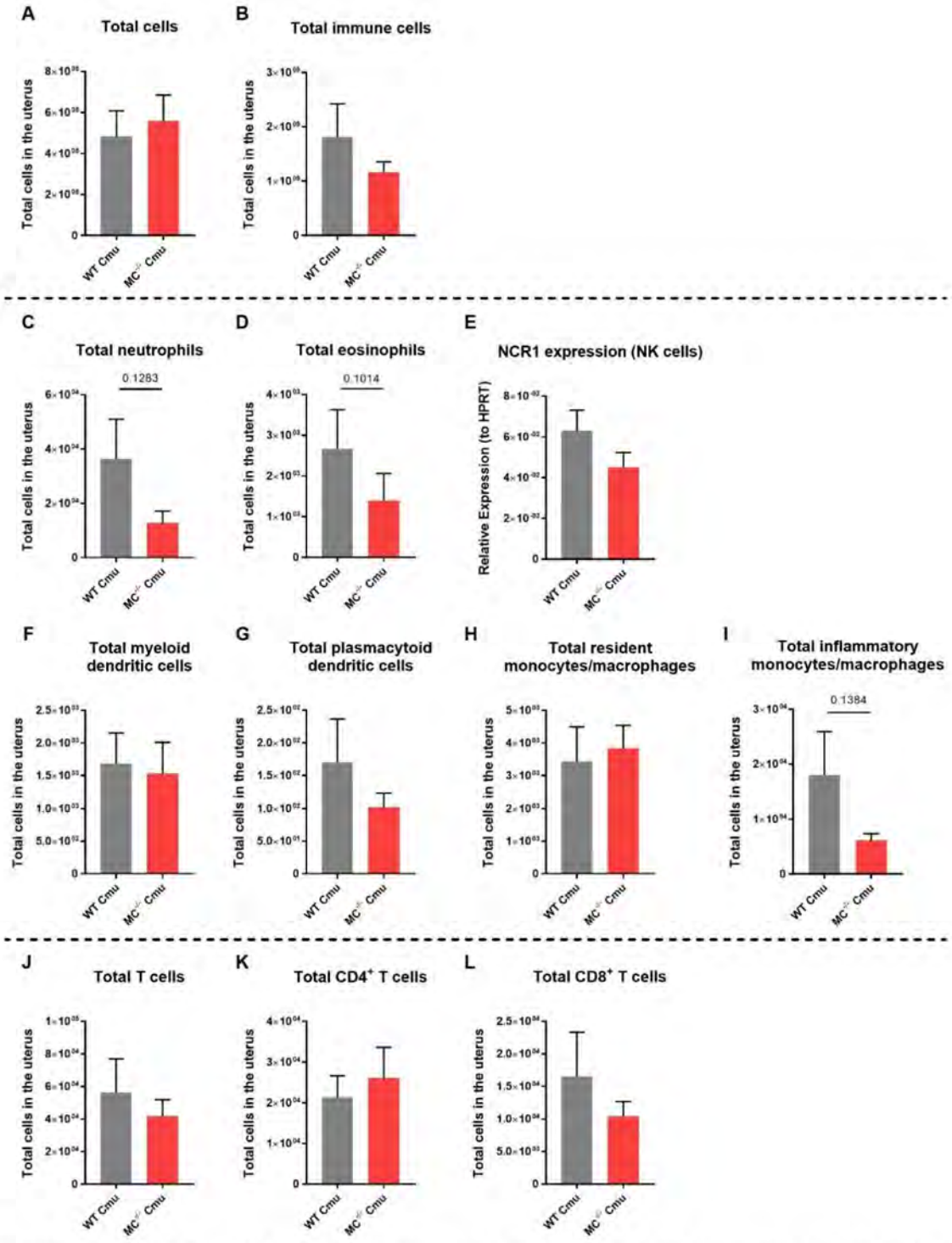


Figure 2.7 - Mast cell (MC)-deficient mice have a reduced number of neutrophils, eosinophils, inflammatory monocytes and macrophages present in their uterus during *Chlamydia* infection. MC-deficient (*MC$^{-/-}$*, *Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. RNA was extracted from the uterus and the level of expression of **(E)** NCR1 was quantified by qPCR and normalised using the expression of HPRT. Moreover, **(A)** single cell suspensions from the uterus were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the total number of **(B)** immune cells, **(C)** neutrophils, **(D)** eosinophils, **(F)** myeloid and **(G)** plasmacytoid dendritic cells, **(H)** resident and **(I)** inflammatory monocytes and macrophages, **(J)** T cells, **(K)** CD4$^+$ T cells and **(L)** CD8$^+$ T cells in the uterus. All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed.

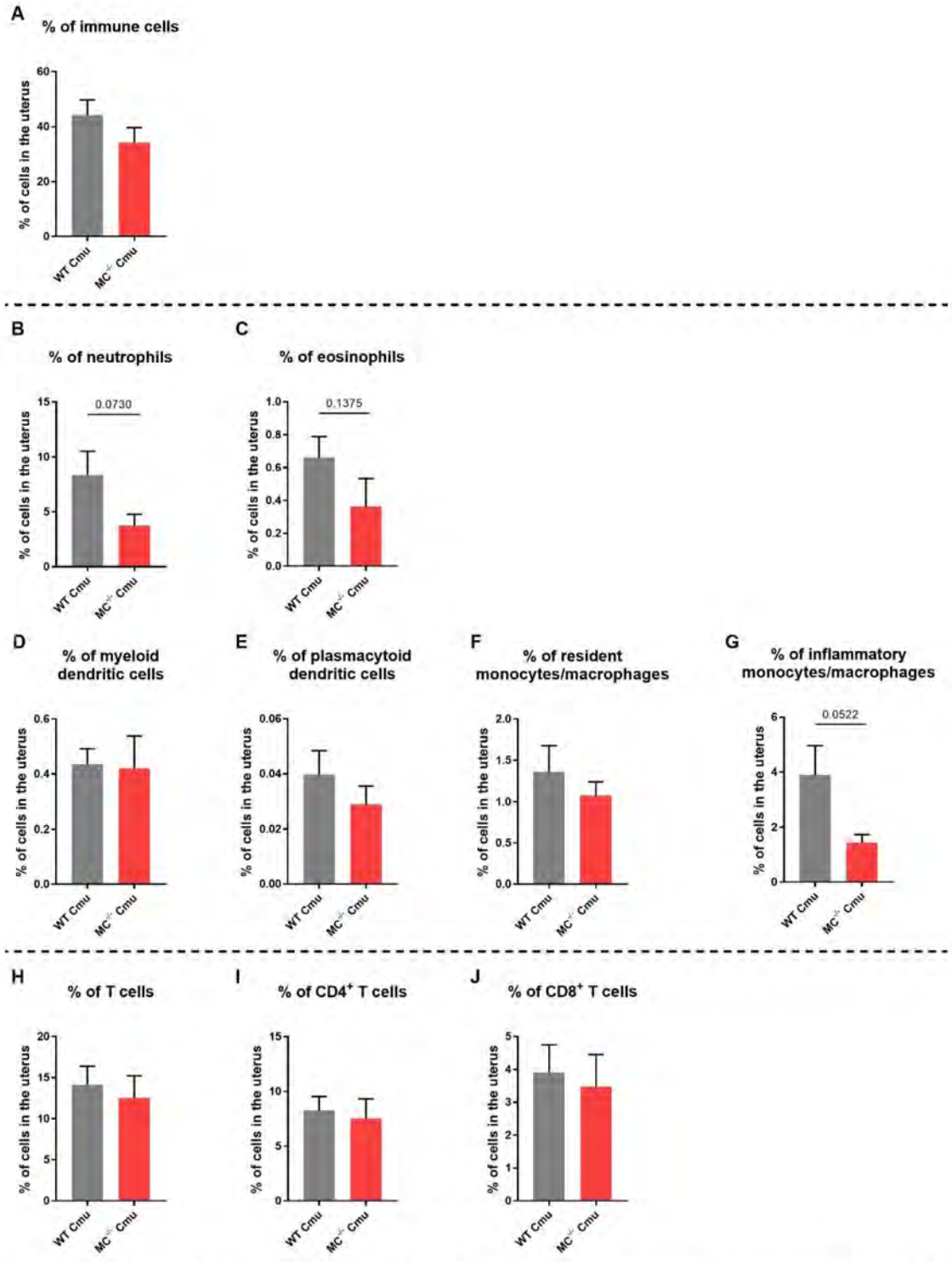


Figure 2.8 - Mast cell (MC)-deficient mice have a decline in the percentage of neutrophils, eosinophils, inflammatory monocytes and macrophages present in their uterus during *Chlamydia* infection. MC-deficient (*MC$^{-/-}$, Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Single cell suspensions from the uterus were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the percentages of **(A)** immune cells, **(B)** neutrophils, **(C)** eosinophils, **(D)** myeloid and **(E)** plasmacytoid dendritic cells, **(F)** resident and **(G)** inflammatory monocytes and macrophages, **(H)** T cells, **(I)** CD4$^+$ T cells and **(J)** CD8$^+$ T cells among total viable uterine cells. All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed.

2.4.7. MC-deficient mice exhibit a decline in the eosinophils, γδ T cells and B cells but an increase in inflammatory monocytes and macrophages in their bone marrow during *Chlamydia* FRT infection

My data provides evidence to show that MCs contribute to the development of *Chlamydia*-induced pathology in the FRT and that they may slightly affect the number of innate immune cells into the uterus. In order to assess if MCs have an effect on immune cell haematopoiesis, bone marrow cells were assessed in MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 2.1 C**). The bone marrow of these mice was collected and stained for extracellular markers specific and key immune cells were identified by flow cytometry. The number of cells in the bone marrow was expressed both as the total number of each immune cell type and as a percentage of each immune cell type among all viable cells

The total number of cells in the bone marrow are similar between MC-deficient mice and their WT controls (**Figure 2.9 A**). Although the percentage of immune cells in the bone marrow are slightly increased in MC-deficient mice (**Figure 2.10 A**), the total number of immune cells are similar between MC-deficient mice and their WT controls (**Figure 2.9 B**).

The levels of some innate immune cell present in the bone marrow appear to be modified in MC-deficient mice. For example, although the number and percentage of neutrophils (**Figures 2.9 C and 2.10 B**), NK cells, (**Figures 2.9 E and 2.10 D**) and resident monocytes and macrophages (**Figure 2.9 F and 2.10 E**) remained similar in the bone marrow of *Chlamydia*-infected MC-deficient mice and their WT controls, the number and percentage of eosinophils were decreased (**Figures 2.9 D and 2.10 C**), while the number and percentage of inflammatory monocytes and macrophages (**Figure 2.9 G and 2.10 F**) were increased, in the bone marrow of infected MC-deficient mice compared to WT mice.

Similarly to what was observed in the uterus, no difference in the numbers and percentages of total T cells (**Figures 2.9 H and 2.10 G**), $CD4^+$ T cells (**Figures 2.9 I and 2.10 H**), $CD8^+$ T cells (**Figures 2.9 J and 2.10 I**) and NKT cells (**Figures 2.9 JK and 2.10 J**) are present between MC-deficient mice and their WT controls. However, the number and percentage of γδ T cells

(**Figure 2.9 L and 2.10 M**; $p<0.05$ and $p=0.1325$) and B cells (**Figure 2.9 L and 2.10 L**; $p<0.05$ and $p=0.1805$) are reduced in the bone marrow of infected MC-deficient mice compared to WT mice suggesting a potential role for MCs in mediating γδ T cells and B cell mobilisation during *Chlamydia* infection.

In future experiments, the numbers and percentages of each immune cell type in the bone marrow of sham-infected MC-deficient mice (and WT controls) will be determined in order to establish if the differences in numbers and/or percentages of eosinophils, inflammatory monocytes and macrophages, γδ T cells and B cells are caused by MC deficiency alone or if they are mediated by *Chlamydia* infection.

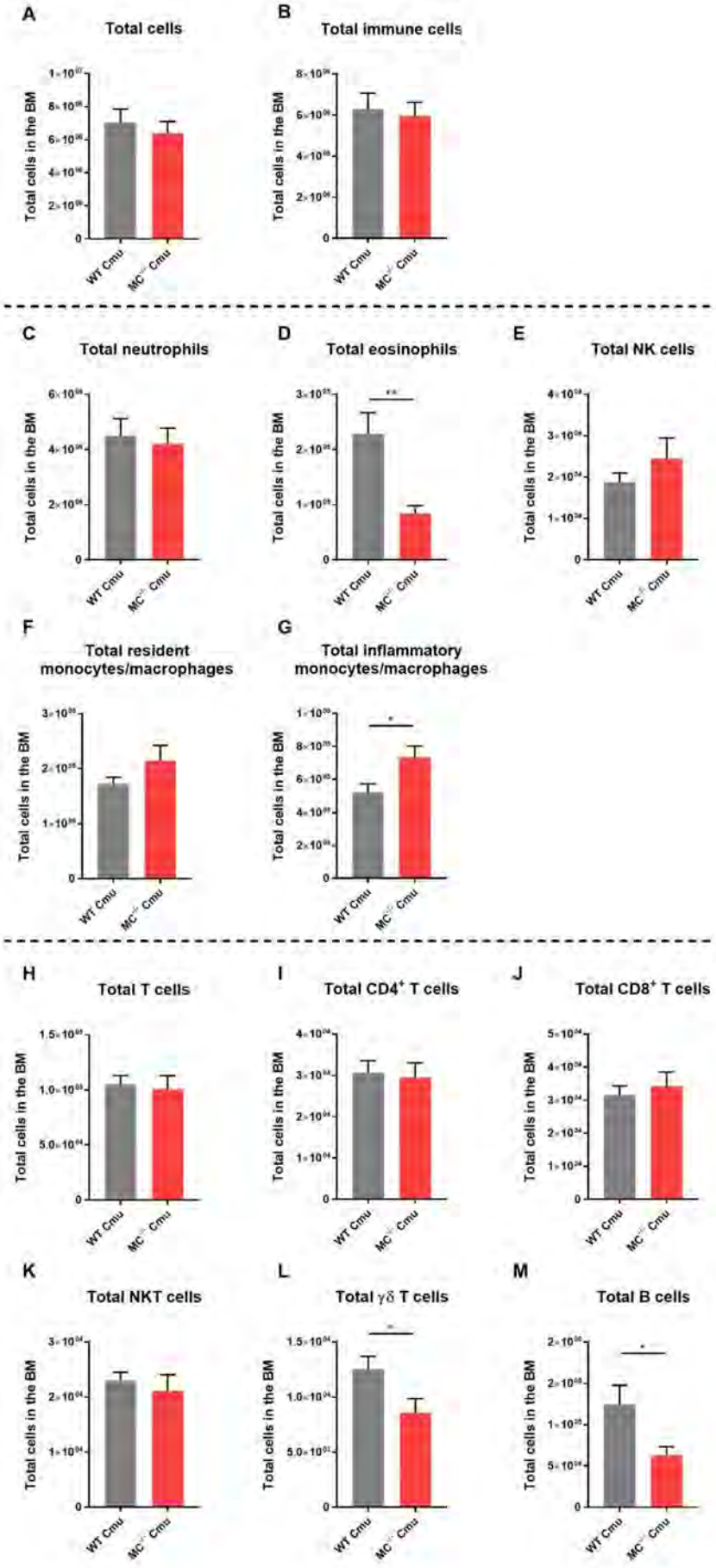

A
Total cells
Total cells in the BM
WT Cmu
MC-/- Cmu
B
Total immune cells
Total cells in the BM
WT Cmu
MC-/- Cmu
C
Total neutrophils
Total cells in the BM
WT Cmu
MC-/- Cmu
D
Total eosinophils
**
Total cells in the BM
WT Cmu
MC-/- Cmu
E
Total NK cells
Total cells in the BM
WT Cmu
MC-/- Cmu
F
Total resident monocytes/macrophages
Total cells in the BM
WT Cmu
MC-/- Cmu
G
Total inflammatory monocytes/macrophages
*
Total cells in the BM
WT Cmu
MC-/- Cmu
H
Total T cells
Total cells in the BM
WT Cmu
MC-/- Cmu
I
Total CD4+ T cells
Total cells in the BM
WT Cmu
MC-/- Cmu
J
Total CD8+ T cells
Total cells in the BM
WT Cmu
MC-/- Cmu
K
Total NKT cells
Total cells in the BM
WT Cmu
MC-/- Cmu
L
Total γδ T cells
Total cells in the BM
WT Cmu
MC-/- Cmu
M
Total B cells
*
Total cells in the BM
WT Cmu
MC-/- Cmu

Figure 2.9 - Mast cell (MC)-deficient mice have a lower number of eosinophils, γδ T cells and B cells, and a higher number of inflammatory monocytes and macrophage,s in their bone marrow (BM) during *Chlamydia* infection. MC-deficient (*MC*$^{-/-}$, *Cpa3-Cre;Mcl-1*$^{fl/fl}$) mice and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. **(A)** Single cell suspensions from the bone marrow were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the total number of **(B)** immune cells, **(C)** neutrophils, **(D)** eosinophils, **(E)** NK cells, **(F)** resident and **(G)** inflammatory monocytes and macrophages, **(H)** T cells, **(I)** CD4^{+} T cells, **(J)** CD8^{+} T cells, **(K)** NKT cells, **(L)** γδ T cells and **(M)** B cells in the bone marrow. All data are presented as mean±SEM (n≥8) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$, ** representing $p<0.01$, *** representing $p<0.001$ and **** representing $p<0.0001$.

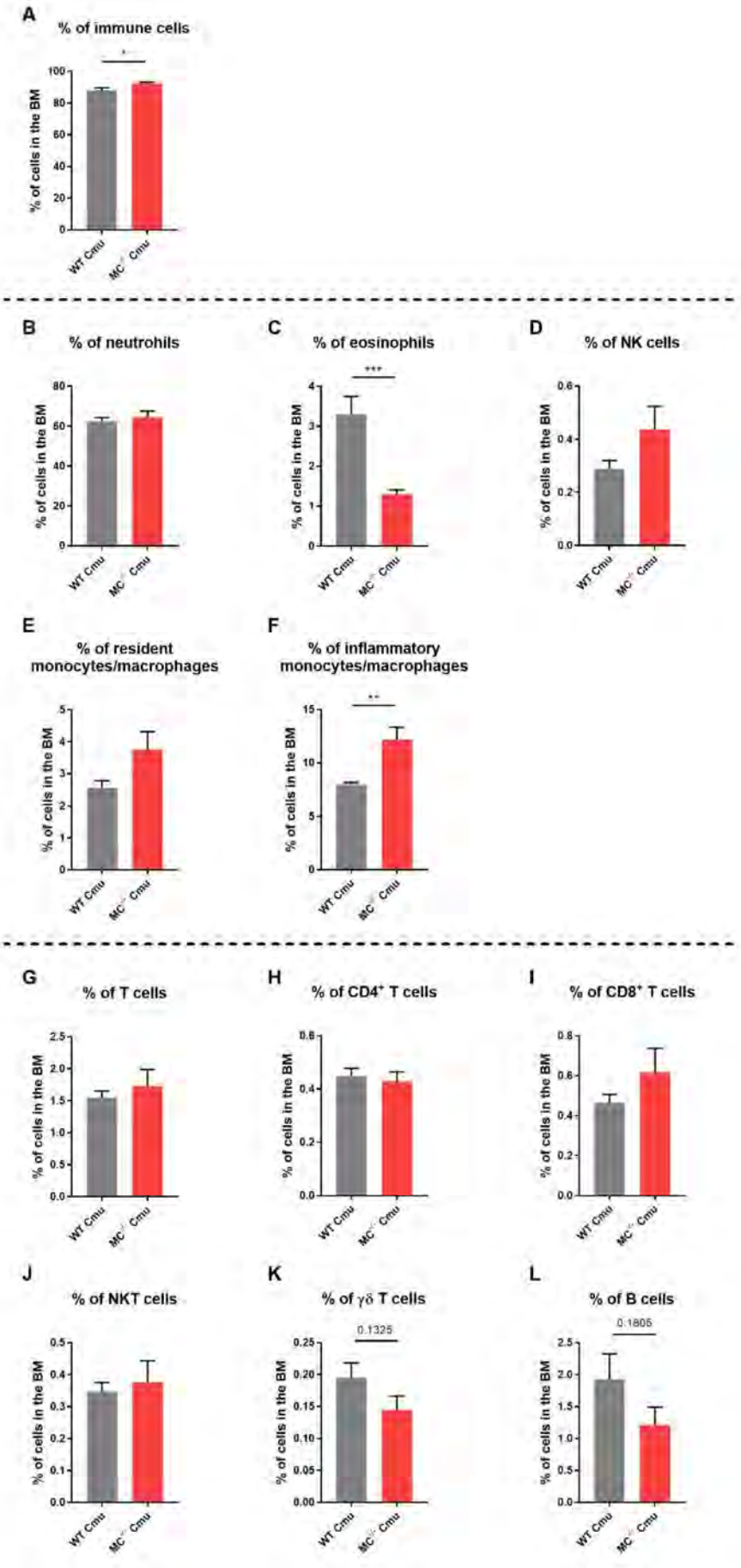

A
% of immune cells
*
% of cells in the BM
WT Cmu
MC-/- Cmu
B
% of neutrohils
C
% of eosinophils
***
D
% of NK cells
E
% of resident monocytes/macrophages
F
% of inflammatory monocytes/macrophages
**
G
% of T cells
H
% of CD4+ T cells
I
% of CD8+ T cells
J
% of NKT cells
K
% of γδ T cells
0.1325
L
% of B cells
0.1805

Figure 2.10 - Mast cell (MC)-deficient mice have a significant higher percentage of immune cells and inflammatory monocytes and macrophages, but a significant lower percentage of eosinophils in their bone marrow (BM) during *Chlamydia* infection. MC-deficient (*MC$^{-/-}$*, *Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and their associated WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Single cell suspensions from the bone marrow were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the percentages of **(A)** immune cells, **(B)** neutrophils, **(C)** eosinophils, **(D)** NK cells, **(E)** resident and **(F)** inflammatory monocytes and macrophages, **(G)** T cells, **(H)** CD4$^+$ T cells, **(I)** CD8$^+$ T cells, **(J)** NKT cells, **(K)** γδ T cells and **(L)** B cells among total viable cells in the bone marrow. All data are presented as mean±SEM (n≥8) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$, ** representing $p<0.01$, *** representing $p<0.001$ and **** representing $p<0.0001$.

2.4.8. MC-deficient mice did not have changes in the expression of Th cell polarising transcription factors during *Chlamydia* FRT infection

During *Leishmania* infection, MCs have been shown to induce Th1 and Th17 responses, while during helminth infections, MCs have been shown to regulate Th2 responses (260, 393). Therefore, in order to assess if MCs affect the Th1, Th2, Th17 and/or Treg responses in the FRT of *Chlamydia*–infected mice, MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 2.1 C**). The left uteri were collected for subsequent RNA extraction and reverse transcription. The levels of expression of the transcription factors Tbet, GATA3, RORγt and FOXP3, associated respectively with Th1, Th2, Th17 and Treg responses (394), were assessed by qPCR.

Overall, the expression of these markers was not affected between MC-deficient mice and WT controls, suggesting that the absence of MCs might not affect Th cell polarisation during *Chlamydia* infection (**Figure 2.11**). However, it is also possible that MCs may affect Th responses at earlier time point, but, due to a limited number of mice available, it was not possible to realise another time point. It is also possible that the protein expression of these factors might be affected. As all of the FRT tissues of these mice were used for flow cytometry analyses and a small quantity for qPCR, this experiment will need to be repeated for protein analyses and analyse the expression of these factors in T cells using intracellular staining for flow cytometry.

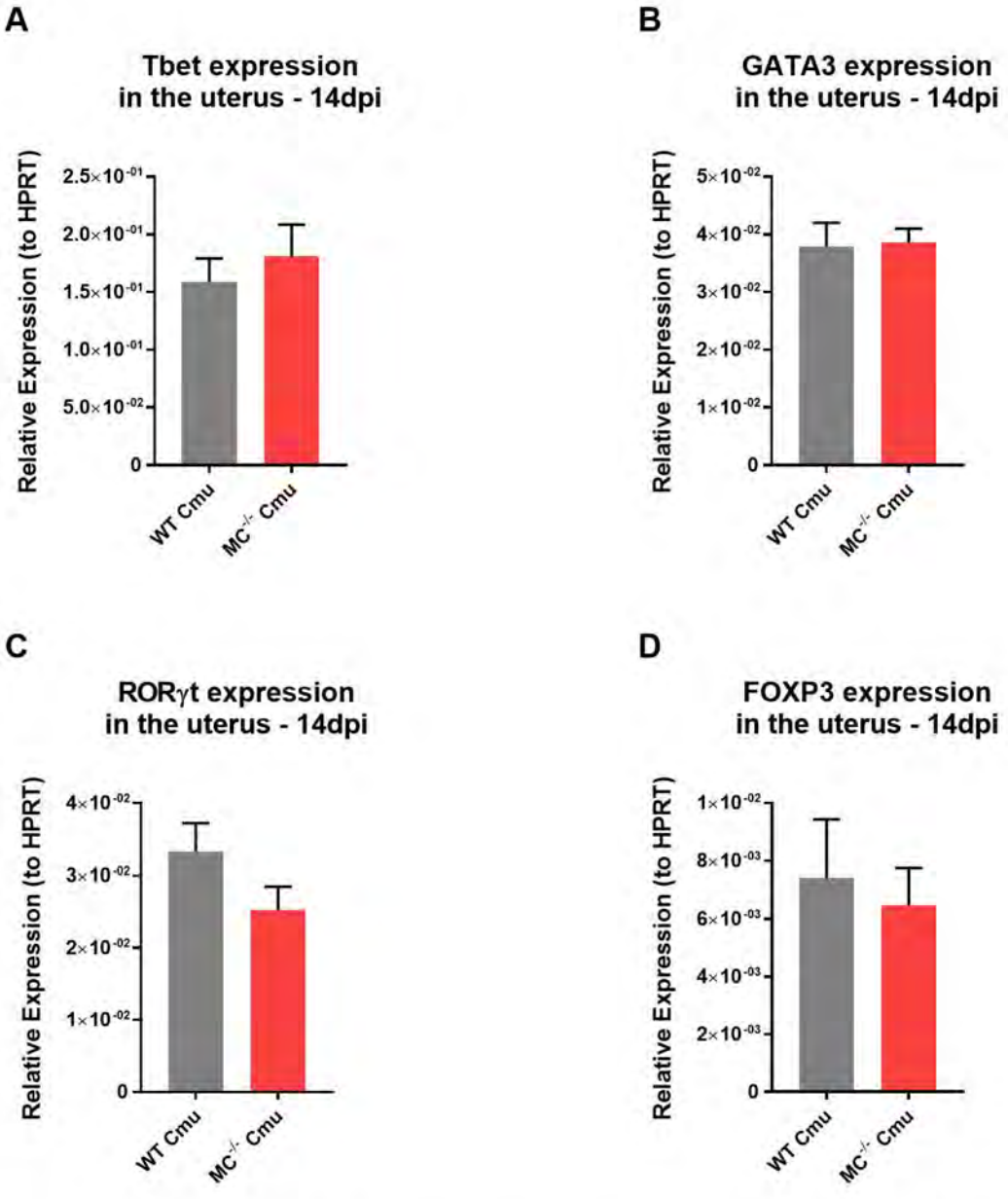


Figure 2.11 – The expression of the transcription factors Tbet, GATA Binding Protein (GATA)3, RAR-related orphan receptor (ROR)γt and forkhead box P3 (FOXP3) is not altered in the uterus of *Chlamydia*-infected mast cell (MC)-deficient mice compared to WT controls. MC-deficient (*MC$^{-/-}$*, *Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and their associated WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection (dpi). RNA was extracted from the uterus and the levels of expression of the transcription factors **(A)** Tbet, **(B)** GATA Binding Protein (GATA)3, **(C)** RAR-related orphan receptor (ROR)γt and **(D)** forkhead box P3 (FOXP3) were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥8) and Student's t-tests were performed.

2.4.9. The expression of the pro-inflammatory chemokine CXCL15 is reduced in the *Chlamydia*-infected uterus of MC-deficient mice

Although I show that there are no changes in the expression the transcription factors associated with Th1/Th2/Th17/Treg polarisation in MC-deficient mice, the level of expression of key immune mediators of *Chlamydia* infection may be affected. To assess this, MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 2.1 C**). The left uterus were collected for subsequent RNA extraction and reverse transcription. The levels of the key immune mediators associated with clearance of infection and *Chlamydia*-induced pathology were assessed using qPCR.

The levels of expression of IFNγ, TNFα, IL-1β, IL-4rα, IL-6, IL-10, IL-12p40, IL-13, IL-17a, CXCL1, CXCR2, GM-CSF, MMP9, TLR3 and PAR2 are similar between MC-deficient mice and WT controls (**Figure 2.12**). However, the expression of the neutrophil-chemoattractant chemokine CXCL15 is significantly reduced in the uterus of MC-deficient mice compared to WT controls (**Figure 2.12 K**). This observation agrees with the slight reduction in the number of neutrophil observed in the uterus of *Chlamydia*-infected MC-deficient mice compared to WT controls (**Figure 2.7 C**).

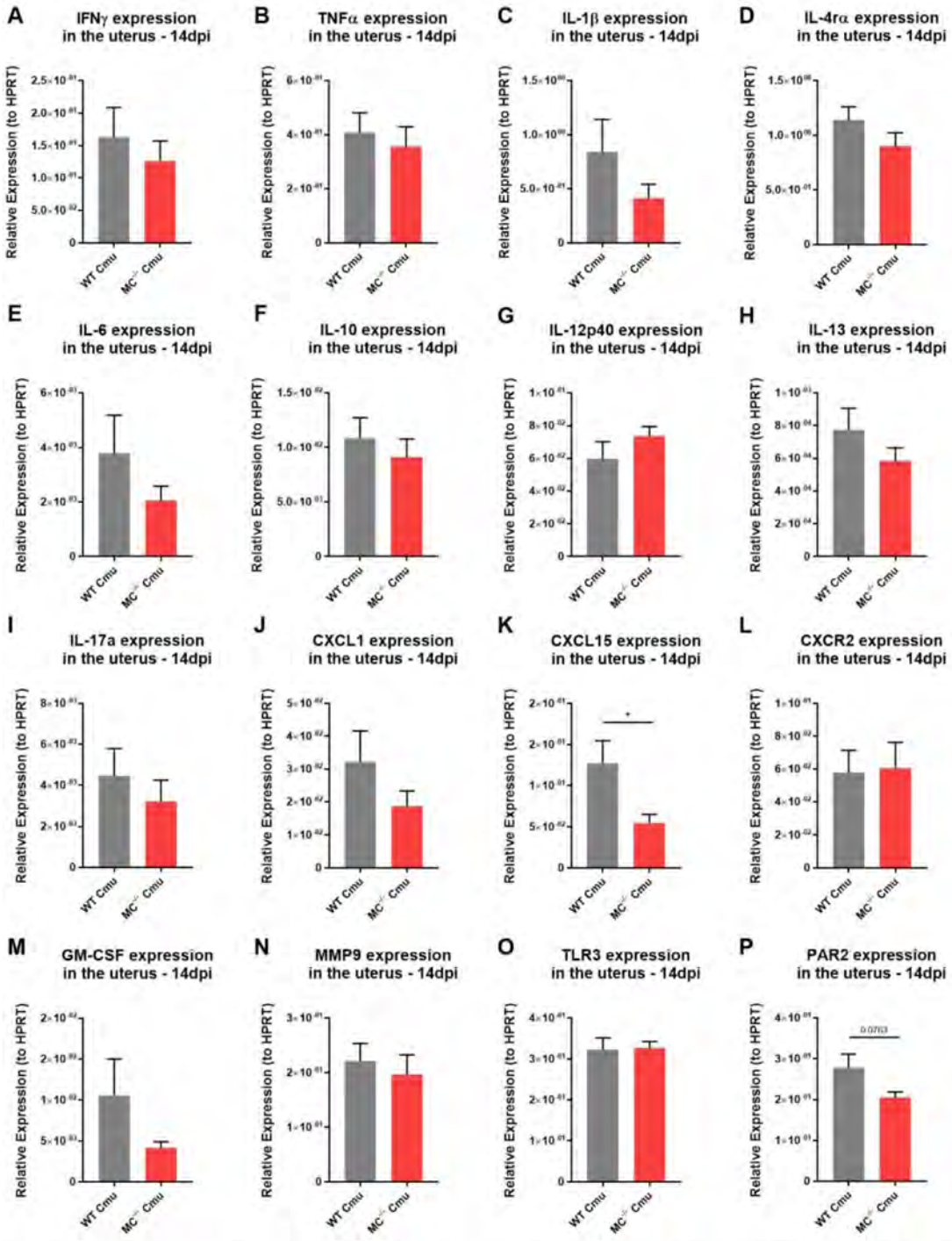


Figure 2.12 - The expression of the pro-inflammatory chemokine (C-X-C motif) ligand (CXCL)15 is reduced in the *Chlamydia*-infected uterus of mast cell (MC)-deficient mice compared to WT controls. MC-deficient (*MC$^{-/-}$*, *Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14dpi. RNA was extracted from the uterus and the expression of the cytokines, chemokines and immune mediators **(A)** interferon (IFN)γ, **(B)** tumor necrosis factor (TNF)α, **(C)** interleukin- (IL-)1β, **(D)** IL-4rα, **(E)** IL-6, **(F)** IL-10, **(G)** IL-12p40, **(H)** IL-13, **(I)** IL-17a, **(J)** CXCL1, **(K)** CXCL15, **(L)** chemokine (C-X-C motif) receptor (CXCR)2, **(M)** granulocyte-macrophage colony-stimulating factor (GM-CSF), **(N)** matrix metallopeptidase (MMP)9, **(O)** toll-like receptor (TLR)3, **(P)** protease-activated receptor (PAR)2 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥8) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$.

2.4.10. Inhibition of MC degranulation potently protects against the early stages of *Chlamydia* FRT infection but is largely detrimental to infection during the later stages

Using MC-deficient mice, I have shown that MCs play an important role in the pathogenesis of *Chlamydia* FRT infection, particularly in the development of upper FRT pathology. I show that this may be due to the effects of MCs on mediating pro-inflammatory responses and innate immune cell recruitment. However, the effects that I have observed are not large and the mechanisms that underpin the effects of MCs on infection are difficult to discern. As outlined previously, MCs produce and release a wide variety of mediators through both degranulation and *de novo* synthesis. Many of these factors may have conflicting/counteracting effects on the pathogenesis of infection and infection-induced pathology.

In order to assess the effects of factors that are released during MC degranulation, I next sought to assess the effects of intravaginal cromolyn treatment on infection and *Chlamydia*-induced pathology. Starting 2 days before infection, WT mice were treated intravaginally each day with the MC stabiliser cromolyn or vehicle alone, subjected to a murine model of *Chlamydia* FRT infection and sacrificed at 3dpi and 14dpi. Treatments were continued on a daily basis throughout the entire time-course of infection (**Figure 2.2 A**).

The levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

I show that at 3dpi, the levels of infection are strongly reduced in the vagina (**Figure 2.13 A**) and in the uterus (**Figure 2.13 B**) of cromolyn-treated mice, suggesting a detrimental role of MC degranulation during the early stages of infection. Whilst the levels of MC degranulation were slightly reduced in the uterus of *Chlamydia*-infected mice compared to non-infected control mice at 3dpi (**Figure 2.5 D**), it is possible that *Chlamydia* infection prompts MC degranulation at earlier time points, hence explaining the protective effect of cromolyn during the early stages of

infection. These protective effects were not maintained when treatment was continued into the later stages of infection, with increased *Chlamydia* observed in the vagina of cromolyn-treated mice (**Figure 2.13 C**). Interestingly, similar levels of pathology are observed in the oviducts of vehicle- and cromolyn-treated mice at 14dpi (**Figure 2.13 E-F**).

Taken together, these results suggest that inhibition of MC degranulation using cromolyn may protect against bacterial proliferation in the early stages of *Chlamydia* infection. At later stages, daily cromolyn treatments may amplify bacterial proliferation in the FRT, without affecting the development of pathology. In future studies, longer time points will need to be performed in order to verify if cromolyn treatments affect the development of *Chlamydia*-induced pathology at very late stages of infection.

Taken together, my findings reveal that inhibition of MC degranulation using cromolyn does not recapitulate the phenotype of reduced hydrosalpinx observed in MC-deficient mice, indicating that factors released specifically during MC degranulation might have different effects on infection *versus* pathology and/or have different effects during different stages of infection.

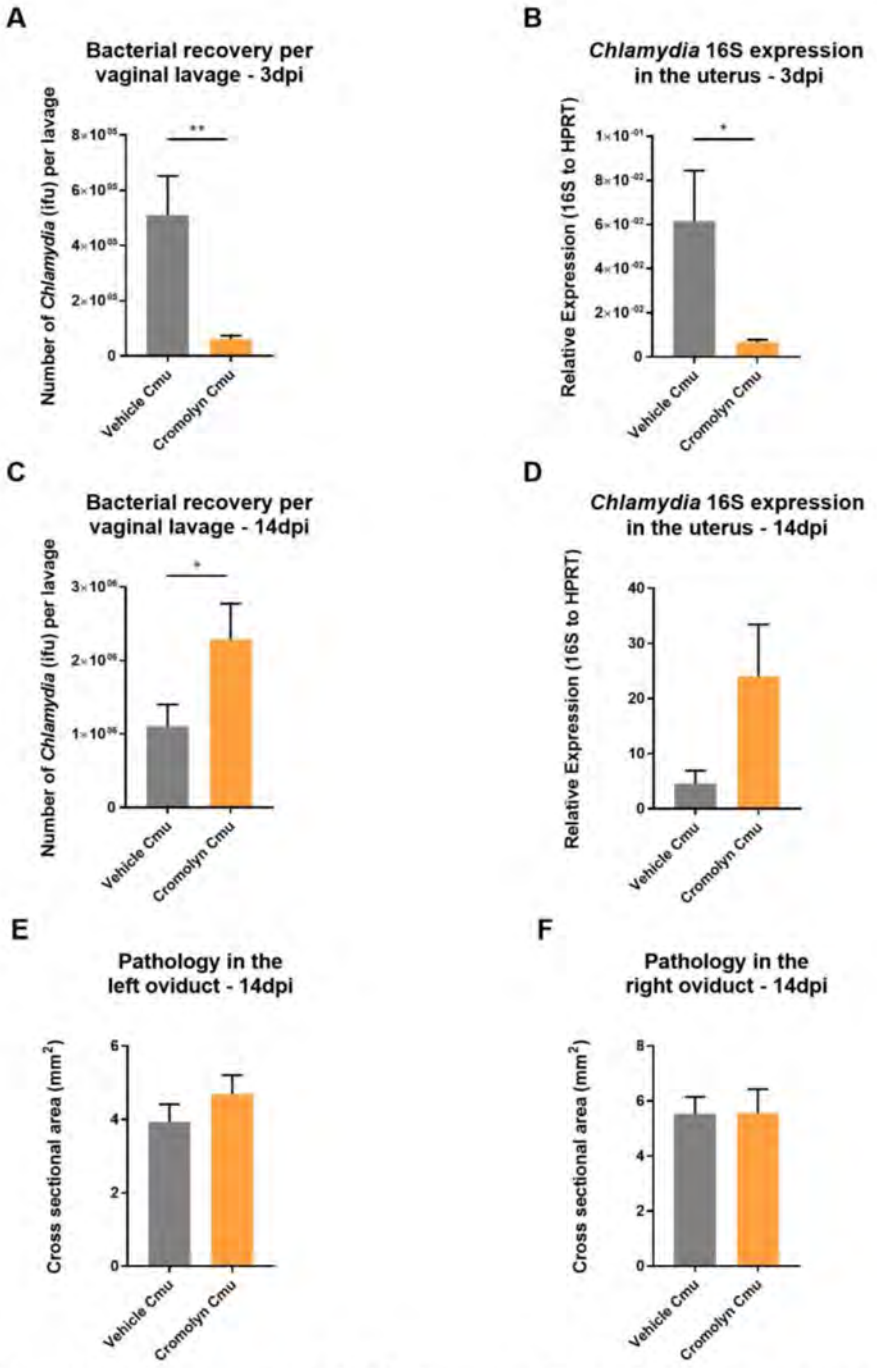


Figure 2.13 – Inhibition of mast cell (MC) degranulation by cromolyn treatments throughout infection protects against the early stages of *Chlamydia* FRT infection but is largely detrimental at later stages. Female wild-type C57BL/6 mice were treated with progesterone and infected intravaginally with *Chlamydia muridarum* (Cmu). Starting 2 days before infection, mice were treated daily intravaginally with either 5mg/kg cromolyn or vehicle control. Mice were sacrificed at **(A, B)** 3 days post infection (dpi) and **(C, D, E, F)** 14dpi. **(A, C)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per lavage. **(B, D)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross section area of the **(E)** left and **(F)** right oviducts of were measured using a calliper to evaluate the levels of *Chlamydia*-associated pathology. All data are presented as mean±SEM (n≥7) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and ** representing $p<0.01$.

2.4.11. Inhibition of MC degranulation specifically during the early stages of *Chlamydia* FRT infection protects against *Chlamydia*-induced pathology

I have shown that although cromolyn treatment protects against the early stages of infection, continued treatment is detrimental during the later stages. The ability to administer cromolyn during the different stages of infection allows for the delineation of the early *versus* late effects of MC degranulation on the pathogenesis of infection and infection-induced pathology.

In order to validate the protective effects of cromolyn treatments during the early stages of *Chlamydia* FRT infection, WT mice were subjected to a murine model of *Chlamydia* FRT infection and treated daily intravaginally with the MC stabiliser, cromolyn, or vehicle alone, starting 2 days before infection until 3dpi at which point treatment was ceased (**Figure 2.2 B**). The mice were sacrificed at 14dpi and the levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

Despite observing no significant difference in infection in the vagina (**Figure 2.14 A**) and a small increase in infection in the uterus (**Figure 2.14 B**, p=0.0683), cromolyn-treated mice have reduced *Chlamydia*-induced hydrosalpinx (**Figure 2.14 C-D**; p=0.1159 and p<0.05) at 14dpi.

These results suggest that the early inhibition of MC degranulation using cromolyn confers partial protection against *Chlamydia*-induced pathology. Whilst the observation of (slightly) increased *Chlamydia* burden and lower level of pathology might appear contradictory, some of the immune responses induced during *Chlamydia* FRT infection mediate clearance of infection but are also responsible for infection-induced pathology. Therefore, in future studies, the immune cells present in the uterus of vehicle- and cromolyn-treated mice will be analysed using flow cytometry, in order to determine if early cromolyn treatments alter the recruitment of key immune cells to the uterus of *Chlamydia*-infected mice.

The protective effect of cromolyn appears stronger in the right oviduct compared to the left oviduct, which could be due to the complex architecture of the FRT. Similarly, small differences

in infection, pathology and/or immune responses have already been identified between the left and right sections of the FRT, as shown in previous studies in our laboratory (data not shown) and by others (395).

Whilst my findings using cromolyn might be explained by the fact that intravaginal treatments may not cross the cervix and, therefore, are only affecting MCs in the lower FRT, the differential effects observed in MC-deficient and cromolyn-treated mice is congruent with the fact that MCs release a wide variety of compounds that have pleiotropic of effects. In this regard, my findings highlight the importance of further studying the function of individual MC mediators, especially those released during degranulation, on *Chlamydia* infection.

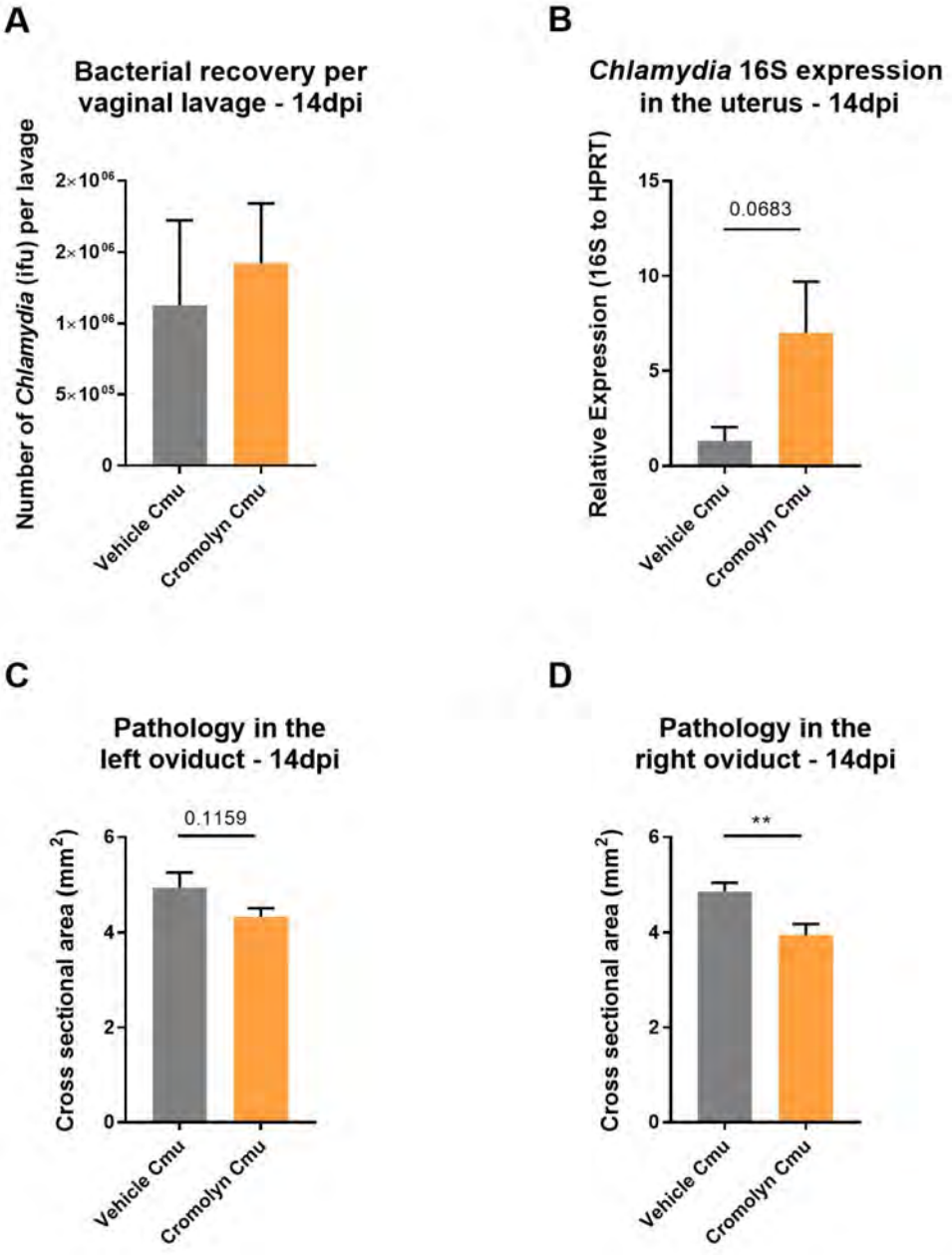


Figure 2.14 - Inhibition of mast cell (MC) degranulation by cromolyn treatments during the early stages of *Chlamydia* FRT infection protects against *Chlamydia*-induced pathology but not alter levels of infection in the vagina and uterus. Female wild-type C57BL/6 mice were treated with progesterone and infected intravaginally with *Chlamydia muridarum* (Cmu). From 2 days before infection until 3dpi, mice were treated daily intravaginally with either 5mg/kg cromolyn sodium salt or vehicle control. Mice were sacrificed at 14dpi. **(A)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per vaginal lavage. **(B)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross section area of the **(C)** left and **(D)** right oviducts were measured using a calliper to evaluate the levels of Chlamydia-associated pathology. All data are presented as mean±SEM (n≥7) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with ** representing $p<0.01$.

2.5. Discussion

The balance between different immune responses in the FRT has been shown to be critical in determining the clearance of *Chlamydia* infection and development of *Chlamydia*-associated pathology. MCs are long-lived tissue-resident cells that can be found in the FRT and have been shown to mediate immune responses during a number of bacterial infections (189, 191, 262, 367). In this chapter, I sought to firstly confirm the presence of MCs in the FRT and then investigate the roles of these MCs and their degranulation in *Chlamydia* FRT infection and infection-induced pathology.

Firstly, I show that MCs are widespread throughout the FRT (**Figure 2.3**). These findings are supported by studies identifying MCs in different organs of the FRT in several species, including humans (318, 322-325, 329, 335). In the vagina, MCs are located just beneath the epithelium while, in the uterus, MCs are mostly situated in the myometrium rather than in association with the endometrium. I show that MCs form a heterogeneous population within the FRT, with alcian blue/safranin staining revealing heparin-positive MCs, chondroitin sulfate-positive MCs and an intermediate population of double positive MCs. This is in accordance with what has already been observed in the FRTs of mice and rats (329, 335). In rodents, two distinct population of mature MCs have been described, with [1] CTMCs, which store the tryptases mMCP6 and mMCP7 and the chymases mMCP4 and mMCP5 bound to heparin proteoglycans, constitutively present and [2] MMCs, which store the chymases mMCP1 and mMCP2 bound to chondroitin sulfate proteoglycans, present following T cell-mediated signalling (200, 396, 397). However, MCs appear to be much more complicated than this simple classification, with MC micro-environment, tissue location, inflammatory states and mouse strain major determining factors that regulate the phenotype of MCs (252, 398, 399). Indeed, both tracheal MMCs and CTMCs of helminth-sensitized and challenged BALB/c mice store mMCP1, mMCP2, mMCP4, mMCP5, mMCP6, mMCP7 and CPA3 (252). An intermediate population of MCs that is positive for both heparin and chondroitin sulfate could represent a different stage in maturation and/or differentiation or could represent MCs that are undergoing trans-differentiation, a process by which the

glycosaminoglycans and MC proteases stored in MC granules change upon environmental stimuli (329, 400). In the FRT, MCs appear widespread, heterogeneous and highly plastic, which could allow them to carry out a variety of functions, from supporting pregnancy (329, 331, 339, 349), to mediating immune responses to infection (367, 368).

The precise nature of the influence of the menstrual cycle on MC number and activation in the FRT remains unclear (318, 324, 327, 332, 333). Similarly, the presence of the female sex hormone receptors ERα, ERβ and PR on MCs is still largely uncertain as different studies have shown different results (318, 332-334). Furthermore, regardless of whether MCs express these receptors or not, they could still indirectly be affected by female sex hormones through the changes in environmental cytokine and chemokine release driven by these hormones (401-403). I enumerated MCs in the myometrium of mice seven days following female sex hormone treatments and observed that oestrogen-treated mice have less MCs in their uterus compared to progesterone-treated mice. I also showed that the degranulation of uterine MCs was not affected by hormone treatment. These results suggest that progesterone may induce recruitment and/or local expansion of MCs in the FRT, or that oestrogen may suppress these responses (**Figure 2.4**). The increase in MCs seven days following progesterone treatment (*i.e.* the day of infection in the mouse model of *Chlamydia* infection) might suggest a detrimental role of MCs in host immune responses to *Chlamydia muridarum*, as oestrogen-treated mice are resistant to infection (90, 388). However, it is likely that oestrogen-mediated protection against *Chlamydia* is connected to a wide number of changes in the FRT, ranging from tissue morphology, epithelial cell responses, release of cytokines and chemokines or immune cell recruitment, rather than a decrease in MC number (401-404). The lower number of uterine MCs in oestrogen-treated mice was unexpected as some studies did not find differences in the number of MCs during the menstrual cycle and/or following hormonal treatments, while others show increased numbers of MCs in the FRT when oestrogen levels are high. Indeed, Jensen et *al.* showed that the number of uterine MCs was slightly higher following oestrogen treatment than with progesterone treatment (332). Similarly, other studies have shown higher number of uterine MCs during oestrogen-dominated oestrus compared to progesterone-dominated diestrus (329, 335), while the use of progesterone contraceptive has been

shown to decrease the number of endometrial MCs (334). In addition, oestrogen levels in the blood positively correlate with the number of cervical MCs in horses, even if no correlation was observed in the vagina or uterus (324). While *in vitro* studies reveal that oestrogen can increase MC degranulation (332, 333, 354), few *in vivo* studies have investigated whether MC degranulation is affected by female sex hormones. However, there is evidence to suggest that MC degranulation might be increased in endometrial tissues at the beginning of, and during, menstruation (336, 338). Therefore, my results appear to differ from what has been shown in the literature regarding number and degranulation of MCs in the FRT. However, no other study followed the same experimental protocol, using a variety of different animal models (323, 324, 329, 332, 335), no female sex hormone treatment (324, 329, 335) or different female sex hormone treatment regimens (332). In addition, differences in results could also be caused by the tissue fixation method used (405-407). While using flow cytometry could be used to measure the number of MCs as well as to evaluate their activation status (262), we did not obtain reliable staining for uterine MCs in our flow cytometry experiments (**Appendix, Figure 6.4**). Given the time constraints of my PhD, I was not able to optimise a technique to measure MCs using flow cytometry. In future studies, this technique will be optimised to validate our results. Vaginal smears will also be collected at the endpoints to confirm the stage of oestrus cycle for each mouse (329).

Although MCs are most well documented for their detrimental role in allergy (189), it is now recognised that MCs are involved in a variety of physiological and pathophysiological processes (189, 191). Importantly, MCs have been shown to mediate host responses to a range of bacterial, viral and parasitic infections, in a wide variety of tissues (253-256, 258-261). Although MCs are sometimes recruited to the infectious site, I show that *Chlamydia* infection does not induce the recruitment of MCs in the uterus. Indeed, no change in the number of uterine MCs were present at 3dpi (**Figure 2.5**), concordant with findings in another study that identified no difference in the number of uterine MCs between infected and naïve mice, as well as between mice infected once or five times with *Chamydia muridarum* (368). Similarly, *Chlamydia pneumoniae* infection appear to not alter the number of MCs in the aortas and lungs during a respiratory tract infection

(262, 408). Therefore, we propose that *Chlamydia* infection does not stimulate recruitment and proliferation of MCs in the FRT. The trend towards a reduction in MC number observed at 14dpi in my studies might suggest that adaptive immune responses and/or other effects induced by *Chlamydia* infection may affect MC tissue homeostasis, however, given that my findings contradict those in previous studies, more experiments are required to validate my findings and such studies were not able to be completed within the timeframe of my studies.

Chlamydia pneumoniae infection was shown to amplify the activation of MCs in the aortas and lungs of mice (262, 408). Similarly, group B *Streptococcus* induces MC degranulation after intraperitoneal infection and are proposed to have similar effects following intravaginal infection (367). Although *Chlamydia* infection did not appear to alter MC degranulation at 3dpi and 14dpi in my studies, it is possible that degranulation was altered at different stages of infection, potentially in the very early stages following *Chlamydia* infection, indicating that additional time points might be required in future studies.

Despite MCs being widespread in the FRT, little is known on their roles during STIs. Therefore, to investigate the role of MCs during *Chlamydia* infection, MC-deficient mice were subjected to a *Chlamydia* FRT infection model. Although MC-deficient mice have lower bacterial burden in their vagina and uterus compared to WT mice, high variability between samples meant that the differences I observed did not reach statistical significance (**Figure 2.6**). Another study correspondingly showed that MC-deficient mice $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ and WT controls have similar levels of *Chlamydia* infection in their vagina at 7dpi, 14dpi, 21dpi and 28dpi (368). This finding might appear surprising given [1] the fact that MCs have been shown to promote the clearance of Group B *Streptococcus* vaginal infection (367) and of *Chlamydia pneumoniae* respiratory infection (262) and [2] the range of antibacterial properties of MCs that include secretion of antimicrobial compounds, phagocytoses, ROS production and creation of MC extracellular nets (291-294). However, during *Chlamydia* FRT infection, it is unlikely that MCs are directly in contact with *Chlamydia* given their location at distance of the epithelium in the FRT. It is however possible that MCs indirectly affect susceptibility to *Chlamydia* infection through mediating pro-inflammatory processes. While higher number of mice could not be bred in the timeframe of my

thesis, increasing the number of mice in future studies could allow to definitely conclude on the effect of MCs in mediating clearance of *Chlamydia* infection in the vagina and uterus.

Despite an uncertain role for MCs in mediating clearance of *Chlamydia* infection, my results highlight that MCs might play an important role in the pathogenesis of *Chlamydia* infection-induced pathology. Indeed, MC-deficient mice are protected against *Chlamydia*-induced hydrosalpinx at 14dpi (**Figure 2.6**). Therefore, my work, as well as that performed by others (368), suggest that MCs might be important contributor to the development of *Chlamydia*-induced diseases in the FRT.

Upon activation, MCs degranulate and release biologically active preformed mediators contained in their secretory granules. Shortly after, MCs generate and release neoformed compounds such as the lipid mediators prostaglandins and leukotrienes. Hours later, MCs produce cytokines and chemokines, allowing a more specific response to the stimulatory factor (189, 199). All of these mediators have potent effects on the local environment, conferring a plethora of different functions to MCs. One of the main effects shared by many of those factors is the induction of immune cell recruitment. For instance, the recruitment of neutrophils and eosinophils to the infection site can be induced by release of mMCP6, leukotrienes, IL-6 and TNFα from MCs following bacterial and parasitic infections (253, 272-275, 377). To assess if MCs mediate immune cell recruitment to the upper FRT following *Chlamydia* infection, flow cytometry was performed to profile and enumerate the immune cells in the uterus of MC-deficient mice and WT controls. Overall, the total number of uterine cells, as well as the number and percentage of immune cells in the uterus, were comparable between MC-deficient mice and WT controls (**Figures 2.7 and 2.8**). While similar levels of resident monocytes and macrophages, myeloid DCs and plasmacytoid DCs are present in the uterus of *Chlamydia*-infected, MC-deficient and WT control mice, the numbers and percentages of inflammatory monocytes and macrophages, neutrophils and eosinophils were slightly decreased in MC-deficient mice. Those reductions did not reach significance, most likely because of the small sample size used, which was a result of the need to combine samples from different mice due to the low number of cells obtained after the digestion of the uterine tissue. Increasing the number of mice would allow more reliable

results. I expect that the decrease in inflammatory monocytes and macrophages, neutrophils and/or eosinophils would reach significance with a greater sample size. In support of my findings, MCs are known to be critical in inducing immune cell recruitment following other bacterial infections, as shown in the context of *Klebsiella pneumonia* and *Pseudomonas aeruginosa* respiratory infections or *Escherichia coli* urinary tract infection (253, 254, 262, 392). Moreover, MC-deficient mice have previously been shown to decrease the number of macrophages and neutrophils in bronchoalveolar lavage fluid and lungs of mice with *Chlamydia pneumoniae* respiratory infection (262). In addition, a number of mediators produced by MCs, including histamine, tryptase, leukotrienes B_4 and prostaglandin D_2, have been positively associated with eosinophil migration and activation (265, 409-412). Although IL-4 production by eosinophils has previously been shown to be protective against uterine pathology following *Chlamydia trachomatis* infection (140), the role of eosinophils during *Chlamydia* infection is yet to be fully characterised. Given that both excessive infiltration of neutrophils (127, 133) and secretion of pro-inflammatory cytokines such as IL-1β and TNFα by monocytes and macrophages (149, 413) have been associated with induction of immunopathology in the upper FRT, I propose that MC-mediated infiltration of innate immune cells to the *Chlamydia*-infected upper FRT may be a key trigger for infection-induced oviduct pathology.

MCs are also recognised for mediating adaptive immune responses following infection. Indeed, they can directly present antigens to T cells *via* their MHC complexes (272, 279). In my studies, similar numbers of uterine T cells, $CD4^+$ T cells and $CD8^+$ T cells are present in MC-deficient mice and WT controls (**Figures 2.7 and 2.8**). Previous studies have shown that MCs can recruit DCs to infected tissues and prompt their migration to the draining lymph nodes, thereby allowing presentation of antigens to naïve T cells and priming specific T cell responses (254, 260, 279, 281, 282, 379). While I did not observe changes in the number of DCs and T cells present in the uterus of MC-deficient mice (**Figures 2.7 and 2.8**), another study has revealed that *Chlamydia*-infected, MC-deficient mice ($Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$) have a reduced infiltration of DCs in their FRT-draining lymph nodes (368). Moreover, they also show that splenocytes isolated from *Chlamydia*-infected MC-deficient mice release less IFNγ, IL-10, IL-13 and IL-17 upon

stimulation by inactivated *Chlamydia muridarum* compared to WT controls (368), indorsing the hypothesis that MCs might contribute to the migration of antigen presenting cells into the FRT-draining lymph nodes, hence potentially promoting the induction of specific adaptive immune responses against *Chlamydia* infection (368). The other study investigating the roles of MCs during *Chlamydia* FRT infection became available just recently, therefore their results did not orientate my research (368). However, in future studies, profiling the DCs and T cells in the uterus and FRT-draining lymph nodes, at early and late stages of *Chlamydia* infection, could be informative to assess the dynamic of MC-mediated DC recruitment to the lymph nodes and potential effect on priming naïve T cells.

Although I did not observe any differences in the expression the transcription factors Tbet, GATA3, RORγt and FOXP3 in the FRT of MC-deficient mice infected with *Chlamydia* compared to WT controls (**Figure 2.11**) (394), suggesting that MCs do not alter Th phenotypes during *Chlamydia* infection, additional studies are required to investigate the specific expression of these factors in uterine T cells using flow cytometry and characterise if MCs mediate Th-dominant phenotype in the uterus during *Chlamydia* FRT infection.

To establish the mechanism by which MCs might contribute to the proliferation and/or recruitment of innate immune cell to the uterus following *Chlamydia* infection and/or contribute to oviduct pathology, the levels of expression of various cytokines and chemokines were assessed. However, the expression of the main mediators that have been shown to be involved in mediating protective or detrimental responses for clearance of *Chlamydia* infection and in infection-induced pathology, such as IFNγ, TNFα, IL-1β, IL-12 and IL-17 remained similar between MC-deficient mice and WT controls, with only the chemokine CXCL15 reduced in MC-deficient mice (**Figure 2.12 A-M**). CXCL15 is a chemokine important in neutrophilic inflammation (414-416), that is expressed in the epithelium, endometrium and endometrial crypts of the uterus (417). I propose that MCs might directly or indirectly stimulate the expression of CXCL15 in the uterus, thereby favouring recruitment of neutrophils and causing immunopathology. While MMP9 has been extensively associated with development of *Chlamydia*-induced pathology in the oviduct and is known to be released by neutrophils and MCs, similar levels of expression is observed between

MC-deficient mice and WT controls (**Figure 2.12 N**) (135, 136, 313). However, some MC mediators, including MC proteases, are known to activate MMP9. Therefore analysing the activation of MMP9 sould be performed in order to determine whether MC-mediated activation of MMP9 is contributing to pathology (313). Whilst only a slight decrease in the expression of the protease-activated receptor (PAR) 2 is observed in MC-deficient mice (**Figure 2.12 R**), this receptor, expressed by a variety of cells such as epithelial cells and fibroblasts, can be activated by MC proteases and mediate a range of immune responses to infection (418, 419). Therefore, it is possible that a decrease in PAR2 activation in MC-deficient mice is responsible for decreasing the immune cell recruitment to the uterus.

Despite observing some changes in immune factor expression during *Chlamydia* infection in MC-deficient mice compared to WT controls, overall, I did not observe any pronounced effects that might explain the decreased cellular infiltration and pathology observed in these mice. Therefore, the mechanisms by which MCs might induce infiltration of neutrophils, eosinophils, monocytes and macrophages to the uterus remains uncertain. However, it should be noted that I only examined gene expression. It is possible that changes at the protein levels are present and could explain the decrease in recruitment of eosinophils, monocytes and macrophages to the uterus of MC-deficient mice and/or the reduced hydrosalpinx in MC-deficient mice. Therefore, in future studies, evaluating the protein levels of key immune factors will be necessary to verify if MC deficiency is associated with changes in immune factors associated with protection and/or susceptibility to infection and/or *Chlamydia*-induced pathology. It is possible that some of the potent preformed and neo-formed mediators of MCs, such as biogenic amines, lipid mediators and MC proteases, directly contribute to innate immune cell infiltration as MC proteases, serotonin, prostaglandin E_2 and leukotrienes B_4 have been shown to favour recruitment of neutrophils, monocytes or eosinophils (265, 271, 274, 275, 420-422).

The bone marrow haematopoietic environment plays a key role in the mediating innate and adaptive immune cell responses (423). To establish if the reduction in innate immune cells in the uterus was caused by a decrease in haematopoiesis, I profiled immune cell responses in the bone marrow of MC-deficient and WT mice, infected with *Chlamydia*. The number and percentage of

inflammatory monocytes and macrophages was increased, and the number and percentage of eosinophils decreased in MC-deficient mice (**Figures 2.9 and 2.10**). It is possible that the decrease in the recruitment of inflammatory monocytes and macrophages cells into the FRT during infection in MC-deficient mice may result in their increased accumulation in the bone marrow. The reduced numbers of eosinophils in the FRT of MC-deficient mice could be the result of decreased eosinophil haematopoiesis that I observed. Supporting this, MCs are known to be a major source of chymase and of cytokines and chemokines, such as IL-3, IL-5 and GM-CSF, which have been shown to be important for eosinophils differentiation, survival, mobilisation in the blood stream, tissue-infiltration and/or activation (424-426).

In addition to eosinophils, the numbers of γδ T cells and B cells are reduced in the bone marrow of MC-deficient mice during *Chlamydia* infection compared to WT controls (**Figures 2.9 and 2.10**). It is possible that both B cells and γδ T cells are strongly recruited to the uterus, leading to a reduction in the number of those cells in the bone marrow, or that these cells might be reduced in the bone marrow because of a decrease in haematopoietic signalling in MC-deficient mice. Regretfully, these two cell populations were not analysed in the uterus. While not investigated in the context of *Chlamydia* FRT infection, γδ T cells are known to prompt maturation of macrophages and DCs, as well as to induce macrophages recruitment through regulation of specific chemotactic mediator and secretion of IFNγ, TNFα and IL-17 (427). Importantly, B cells are known to confer protection against reinfection with *Chlamydia*, through the production of *Chlamydia*-specific antibodies and/or priming of $CD4^+$ T cells by antigen presentation in the draining lymph nodes (160, 162, 428). Therefore, it is possible that the reduction in B cells in MC-deficient mice may result in an increase in susceptibility to reinfection.

One of the limitation of this study is the lack of sham-infected controls. To address this issue, in future studies, the differences in immune responses between sham-infected, MC-deficient mice and WT controls, will be analysed in order to determine if the differences observed between *Chlamydia*-infected MC-deficient mice (and WT controls) are caused by MC deficiency alone or if they are mediated by *Chlamydia* infection. Another limitation of this study is the use of the genetically modified *Cpa3-Cre;Mcl-1*$^{fl/fl}$ strain of mice to assess the role of MCs during

Chlamydia infection. Indeed, even if these mice have 92% to 100% MC deficiency, they also have a mild anaemia and a secondary diminution in basophils (237). Therefore, basophils could be involved in mediating some of the effects observed in this study, as they have been shown to alter immune responses to infections (429, 430). However, one of the advantage of this strain of mice is that the deficiency in MCs is independent of the loss of the *Kit* gene, unlike for $Kit^{W}/Kit^{W\text{-}v}$ or $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice (237, 431). Indeed Kit has multiple functions unrelated to MCs, therefore loss of this gene have a plethora of cascading consequences that can affect immune and other responses independently of MCs. For instance, $Kit^{W}/Kit^{W\text{-}v}$ mice are sterile, anaemic and have reduced neutrophils. Whilst $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice are fertile and not anaemic, their myeloid and megakaryocytic cells proliferate in the spleen, and higher numbers of Tregs and neutrophils are reported in these mice (431-435). In *Cpa3-Cre;Mcl-1*$^{fl/fl}$ mice, monocytes, eosinophils and neutrophils have been shown to be not altered in blood and bone marrow, with only a small increase in splenic neutrophils observed (237). Therefore, these mice are considered as a better model for assessing the effects of MCs. However, since the mice used in my study are deficient in MCs throughout their entire life, major effects of MC deficiency on normal immune development in these mice, rather than a local effect of MCs in the FRT during infection, cannot be ruled out as the cause for the phenotypic changes that I saw in with MC-deficient mice in response to *Chlamydia* FRT infection.

To assess the role of MC degranulation during *Chlamydia* infection, mice were treated intravaginally with the MC stabiliser cromolyn. I show that treatment with cromolyn protects against the early stage of *Chlamydia* infection, revealing a potential detrimental role of MC degranulation during the early stages of infection. However, contrary to what I observed in MC-deficient mice, cromolyn did not protect against *Chlamydia*-induced pathology, and even resulted in increased bacterial burden in the vagina at 14dpi (**Figure 2.13**). The difference in responses between MC-deficient mice and cromolyn-treated mice could be due to the fact that the MC-deficient mice, *Cpa3-Cre; Mcl-1*$^{fl/fl}$, also have a reduction in basophils (237). Indeed, basophils have been shown to be important in immune response to pathogens, such as *Nippostrongylus brasiliensis*, and are known to mediate Th responses (436, 437); therefore they could contribute,

at least partially to the phenotype observed in MC-deficient mice. The differential effects may also be due to the fact that cromolyn treatment is likely to only target MCs in the vagina and cervix as we have observed that intravaginal treatments cannot reach the upper FRT (data not shown). However, the differences in response between MC-deficient and cromolyn-treated mice may also reveal a differential role for MCs and MC degranulation, as the variety of factors released by MCs might have differential effects during *Chlamydia* FRT infection. Since a strong protective effect of cromolyn treatments was observed in the early stages of infection, we decided to alter the treatment regimen aiming at only inhibiting MC degranulation during the early stages of infection. This treatment conferred partial protection against *Chlamydia*-associated pathology, hence reinforcing the idea of a detrimental role of MC degranulation during the early stages of *Chlamydia* infection (**Figure 2.14**). Overall, these results represent a proof of principle study showing that MCs can be pharmaceutically targeted to protect against *Chlamydia* FRT infection. In future studies, intravaginal treatments with the compound 48/80 could be given to assess the effect of MC degranulation and validate results obtained with MC degranulation.

Overall, my results indicate a possible differential function of MCs and MC degranulation. Indeed, MCs are expected to have a plethora of different pro- and anti-inflammatory activities *in vivo*, with redundancy and antagonism between preformed mediators, neoformed mediators and secreted cytokines and chemokines. For instance, some of the factors produced by MCs can enhance vascular permeability (268, 438), alter cell junctions (296), activate MMPs (313, 314) and degrade extracellular matrix components (233, 439), hence enhancing the pro-inflammatory environment. By contrast, some MC mediators exhibit anti-inflammatory activities. For instance the β-chymase mMCP4 degrades the cytokines IL-33 and TNFα, which is has been shown to protect against airway inflammation in the lung and promote survival during sepsis (307, 309). Similarly, heparin has been shown to be able to reduce eosinophil infiltration following nasal allergen challenge and during oedema formation (277, 278, 440). Therefore, studying the effects of individual factors produced by MCs is necessary to fully characterise the function of MCs during *Chlamydia* infection. Consequently, Chapter 3 will focus on investigating specifically the roles of some MC proteases during *Chlamydia* FRT infection.

Altogether, my data reveal that MCs and/or MC degranulation play a detrimental role during *Chlamydia* FRT infection by increasing susceptibility to infection during the early stages, and contributing to the development of *Chlamydia*-associated pathology in the later stages of infection. The mechanisms by which *Chlamydia* infection induces the activation of MCs remain unclear. Although *Chlamydia* infection does not increase the number and the degranulation of uterine MCs at 3 or 14dpi, this does not rule out effects at different time points not examined during my studies. It is also possible that MCs act through their release of cytokines and chemokines such as TNFα, CXCL1 and CXCL2 (191, 441). *In vitro* studies show that degranulation of human cord blood derived MCs does not occur following incubation with *Chlamydia trachomatis*, but that these MCs produce a range of cytokine and chemokines, including TNFα, IL-1β, IL-6, GM-CSF and IL-8/CXCL8, in response to the bacteria (368). However, it is unlikely that the activation of MCs only occurs through contact between MCs and *Chlamydia in vivo*. Instead, it is likely that infection-induced MC activation occurs following stimulatory signals produced by infected epithelial cells and/or resident immune cells following detection of *Chlamydia* infection. Therefore, *in vivo*, *Chlamydia*-induced activation of MCs is likely mediated by complex interactions between a number of potential pathways including IgE and IgG binding to FcεRI or FcγR, PAMP-induced TLR signalling, complement-mediated signalling in MCs as well as cytokines and chemokines induced in response to *Chlamydia* infection in the FRT (188, 191).

2.6. Conclusion

In summary, my study reveals roles for MCs and MC degranulation in *Chlamydia* FRT infection and in the development of *Chlamydia*-induced pathology. Even if the mechanism that underpins these effects is yet to be elucidated, this study has improved our understanding of the roles of MCs during FRT infection by highlighting that MCs are an important, previously underappreciated, player in mediating immune responses during infection. Given that MC degranulation appears to play a very important role in MC-mediated response, and that MCs granules contain a wide number of proteases that may have differential effects on infection and

infection-induced pathology, the next chapters of my thesis will focus on determining the roles that individual MC proteases play in the pathogenesis of *Chlamydia* FRT infection

Chapter three: The role of mast cell proteases during *Chlamydia* female reproductive tract infection

3.1. Abstract

Infections by *Chlamydia trachomatis* are responsible for severe diseases in women, including pelvic inflammatory disease and tubal factor infertility. The complexity of the immune responses in the FRT and the fact that some immune responses can be protective and detrimental have hampered the development of novel preventions and/or treatments. In Chapter 2, the presence of MCs in the FRT was characterised and a new role for MCs in mediating *Chlamydia* FRT infection and pathology was revealed. Given that MCs possess numerous secretory granules containing a range of potent mediators, with MC proteases, comprising mMCP4, mMCP5, mMCP6, mMCP7 and Cpa3, being the most abundant mediators in MC granules, this chapter will aim at identifying the roles of some of these MC proteases during *Chlamydia* FRT infection to further characterise the function of MCs during infection.

Firstly, the pattern of expression of key MC proteases was investigated. The expression of mMCP4, mMCP5, mMCP6 and Cpa3 was increased by oestrogen treatments suggesting that menstrual cycle/reproductive state regulates MC phenotype and/or protease expression in the FRT. While the expression of those MC proteases was not altered in the early stages of *Chlamydia* infection, at later stages, their expression was reduced. The expression of Ndst2, an enzyme necessary for sulfation of heparin, remain unchanged by female sex hormones and *Chlamydia* infection.

To investigate the roles of MC proteases during *Chlamydia* infection, Ndst2-deficient mice, that lack the fully sulfated heparin required for storage of many MC proteases in the secretory granules of MCs, were subjected to intravaginal *Chlamydia muridarum* infection. These mice displayed increased *Chlamydia* burden in their uterus but were protected against hydrosalpinx. Moreover, a reduction in the number of both innate and adaptive immune cells was observed in

their uterus, suggesting that heparin-protease complexes might contribute to the clearance of *Chlamydia* by mediating the migration of protective cells, such as CD4+ T cells, but might also contribute to the development of immunopathology by mediating the infiltration of detrimental cells, such as neutrophils. Ndst2-deficient mice also displayed a decrease in the expression of the transcription factors Tbet and RORγt at 3dpi, suggesting a potential role of heparin-proteases complexes in inducing type 1 and type 17 responses, that have been associated with protection against *Chlamydia* infection and development of pathology, respectively.

To investigate the individual roles of specific MC proteases, the chymase mMCP5 and the tetramer-forming tryptases mMCP6 and mMCP7, a serie of genetically modified mice that are deficient for some of these factors were subjected to intravaginal *Chlamydia muridarum* infection Overall, the effect of deficiency for each MC protease is diverse. While, mMCP6 might not play a major role during *Chlamydia* infection, mMCP7 might offer slight protection against pathology and mMCP5 might be detrimental to the early stages of infection. Taken together, these data suggest that MCs proteases mediate either protective or detrimental responses to *Chlamydia* infection and that individual proteases differentially affect the course of infection and/or the development of associated pathology.

Characterising the mechanisms of action and elucidating the exact MC mediator(s) that mediate protection against infection, *versus* those contribute to the development of *Chlamydia*-associated pathology, may identify new therapeutic targets for the prevention and/or treatment of *Chlamydia* infection and associated disease.

3.2. Introduction

As previously described, infection by *Chlamydia trachomatis* can cause irreversible damages to the fragile FRT tissues of women (23). The complexity of the host immune responses associated with clearance/susceptibility to *Chlamydia* infection and/or development of associated pathology have prevented the development of a novel prophylaxis and/or alternative therapies against *Chlamydia* infection. MCs are large tissue-resident immune cells that I and, recently others, have identified as playing a role during *Chlamydia* FRT infection and in the development of *Chlamydia*-induced pathology (**Chapter 2** and (368)), however, the mechanisms involved remain to be fully resolved.

MCs are involved in a variety of immune functions, including immune cell recruitment (188, 266, 273, 275, 442, 443), and activation of DC maturation and migration to the lymph nodes to promote adaptive immune responses (260, 281, 282, 284). While largely recognised for their roles in Th2-mediated allergic responses (284-286), MCs have been shown to stimulate the conversion of Th2 cells into Tregs (444), as well as to promote Th1 and Th17 responses in response to *Leishmania* infections (260). Whilst MCs are relatively scarce in most tissues, they contain numerous secretory granules that are full of a wide variety of preformed mediators including serglycin proteoglycans, histamine, serotonin, cytokines and MC proteases that have very potent effects (190 135). Given the sheer number of effects that MCs have been shown to play important roles in, a greater understanding of the effects specific MC factors have during *Chlamydia* FRT infection is required to better understand the mechanisms that underpin the effects of MCs on infection and infection-induced pathology. My PhD studies have focused on the effects that MC proteases play in MC-mediated responses.

MC proteases are the main preformed mediators stored in MCs. They are estimated to represent approximately 50% of all proteins in MCs (199). MC proteases are synthesized as inactive pre-pro-enzymes. Their pre-peptides, that serve as signal peptides, and their pro-peptides, that serve as activation peptides, are proteolycally cleaved before the storage of MC proteases in the secretory granules of MCs in their mature enzymatically active form (202-204).

In humans, MC proteases include the α-chymase, CMA1, α- and β-tryptases as well as the metalloprotease, Cpa3. In mice, the main MC proteases are the α-chymase, mMCP5, the β-chymases, mMCP1, mMCP2 and mMCP4, the tetramer-forming tryptases mMCP6 and mMCP7 and the metalloprotease, Cpa3 (199, 200). While MCs have often been classified as MC_T, MC_C and MC_{TC} in humans based on their MC protease content, and as CTMC and MMC in rodents based their tissue location (200, 318), the phenotype of MCs is highly heterogeneous. MC phenotype is mediated by local environmental conditions, which are determined by specific tissue location, cytokine levels and infection/inflammatory status (202, 252, 305). This means that the protease content of MCs varies greatly from tissue to tissue and is heavily dependent upon the status of the local tissue environment.

Most of the proteases that are stored in the secretory granules of MCs are tightly bound to highly negatively charged serglycin proteoglycans formed by heparin or chondroitin sulfate chains (244, 245, 445) with mMCP1 forming bonds with chondroitin sulfate proteoglycans, whilst mMCP4, mMCP5, mMCP6, mMCP7 and Cpa3 form bonds with heparin proteoglycans (202, 244, 245, 446). Upon degranulation, most of the proteoglycan-protease complexes remain intact, which protects the proteases from protease inhibitors in the surrounding extracellular environment for a relatively long period of time (202, 221). However, some MC proteases, such as mMCP7, have a lower positive charge in their heparin-binding sites, and, upon MC degranulation, they dissociate from their proteoglycans, diffuse and reach the blood circulation (221, 223). Importantly, the enzyme Ndst2 is key for the sulfation of heparin sulfate and heparin in MCs, and Ndst2 deficiency result in abnormal storage of the MC mediators that are normally bound to heparin in the secretory granules of MCs, including histamine and the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3 (244, 245).

The major MC proteases are trypsin-like serine proteases termed tryptases that can specifically cleave substrates after positively charged amino acids such as arginine and lysine (208, 214). In humans, the tryptase locus is positioned on the chromosome 16p13.3 with genes coding for a variety of tryptases, including the αI-, βI-, βII-, βIII-, γ- and δ-tryptases. In mice, the corresponding locus is found on the chromosome 17A3.3 and encodes for 13 functional tryptases

including mMCP6 and mMCP7 (447, 448). They are expressed in a strain-dependent manner, with C57BL/6 mice naturally deficient in mMCP7 protein due to the presence of an early stop codon in the *Mcpt7* gene (227, 449). In the secretory granules of MCs, α- and β-tryptase (human) and mMCP6 and mMC7 (mice) are stored as homo- or hetero-tetramers bound to serglycin proteoglycans (450). Both mMCP6 and mMCP7 have been shown to have some overlapping substrate specificity, including the capacity to cleave the α-chain of fibrinogen in plasma (222, 303).

Other MC proteases are chymotrypsin-like serine proteases termed chymases that possess a wide S1-pocket allowing cleavage of large and aromatic non-polar hydrophobic residues, such as phenylalanine or methionine (208, 234). They are often stored in the secretory granules of MCs as active monomers tightly bound to serglycin proteoglycans (451). In humans, the chymase locus is located on chromosome 14 and encodes only 4 functional genes, with only 1 coding for a MC protease, the α-chymase, CMA1. The mouse locus is approximately 3 times bigger and encodes for 14 genes, including the MC-specific α-chymase, mMCP5, and β-chymases mMCP1, mMCP2, mMCP4 and mMCP9 (230, 231). While mMCP5 has the most similar amino acid structure to the human CMA1, a mutation in its active site changes its enzymatic activity from chymotrypsin-like to elastase-like, characterised by a smaller S1-pocket allowing only restricted cleavage after small-uncharged amino acids (210). On the other hand, mMCP4 possesses the closest substrate specificity to human CMA1 in terms of enzymatic activity and tissue distribution, and is therefore often considered as the functional homolog of human chymase (200, 233, 234).

The MC protease, Cpa3, is a zinc-dependent metalloprotease that cleaves peptide bonds at the end of a substrate, with high affinity for aromatic hydrophobic amino acids (452, 453). Cpa3 is mainly expressed in MCs, but can also be found in basophils (237). It is stored in the secretory granules of MCs as a mature protease bound to serglycin proteoglycans. While little is known with regards to its biological function, Cpa3 could be part of similar enzymatic cascade as chymases, given that chymase-mediated cleavage create Cpa3-susceptible substrate (characterised by hydrophobic amino acids on their end) (208, 234, 452, 453). Moreover, storage

of Cpa3 appears to be strongly associated with storage of α-chymase, as deficiency in one causes a deficiency in the other (239, 240).

The different MC proteases have been shown to mediate a variety of functions *in vivo*. Most MC proteases, including mMCP4, mMCP5, mMCP6 and mMCP7, can proteolytically cleave fibrinogen (222, 233, 239, 303, 305, 306), which can regulate coagulation (222, 303) as well as create small fragments of fibrinogens, that can active pro-inflammatory responses and recruitment of innate and adaptive immune cells (454, 455). For example, mMCP4-mediated fibronectin cleavage has been shown to induce recruitment of macrophages and T cells to the kidney during unilateral urethral obstruction (306), mMCP7-mediated fibronectin cleavage is believed to contribute to IL-6-mediated neutrophil and eosinophil infiltration into the conjunctiva (202). Moreover, MC proteases regulate the activity of diverse range of cytokines and chemokines in both humans and mice and play important roles in the induction and/or modulation inflammatory responses (308-311, 456), with for example, mMCP4 protects against eosinophilic inflammatory responses in the airways through degradation of IL-33 (309). In addition, the chymases mMCP4 and mMCP5 have been shown to activate MMP9, a well-recognised factor associated with both tissue remodelling and inflammation (239, 315), and to contribute to disruption of epidermal tight junctions and inflammation during injury (296, 297).

MC protease-mediated inflammatory responses have been shown to play important roles in the context of a number of infections. For example, chymases have associated with enhanced cleavage of tight junction proteins, increased breakdown of the blood-brain-barrier and exacerbated levels of infection and mortality during Japanese encephalitis virus infection (295). Furthermore, mMCP1 has been shown to be protective against *Trichinella spiralis* gastrointestinal infection, by contributing to the induction of pro-inflammatory responses. Whilst the mechanisms involved were not fully elucidated, mMCP1 could participate in inflammation by activating MMPs or by affecting the epithelium and its permeability through induction of apoptosis and/or proteolysis of tight junctions (300, 301).The chymase mMCP4 has been shown to protect against group B *Streptococcus* infection in the FRT by preventing bacterial attachment to host tissues by cleaving fibronectin (305) and the tryptase mMCP6 plays protective roles during *Klebsiella*

pneumoniae and *Trichinella spiralis* infections by inducing the infiltration of neutrophils and eosinophils to the infectious sites (274, 275).

Given that I have shown a role for MCs in mediating the pathogenesis of *Chlamydia* FRT infections (**Chapter 2**) and the wide variety of function of the different MC proteases, this chapter will investigate the role of some MC proteases during *Chlamydia* FRT infections and determine if they could be mediating the induction of *Chlamydia*-induced immunopathology. For this, the expression of the MC proteases, mMCP4, mMCP5, mMCP6 and CPA3, and of Ndst2 in the FRT will be characterised according to female sex hormones and *Chlamydia* infection. To identify the roles of heparin-protease complexes, Ndst2-deficient mice will be subjected to a mouse model of *Chlamydia muridarum*. The *Chlamydia* burden, the levels of *Chlamydia*-induced pathology and the *Chlamydia*-induced immune responses will be characterised. To characterise the individual effect of mMCP5, mMCP6 and mMCP7 during *Chlamydia* infection, mMCP5-deficient, mMCP6-deficient and mMCP6-deficient/mMCP7-sufficient mice will be subjected to a mouse model of *Chlamydia muridarum* and the *Chlamydia* burden, as well as the levels of *Chlamydia*-induced pathology will be evaluated.

3.3. Material and methods

3.3.1. Ethics statement

All the animals and procedures used in this study were approved by the Animal Care and Ethics Committee at the University of Newcastle (Callaghan, NSW, Australia).

3.3.2. Mouse strains used to investigate the roles of MC proteases during *Chlamydia* infection

WT C57BL/6 female mice were obtained from the ABR facility (Moss Vale) in order to evaluate the effects of female sex hormones and *Chlamydia* FRT infection on the expression of MC proteases and Ndst2 (**Table 3.1**). In addition, MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) female mice and their appropriate controls (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) were obtained from the UniSA animal facility (Adelaide) to assess if the expression of MC proteases and Ndst2 is MC-dependent (**Table 3.1**) (237).

Ndst2-deficient (*Ndst2*$^{-/-}$), mMCP5-deficient (*mMCP5*$^{-/-}$), mMCP6-deficient (*mMCP6*$^{-/-}$ *mMCP7*$^{-/-}$), mMCP6-deficient/mMCP7-sufficient (*mMCP6*$^{-/-}$ *mMCP7*$^{+/+}$) female mice and WT controls were obtained from the ABR facility (Moss Vale) to assess the collective role of the MC mediators that are normally bound to heparin in the secretory granules of MCs, including histamine and the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, as well as the individual roles of some of these MC proteases during *Chlamydia* FRT infection (**Table 3.1**) (239, 244, 274, 275, 442, 457, 458). All wild type (WT) mice used in this study are C57BL/6 mice, and therefore express mMCP4, mMCP5, mMCP6, Prss31 and Cpa3 but not mMCP7 (212, 227, 449).

Upon delivery, mice were housed in SPF conditions in the Bioresources facility at the HMRI (New Lambton Heights), with *ad libitum* access to food and water under a 12 hours light-dark cycle.

Transgenic mouse line	Mouse background	Facility of origin	Description of the genetic modification
WT	C57BL/6	ABR	-
WT *Cpa3-Cre; Mcl-1$^{WT/WT}$*	C57BL/6	UniSA	-
Cpa3-Cre; Mcl-1$^{fl/fl}$	C57BL/6	UniSA	Deficiency in the intracellular anti-apoptotic factor myeloid cell leukemia-1 (Mcl-1) required for MC survival
WT *(mMCP6$^{+/+}$ mMCP7$^{-/-}$)*	C57BL/6	ABR	-
Ndst2$^{-/-}$	C57BL/6	ABR	Deficiency in Ndst2 resulting in deficient storage of histamine and MC proteases (mMCP4, mMCP5, mMCP6 and Cpa3) in the secretory granules of MCs
mMCP5$^{-/-}$	C57BL/6	ABR	Deficiency in the α-chymase mMCP5 (and secondary loss of CPA3)
mMCP6$^{-/-}$ mMCP7$^{-/-}$	C57BL/6	ABR	Deficiency in the tetramer-forming tryptases mMCP6 and mMCP7
mMCP6$^{-/-}$ mMCP7$^{+/+}$	C57BL/6	ABR	Deficiency in the tetramer-forming tryptase mMCP6 and presence of the other tetramer-forming tryptase mMCP7

Table 3.1 - Description of the wild type and transgenic mouse strains used to investigate the roles of mast cell proteases during *Chlamydia* infection. *WT: wild type, MC: mast cell, ABR: Australia BioResources, UniSA: University of South Australia, Cpa3: carboxypeptidase A3, Ndst2: N-deacetylase/N-sulphotransferase-2, mMCP5: mouse mast cell protease.*

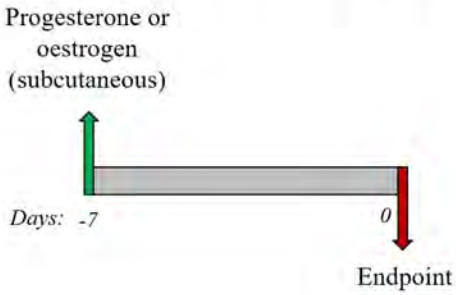
As explained in **Chapter 2** (**Section 2.3.2**), in order to assess the effects of female sex hormones on the expression of MC proteases and Ndst2 in the FRT, WT mice (10-11 weeks old) were subcutaneously administrated with either medroxyprogesterone acetate (Depo-Provera) or 17β-oestradiol (Sigma-Aldrich) to synchronise their oestrus cycle and induce diestrus or oestrus, respectively (332, 388). Seven days later, mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb) and FRT tissues were collected for analyses (**Figure 3.1 A**).

As explained in **Chapter 2** (**Section 2.3.2**), in order to assess the effect of *Chlamydia* infection on the expression of MC proteases and Ndst2 in the FRT (**Figure 3.1 B**), to verify if the expression of MC proteases and Ndst2 is MC-dependent (**Figure 3.1 C**) and to characterise the roles of heparin-protease complexes and individual MC proteases during *Chlamydia* infection

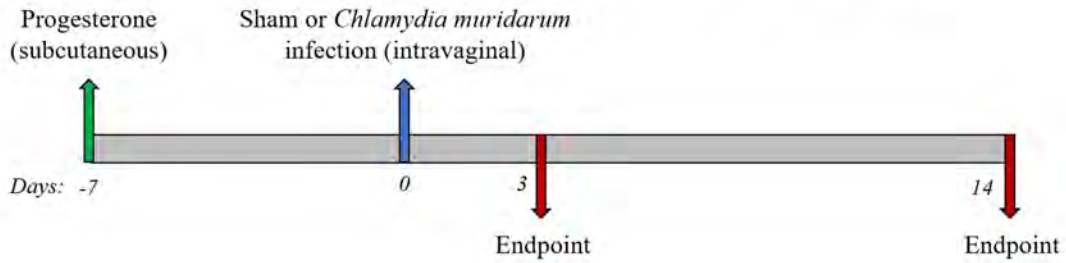
(**Figure 3.1 D**), MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$), Ndst2-deficient (*Ndst2*$^{-/-}$), mMCP5-deficient (*mMCP5*$^{-/-}$), mMCP6-deficient (*mMCP6*$^{-/-}$ *mMCP7*$^{-/-}$), mMCP6-deficient/mMCP7-sufficient (*mMCP6*$^{-/-}$ *mMCP7*$^{+/+}$) mice and appropriate WT controls (6-18 weeks old) were subcutaneously administrated with medroxyprogesterone acetate (Depo-Provera). Seven days later, mice were infected intravaginally with $5x10^4$ ifu of *Chlamydia muridarum* (ATCC VR-123) in 10µl SPG or sham-infected with 10µl SPG alone under ketamine:xylazine anaesthesia (Ilium Ketamil® and Ilium Xylazil-20®) (177, 389). For infections in the MC-deficient mice and their associated WT controls, ketamine:xylazine anaesthesia was replaced by ketamine:medetomidine anaesthesia (with atipamezole reversal; Ilium Ketamil®, Ilium Medetomidine® and Ilium Atipamezole®) in order to conform with best practices*. At 3 and/or 14dpi, mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb) and FRT tissues were collected for analyses.

* All the other infections were approved and/or conducted prior to an update in the Animal Care and Ethics Committee (ACEC) guidelines for best practice for anaesthesia.

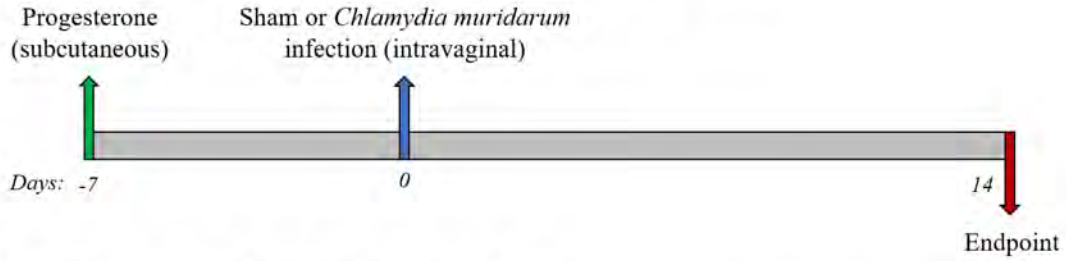
(A) Effect of female sex hormones on the expression of MC proteases and Ndst2 (WT mice)



(B) Effect of *Chlamydia* infection on the expression of MC proteases and Ndst2 (WT mice)



(C) Effect of MC deficiency on the expression of MC proteases and Ndst2 (*Cpa3-Cre;Mcl-1*$^{WT/WT}$ and *Cpa3-Cre;Mcl-1*$^{fl/fl}$ mice)



(D) Role of MC proteases during *Chlamydia* infection (WT, *Ndst2*$^{-/-}$, *mMCP5*$^{-/-}$, *mMCP6*$^{-/-}$ and *mMCP6*$^{-/-}$ *mMCP7*$^{+/+}$ mice)

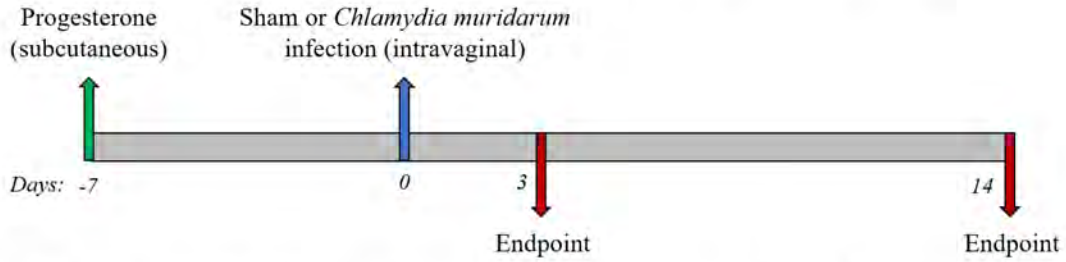


Figure 3.1 - Mouse model of hormonal treatment and *Chlamydia muridarum* female reproductive tract (FRT) infections. (A) Progesterone or oestradiol was administrated subcutaneously to wild type (WT) C57BL/6 female mice. Seven days later, tissues were collected for subsequent analyses to assess the expression of *N*-deacetylase/*N*-sulphotransferase-2 (Ndst2) and mast cell (MC) proteases in the FRT [Chapter 2]. **(B)** WT C57BL/6 female mice were administrated progesterone subcutaneously seven days prior to intravaginal infection with $5x10^4$ inclusion forming units of *Chlamydia muridarum* or sham-infection. Tissues were collected at 3 and 14 days post infection (dpi) for subsequent analyses of the expression of Ndst2 and MC proteases in the FRT [Chapter 2]. **(C)** MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) female mice and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls were administrated progesterone

subcutaneously seven days prior to intravaginal infection with $5x10^4$ inclusion forming units (ifu) of *Chlamydia muridarum*. Tissues were collected at 14dpi for subsequent analyses of the expression of MC proteases and Ndst2 in MC-deficient mice [Chapter 2]. **(D)** Ndst2-deficient (*Ndst2*$^{-/-}$), mouse mast cell protease (mMCP)5-deficient (*mMCP5*$^{-/-}$), mMCP6-deficient (*mMCP6*$^{-/-}$ *mMCP7*$^{-/-}$) and mMCP6-deficient/mMCP7-sufficient (*mMCP6*$^{-/-}$ *mMCP7*$^{+/+}$) female mice and WT controls were administrated progesterone subcutaneously seven days prior to intravaginal infection with $5x10^4$ ifu of *Chlamydia muridarum*. Tissues were collected at 3dpi and 14dpi for subsequent assessment of the roles of proteases-proteoglycan complexes and individual MC proteases during *Chlamydia* reproductive tract infection.

3.3.3. RNA extractions from FRT tissues

As explained in **Chapter 2** (**Section 2.3.5**), the left ovaries, oviducts and uterine horns were harvested at endpoint, snap frozen and stored at -80°C. Total RNA extractions were performed using the TRIzol® method according to the manufacturer's instruction.

3.3.4. Reverse transcription of RNA from reproductive tract tissues

As explained in **Chapter 2** (**Section 2.3.6**), the purity, quality and concentration of the RNA samples were measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The RNA samples were then treated with DNase I and reversed transcribed into cDNA using M-MLV reverse transcriptase enzyme (Life Technologies) according to the manufacturer's instructions.

3.3.5. DNA extraction from vaginal lavage

As explained in **Chapter 2** (**Section 2.3.7**), vaginal lavages were realised at endpoint to evaluate the *Chlamydia muridarum* burden in the lower FRT. The GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies Sdn. Bhd.) was used according to the manufacturer's instruction in order to extract the bacterial DNA from the vaginal lavage fluids.

3.3.6. Real-time quantitative polymerase chain reaction

3.3.6.1. RNA expression analyses

As explained in **Chapter 2** (**Section 2.3.8.1**), qPCR were realised on the cDNA samples using custom designed primers (IDT; **Appendix, Table 6.1**) and iTaq™ Universal SYBR® Green Supermix (Bio-Rad), cycling conditions of 50°C for 2 minutes, 95°C for 2 minutes followed by

40 cycles of 95°C for 15 seconds and 55-65°C for 1 minute in a Mastercycler® ep Realplex2 Real-time PCR System (Eppendorf), a CFX96 or a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Dissociation analyses were performed to verify the specificity of each primer pairs and the relative expression of each target gene was determined by comparison to HPRT.

The gene expression of Ndst2, of the chymases, mMCP4 and mMCP5, of the tetramer-forming tryptase, mMCP6, and of the metalloprotease, Cpa3, were measured in FRT tissues using specific primers (IDT; **Appendix, Table 6.1**) to evaluate if the expression of MC proteases and Ndst2 is regulated by female sex hormones, *Chlamydia* FRT infection and/or by the presence of MCs.

The *Chlamydia* burden in the uterine was evaluated using primers targeting the *Chlamydia muridarum* 16S rRNA (IDT; **Appendix, Table 6.1**) in order to evaluate the levels of ascending infection in Ndst2-deficient, mMCP5-deficient, mMCP6-deficient, mMCP6-deficient/mMCP7-sufficient mice and WT controls (177, 389).

The expression profile of various genes coding for cytokines, chemokines and immune mediators associated with susceptibility/clearance of *Chlamydia* infection and/or associated pathology and/or host immune responses (Tbet, GATA3, RORγt and FOXP3, IFNγ, TNFα, IL-1β, IL-4rα, IL-6, IL-10, IL-13, IL-17, CXCL1, CXCL15, signal transducer and activator of transcription [STAT]1, STAT6, GM-CSF, MMP9, TLR2 and PAR2) were determined using specific primers (IDT; **Appendix, Table 6.1**) to investigate the changes in inflammation status in the *Chlamydia*-infected uterus of Ndst2-deficient and WT controls.

3.3.6.2. DNA expression analyses

As explained in **Chapter 2** (**Section 2.3.8.2**), primers targeting the gene encoding for *Chlamydia* MOMP (IDT; **Appendix, Table 6.1**) were used to estimate the *Chlamydia* burden in the lower FRT. The presence of the *Chlamydia* MOMP gene in each sample was then compared to DNA standards extracted from known concentrations of *Chlamydia muridarum* to evaluate the number of *Chlamydia* in each vaginal lavage fluid sample of Ndst2-deficient, mMCP5-deficient, mMCP6-deficient, mMCP6-deficient/mMCP7-sufficient mice and WT controls.

3.3.7. Evaluation of *Chlamydia*-associated pathology

As explained in **Chapter 2** (**Section 2.3.9**), the level of hydrosalpinx in the oviducts of *Chlamydia*-infected mice at 14dpi was evaluated by measuring the size of their oviducts in two planes using a digital calliper (Sontax) in order to estimate the cross sectional area of each oviduct as a representation of oviduct swelling caused by *Chlamydia* infection.

3.3.8. Characterisation of the immune cell numbers in the uterus during *Chlamydia* FRT infection using flow cytometry

Flow cytometry was used to characterise the immune cells in the *Chlamydia*- infected uterus of Ndst2-deficient mice and WT controls. Briefly, as explained in details in **Chapter 2** (**Section 2.3.10**), uterine horns were dissociated and digested 30 minutes at 37°C with 40U/mL of DNase I and 2mg/mL of collagenase D (Roche) at 37°C. After further dissociation, samples were passed through a 70µm nylon cell strainer, treated with red blood cell lysis buffer and the number of living cells was enumerated using trypan blue exclusion and a Countess™ automated cell counter (Invitrogen). The single cell suspensions were blocked at 4°C for 15 minutes with 10ng/mL of anti-mouse CD16/32 (InVivoMAb).

For staining, the single cell suspensions were incubated at 4°C for 20 minutes with a cocktail of antibodies specific for surface markers conjugated with either a flurorochrome or biotin. Then, they were incubated at 4°C for 20 minutes with a streptavidin antibody conjugated with a BV605 fluorochrome (**Appendix, Table 6.2**). An unstained control was generated as a negative control, and single fluorochrome controls for each antibody used in the panel were produced in order to set the voltages and the compensation for removal of signal overlap between fluorochromes.

Finally, the stained cells were fixed overnight at 4°C in 4% PFA and the fluorescence of each sample was measured using a BD LSRFortessa™ X-20 cell analyzer and examined with the BD FACSDiva™ Software (BD Biosciences) to determine the percentages and total numbers of innate and adaptive immune cells in the *Chlamydia*-infected uterus of Ndst2-deficient mice and WT controls, with the gating strategy based on the specific forward scatter, side scatter and surface markers of each immune cell type (**Table 3.2** and **Appendix, Figure 6.1**).

Cell types	Surface marker expressions
Immune cells	$CD45^+$
Neutrophils	$CD45^+$ $CD11b^+$ $Ly6G^+$
Eosinophils	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^+$
Myeloid dendritic cells	$CD45^+$ $CD11b^+$ $CD11c^+$ $Ly6C^-$ $PDCA^-$
Plasmacytoid dendritic cells	$CD45^+$, $CD11b^-$ $CD11c^+$ $Ly6C^+$, $PDCA^+$
Resident monocytes/macrophages	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^-$ $F4/80^+$ $Ly6C^-$
Infiltrating monocytes/macrophages	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^-$ $F4/80^+$ $Ly6C^+$
T cells	$CD45^+$ $CD3^+$
$CD4^+$ T cells	$CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$
$CD8^+$ T cells	$CD45^+$ $CD3^+$ $CD4^-$ $CD8^+$

Table 3.2 – Surface markers used for identifying immune cells in the uterine tissue of *Chlamydia*-infected Ndst2-deficient mice and WT controls.

3.3.9. Statistical analyses

Data are presented as mean ± SEM. All statistical tests were realised using the GraphPad Prism software (version 7.0; GraphPad Software). Grubbs outlier tests were realised, followed by normality testing using the D'Agostino & Pearson normality test for analysis of 8 or more samples or the KS normality test for analysis of less than 8 samples. Then, as appropriated, Student's t-tests or Mann-Whitney tests were performed for comparisons of two experimental groups. For comparisons of 3 groups, ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons tests or Kruskal-Wallis tests with Dunn's multiple comparisons tests were performed as appropriated, with a $p<0.05$ considered as statistically significant.

3.4. Results

3.4.1. Mice treated with oestrogen have increased expression of MC proteases mMCP4, mMCP5, mMCP6 and Cpa3 in their FRT, in the absence of infection

In **Chapter 2**, female sex hormones were shown to affect the number of MCs in the uterus. In order to examine if female sex hormones also affect the phenotype of MCs regarding their expression of MC proteases, WT female mice were treated with progesterone (diestrus) or oestrogen (oestrous) subcutaneously (**Figure 3.1 A**). Seven days later, the left ovaries, oviducts and uterus were collected for subsequent RNA extraction and reverse transcription. The levels of expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3, were assessed by qPCR.

In the ovary, the expression of mMCP4 and Cpa3 were slightly augmented (**Figure 3.2 A-D**) while the expression of mMCP5 and mMCP6 were significantly increased (**Figure 3.2 B-C**) by oestrogen treatments. Similarly, in the oviduct, the expression Cpa3 was slightly augmented (**Figure 3.2 H**) while the expression of mMCP4, mMCP5 and mMCP6 were significantly increased (**Figure 3.2 E-G**) by oestrogen treatments. Finally, in the uterus, the expression of all the MC proteases were significantly augmented by oestrogen treatments (**Figure 3.2 I-L**).

Overall my results show that oestrogen treatments and/or oestrogen-induced oestrus stimulate the expression of mMCP4, mMCP5, mMCP6 and Cpa3 in the ovary, oviduct and uterus of WT mice. Therefore, female sex hormones and/or oestrous cycle appear to alter the phenotype of MCs in the FRT.

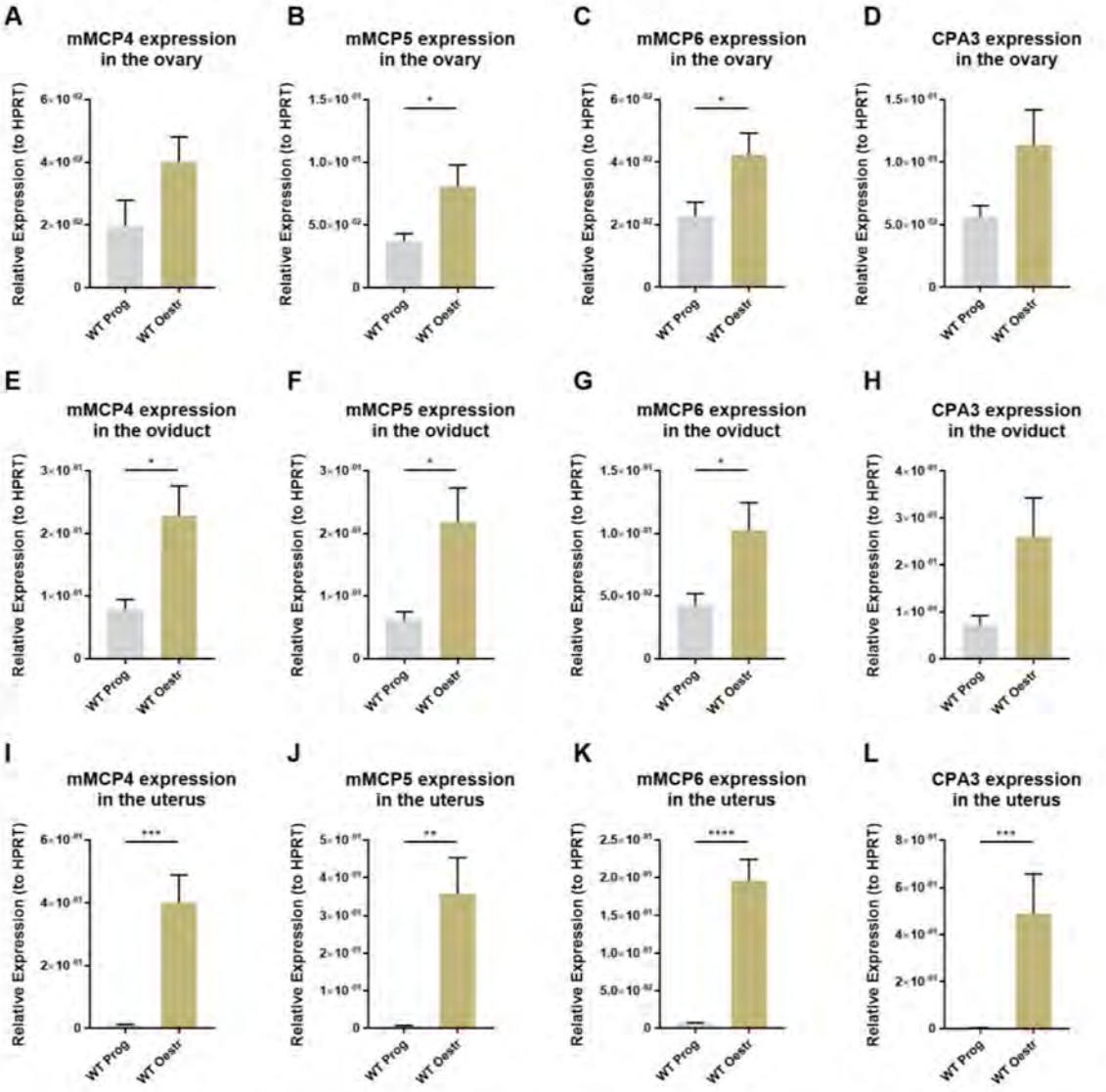


Figure 3.2 – Mice treated with oestrogen have increased expression of the mast cell (MC) proteases mouse mast cell proteases (mMCP)4, mMCP5 and carboxypeptidase A3 (Cpa3) in their ovary, oviduct and uterus compared to mice treated with progesterone. C57BL/6 wild-type (WT) mice were treated with either progesterone (Prog) or oestrogen (Oestr) and sacrificed 7 days later. RNA was extracted from the uterus and the levels of expression of the MC proteases **(A, E, I)** mMCP4, **(B, F, J)** mMCP5, **(C, G, K)** mMCP6 and **(D, H, L)** Cpa3 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥6) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$; ** representing $p<0.01$; *** representing $p<0.001$ and **** representing $p<0.0001$.

3.4.2. Mice infected by *Chlamydia muridarum* have reduced expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3 in the FRT at late stages of infection

In **Chapter 2**, *Chlamydia* infection was shown to induce a slight decrease in the number of uterine MCs. In order to examine if *Chlamydia* infection also affects the phenotype of MCs regarding their expression of MC proteases, female WT mice were treated with progesterone and infected with *Chlamydia muridarum* or sham-infected (**Figure 3.1 B**). At 3dpi and 14dpi, the left uterus were collected for subsequent RNA extraction and reverse transcription. The levels of expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3, were assessed by qPCR.

At 3dpi, the expression of mMCP4, mMCP5, mMCP6 and Cpa3 is similar in the uterus of *Chlamydia*-infected mice and sham-infected controls (**Figure 3.3 A-D**). However, at 14dpi, the expression of mMCP4, mMCP5, mMCP6 and Cpa3 is decreased in the uterus of *Chlamydia*-infected mice compared to sham-infected mice (**Figure 3.3 E-H**).

Therefore, *Chlamydia* infection appear to not alter the phenotype of MCs at early stages but result in decreased expression of MC proteases later. In future studies, the expression of MC proteases will be determined at additional time points in order to more precisely analyse the changes in the phenotype of MCs throughout the course of *Chlamydia* infection.

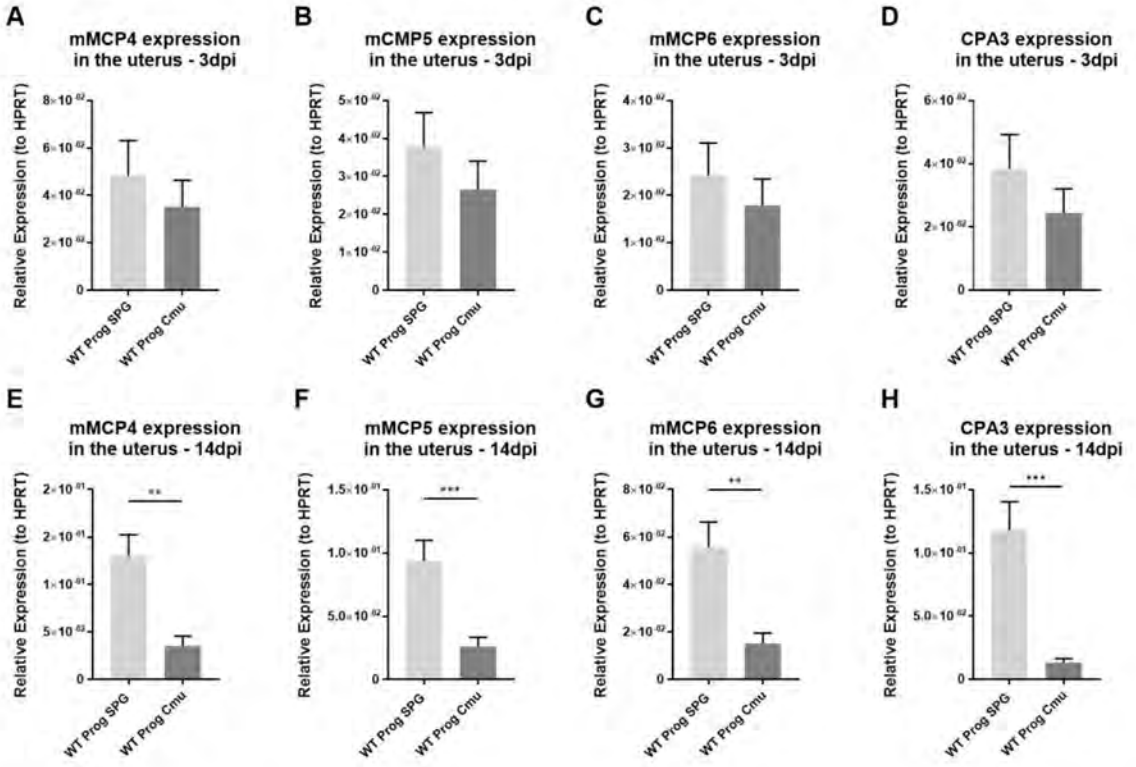


Figure 3.3 – Mice infected with *Chlamydia muridarum* have reduced expression of the mast cell (MC) proteases mouse mast cell proteases 4 (mMCP4), mMCP5 and carboxypeptidase A3 (Cpa3) in the uterus at 14 days post infection (dpi). C57BL/6 wild-type (WT) mice were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) or sham-infected with sucrose-phosphate-glutamate buffer (SPG). Mice were sacrificed at **(A, B, C, D)** 3dpi and **(E, F, G, H)** 14dpi. Total RNA was extracted from the uterus and the levels of expression of the MC proteases **(A, E, I)** mMCP4, **(B, F, J)** mMCP5, **(C, G, K)** mMCP6 and **(D, H, L)** Cpa3 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥6) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with ** representing $p<0.01$ and *** representing $p<0.001$.

3.4.3. MC-deficient mice have strongly reduced expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3 in their uterus during *Chlamydia* FRT infection

My data show that the expression of MC proteases in the FRT is not always correlated with the number of MCs, with oestrogen-treated mice having lower number of uterine MCs but enhanced expression of MC proteases (**Figures 2.4 and 3.2**). This finding could be explained by the fact that oestrogen directly or indirectly stimulate the expression of those factor in MCs or by the fact that other cells in the FRT might express those proteases. Therefore, in order to verify the latest hypothesis and check if MC proteases are specific to MCs in the FRT, MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls were treated with progesterone and infected with *Chlamydia muridarum* (**Figure 3.1 C**). At 14dpi, the left uterus were collected for subsequent RNA extraction and reverse transcription. The levels of expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3, were assessed by qPCR.

As shown in **Figure 3.4**, the expression of all those MC proteases are strongly impaired in the uterus of MC-deficient mice compared to associated WT controls, suggesting that these MC proteases are mostly specific to MCs in the FRT. To confirm this hypothesis, in future studies, immunostaining will be realised to identify the cellular source of mMCP4, mMCP5, mMCP6 and mMCP7, in order to verify that these proteases are definitely specific to MCs in the FRT.

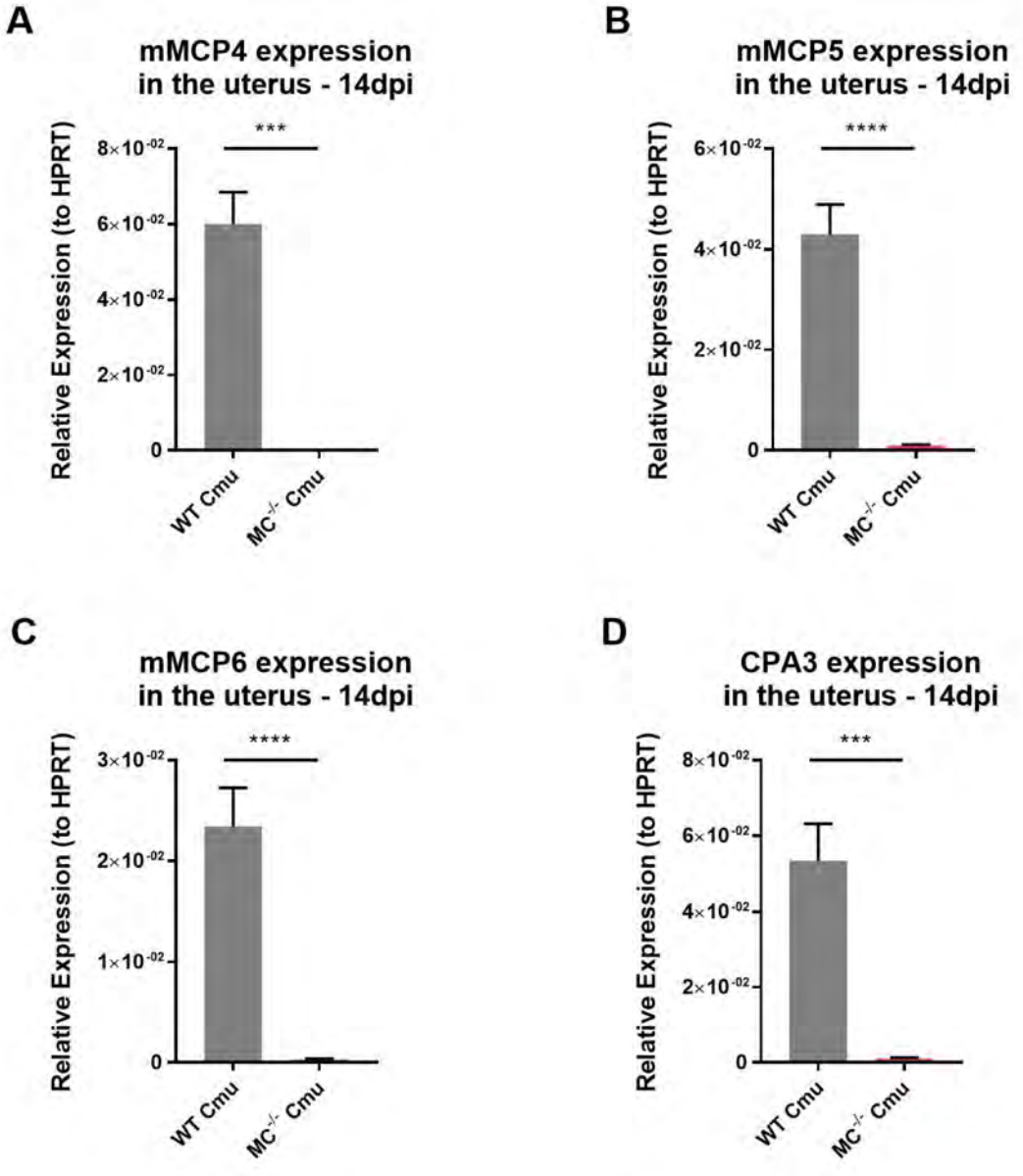


Figure 3.4 - Mast cell (MC)-deficient mice have a reduced expression of the mast cell proteases mouse mast cell proteases (mMCP)4, mMCP5, mMCP6 and carboxypeptidase A3 (Cpa3) in the uterus. MC-deficient (MC$^{-/-}$, *Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and their associated WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection (dpi). RNA was extracted from the uterus and the levels of expression of the MC proteases **(A)** mMCP4, **(B)** mMCP5, **(C)** mMCP6 and **(D)** Cpa3 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥8) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with *** representing $p<0.001$ and **** representing $p<0.0001$.

3.4.4. The expression of Ndst2 is not affected by female sex hormones, *Chlamydia* FRT infection nor MC deficiency

Ndst2 deficiency affects the sulfation of heparan sulfate in MCs and result in alteration of the storage of MC protease in the secretory granules of MCs. Previous data show that female sex hormones and *Chlamydia* infection affect the expression of MC proteases. In order to examine the effects of progesterone and oestrogen on the expression of Ndst2 in the FRT, WT female mice were treated with progesterone (diestrus) or oestrogen (oestrous) subcutaneously and sacrificed 7 days later (**Figure 3.1 A**). In order to examine if *Chlamydia* infection affects the expression of Ndst2 in the FRT, female WT mice were treated with progesterone, infected with *Chlamydia muridarum* or sham-infected and sacrificed at 3dpi and 14dpi (**Figure 3.1 B**). In order to determine if the expression of Ndst2 is dependent on the presence of MCs, MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) were treated with progesterone, infected with *Chlamydia muridarum* and sacrificed at 14dpi. (**Figure 3.1 C**). The left ovaries, oviducts and/or uterus were collected for subsequent RNA extraction and reverse transcription. The levels of expression of Ndst2 were assessed by qPCR.

The expression of Ndst2 in the ovary, oviduct and uterus is not affected by progesterone and oestrogen treatments (**Figure 3.5 A-C**). Similarly, the expression of Ndst2 in the uterus is not affected by *Chlamydia* infection at 3dpi and 14dpi (**Figure 3.5 D-E**). Finally, the expression of Ndst2 in the uterus is not affected by MC deficiency (**Figure 3.5 F**).

Therefore, the expression of Ndst2 in the FRT appear to be constitutive.

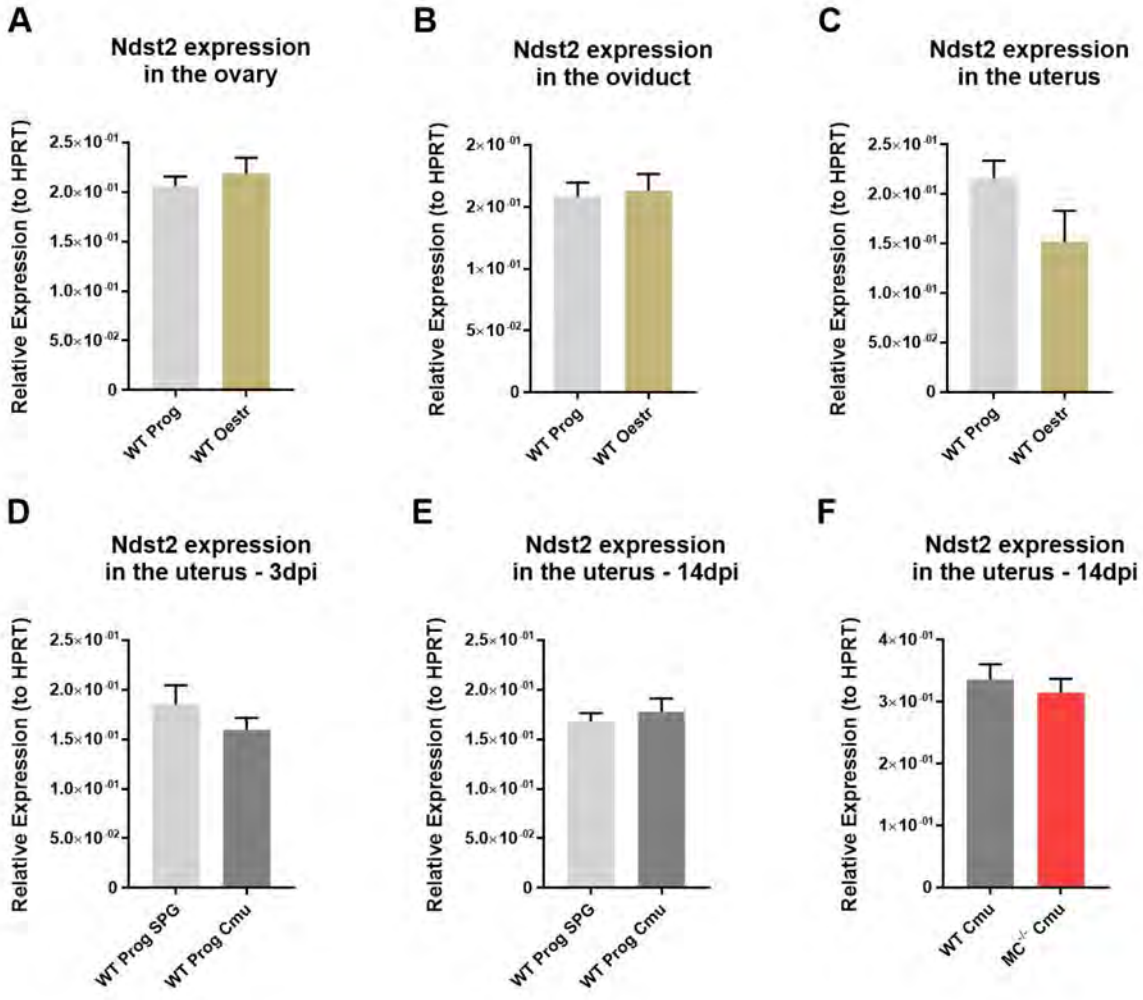


Figure 3.5 –The expression of *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2) in the female reproductive tract (FRT) is not altered by progesterone or oestrogen treatments, *Chlamydia*-infection nor mast cell (MC) deficiency. (A, B, C) C57BL/6 wild-type (WT) mice were treated with either progesterone (Prog) or oestrogen (Oestr) and sacrificed 7 days later. **(D, E, F)** C57BL/6 WT mice were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) or sham-infected with sucrose-phosphate-glutamate buffer (SPG). Mice were sacrificed at **(D)** 3 days post infection (dpi) and **(E)** 14dpi. **(F)** MC-deficient (*MC$^{-/-}$*, *Cpa3-Cre;Mcl-1$^{fl/fl}$*) mice and their associated WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14dpi. RNA was extracted from the uterus and the levels of expression of Ndst2 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥7) and Student's t-tests were performed.

3.4.5. Ndst2-deficient mice have increased *Chlamydia* burden in their uterus while being protected against *Chlamydia*-associated pathology

In Chapter 2, MC-deficient mice were shown to be protected against *Chlamydia*-induced pathology but the mechanism involved was not clearly identified. In order to determine the effect of the MC mediators that are normally bound to heparin in the secretory granules of MCs during *Chlamydia* FRT infection, Ndst2-deficient mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 3dpi and 14dpi (**Figure 3.1 D**). The levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

At 3dpi, the number of *Chlamydia* per vaginal lavage (**Figure 3.6 A**) and the *Chlamydia* burden in the uterus (**Figure 3.6 B**) were similar between Ndst2-deficient mice and WT controls. However, at 14dpi, the *Chlamydia* burden in the uterus was increased in Ndst2-deficient mice compared to WT mice (**Figure 3.6 D**), whilst the number of *Chlamydia* per vaginal lavage remained unchanged between Ndst2-deficient mice and WT controls (**Figure 3.6 C**). The cross sectional area of the left and right oviducts of Ndst2-deficient mice were strongly lower compared to those of WT mice (**Figure 3.6 E-F**).

Overall, these results suggest that heparin-protease complexes and/or other factors affected by Ndst2 deficiency are protective against the development of infection in the uterus but play a detrimental role in the development of *Chlamydia*-induced hydrosalpinx.

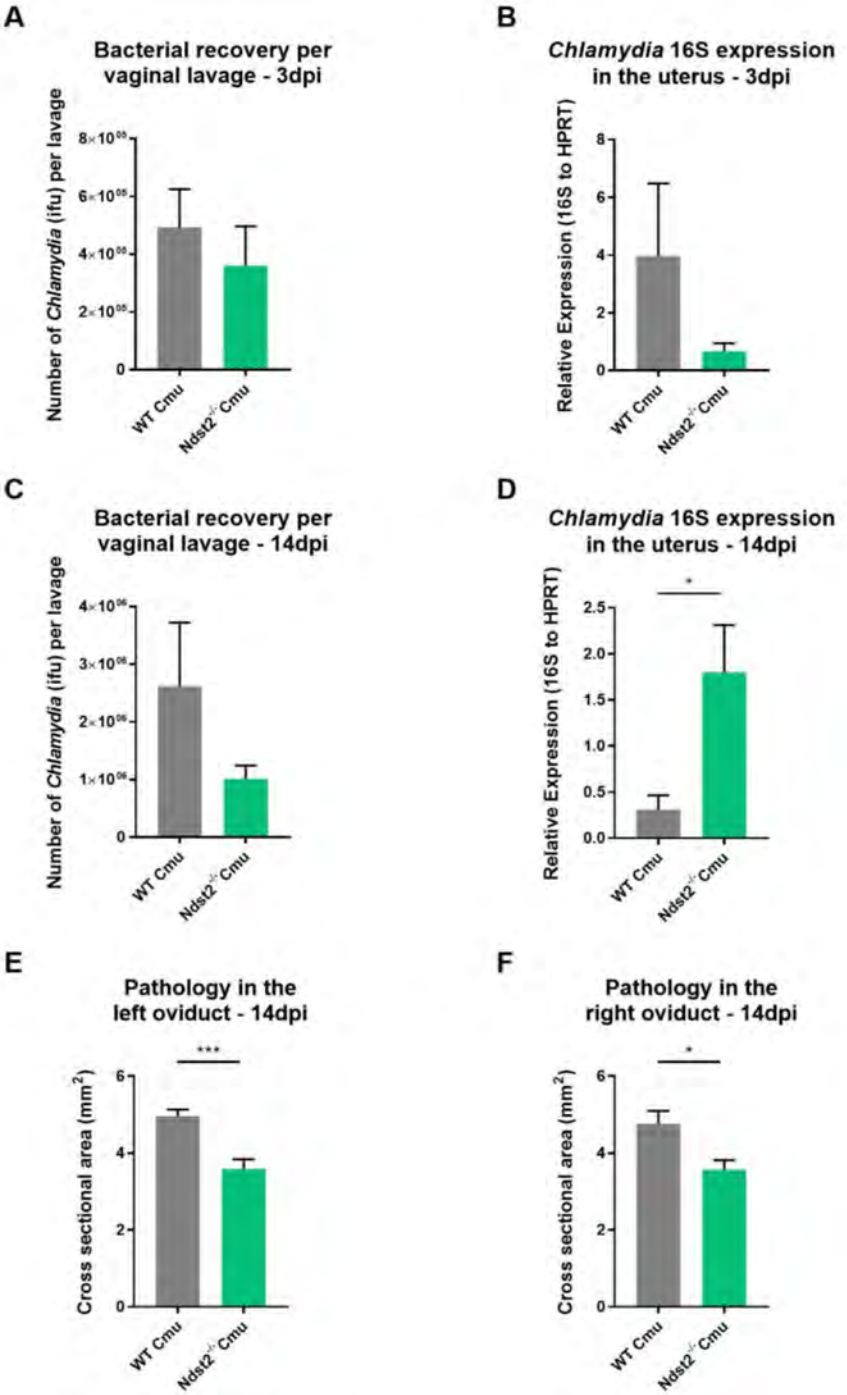


Figure 3.6 – *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice are more susceptible to *Chlamydia* infection while being protective against *Chlamydia*-associated pathology at 14 days post infection (dpi). Ndst2-deficient (*Ndst2*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at **(A, B)** 3dpi and **(C, D, E, F)** 14dpi. **(A, C)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per lavage. **(B, D)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross section area of the **(E)** left and **(F)** right oviducts of were measured using a calliper to evaluate the levels of *Chlamydia*-associated pathology. All data are presented as mean±SEM (n≥6) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and *** representing $p<0.001$.

3.4.6. Ndst2-deficient mice exhibit reduced immune cell mobilisation in their uterus during *Chlamydia* FRT infection

My previous data show that Ndst2-deficient mice have increased levels of infection in their uterus but that they are protected against *Chlamydia* infection. In **Chapter 2**, MC-deficient mice were shown to be protected against *Chlamydia*-induced pathology and to have slightly reduced number of neutrophils, eosinophils, monocytes and macrophages in their uterus. Therefore, in order to assess the effects of the MC mediators that are normally bound to heparin in the secretory granules of MCs, on immune cell recruitment to the FRT during *Chlamydia* infection, Ndst2-deficient mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 3.1 D**). The uteri were collected and digested into a single cell suspension and the total number of cells were enumerated. Single cell suspensions were then stained for extracellular markers and key immune cells were identified by flow cytometry. The number of cells in the uterine tissue was expressed both as the total number of each immune cell type and as a percentage of each immune cell type among all viable cells.

Globally the number of uterine cells is reduced in *Chlamydia*–infected Ndst2-deficient mice compared to WT controls (**Figure 3.7 A**). Moreover, the number and percentage of immune cells in the uterus are both highly diminished in Ndst2-deficient mice (**Figures 3.7 B and 3.8 A**). In more details, at the exception of plasmacytoid DCs and resident monocytes and macrophages whom numbers remain unchanged, the numbers of all investigated immune cells (neutrophils, eosinophils, myeloid DCs, inflammatory monocytes and macrophages, T cells, $CD4^+$ T cells, $CD8^+$ T cells) are strongly reduced in the uterus of *Chlamydia*–infected Ndst2-deficient mice compared to controls (**Figure 3.7**). Whilst the proportion of neutrophils, resident monocytes and macrophages, T cells and $CD8^+$ T cells is similar in the uterus of *Chlamydia*–infected Ndst2-deficient mice and WT controls, an increase in the proportion of plasmacytoid DCs and a reduction in the proportion of eosinophils, myeloid DCs, inflammatory monocytes and macrophages and $CD4^+$ T cells is present the uterus of Ndst2-deficient mice during *Chlamydia* infection (**Figure 3.8**).

Taken together, those findings suggest a potential role for heparin-protease complexes and/or other factors affected by Ndst2 deficiency in mediating the induction of the infiltration of specific innate and adaptive immune cells to the uterus during *Chlamydia* FRT infection. The reduced number of immune cells in the uterus of *Chlamydia*-infected Ndst2-deficient mice could account for the elevated *Chlamydia* burden in their uterus whilst also explicate their lower level of pathology.

Unfortunately, due to restrictions in the breeding of genetically modified mice, the number of mice available was insufficient to include sham-infected control mice in this study. In future experiments, the numbers and percentages of each immune cell type in the uterus of sham-infected Ndst2-deficient mice (and WT controls) will be determined in order to establish if the differences observed in this study are caused by Ndst2 deficiency alone or if they are mediated by *Chlamydia* infection.

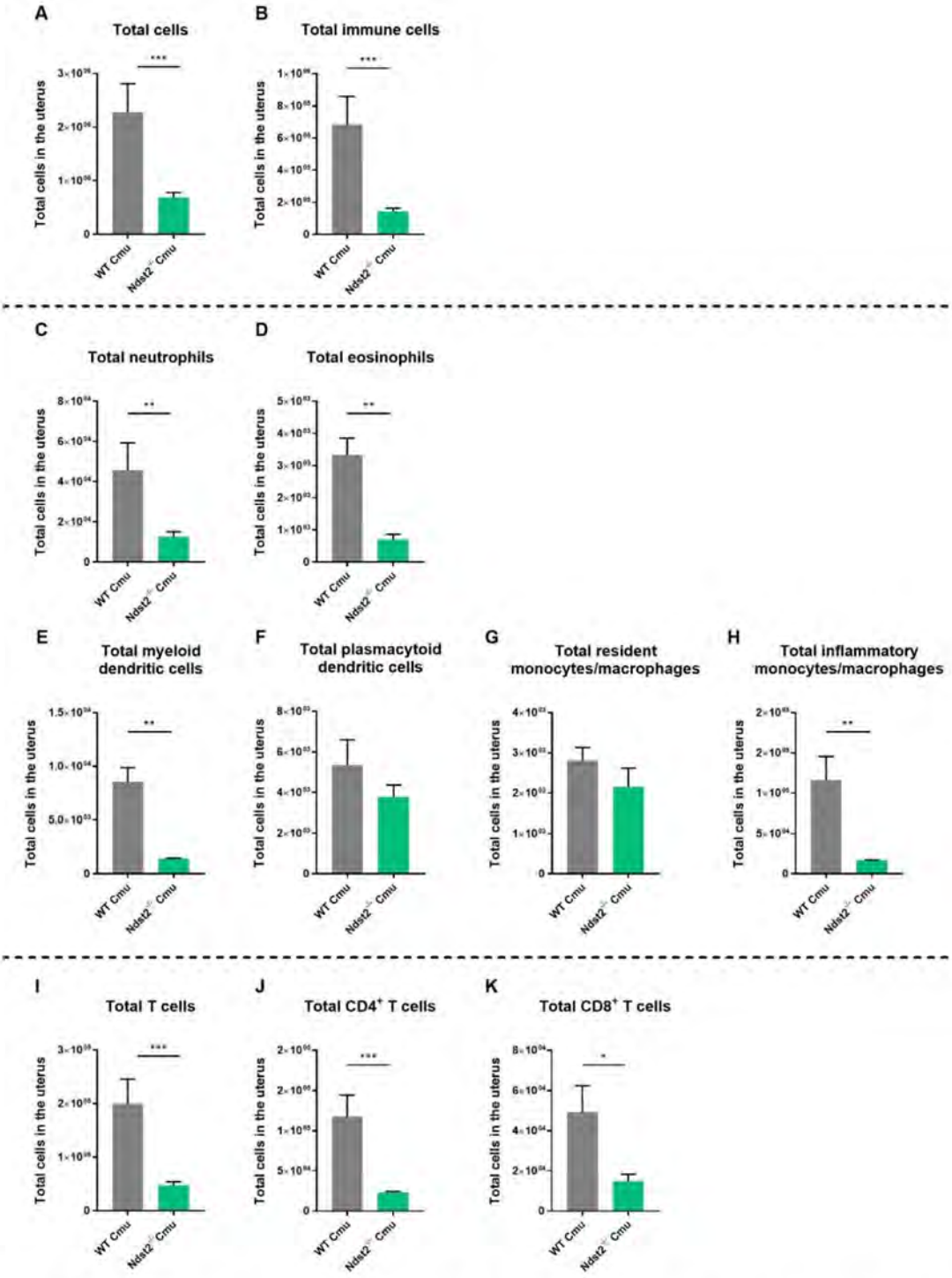


Figure 3.7 – *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice have a reduced number of immune cells, neutrophils, eosinophils myeloid dendritic cells, inflammatory monocytes and macrophages, CD4$^+$ and CD8$^+$ T cells in their uterus during *Chlamydia* infection. Ndst2-deficient (*Ndst2*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. **(A)** Single cell suspensions from the uterus were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the total number of **(B)** immune cells, **(C)** neutrophils, **(D)** eosinophils, **(E)** myeloid and **(F)** plasmacytoid dendritic cells, **(G)** resident and **(H)** inflammatory monocytes and macrophages, **(I)** T cells, **(J)** CD4$^+$ T cells and **(K)** CD8$^+$ T cells in the uterus. All data are presented as mean±SEM (n≥6) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$, ** representing $p<0.01$ and *** representing $p<0.001$.

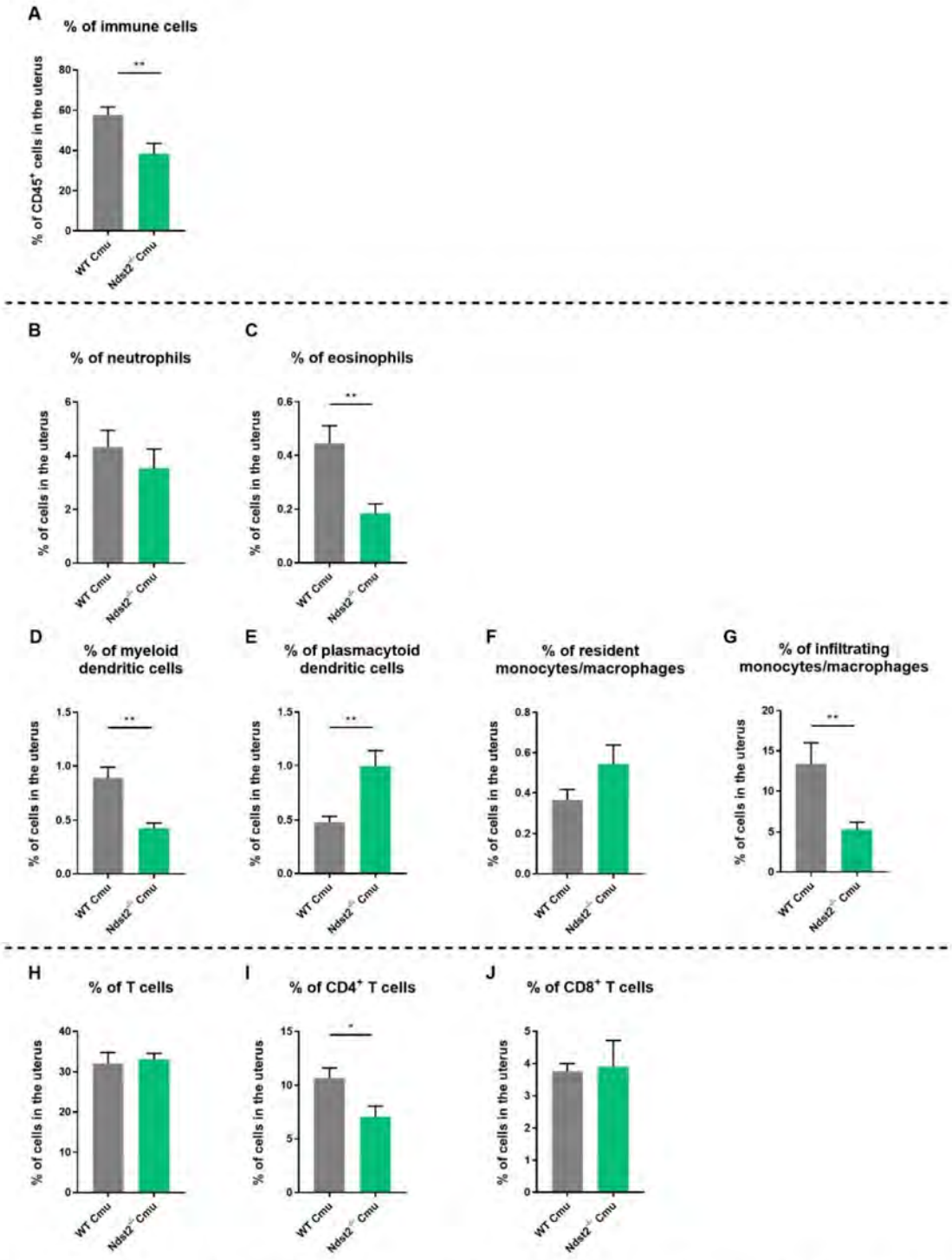


Figure 3.8 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice have a reduced percentage of immune cells, eosinophils, myeloid dendritic cells, inflammatory monocytes and macrophages and CD4$^+$ T cells; while having an increase in plasmacytoid dendritic cells in their uterus during *Chlamydia* infection. Ndst2-deficient (*Ndst2*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Single cell suspensions from the uterus were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the percentages of **(A)** immune cells, **(B)** neutrophils, **(C)** eosinophils, **(D)** myeloid and **(E)** plasmacytoid dendritic cells, **(F)** resident and **(G)** inflammatory monocytes and macrophages, **(H)** T cells, **(I)** CD4$^+$ T cells and **(J)** CD8$^+$ T cells among total viable uterine cells. All data are presented as mean±SEM (n≥6) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and *** representing $p<0.001$.

3.4.7. Ndst2-deficient mice have reduced expression of the transcription factors associated with Th1 and Th17 responses during the early stages of *Chlamydia* FRT infection

In **Chapter 2**, I show that MC deficiency did not affect the expression of the transcription factors Tbet, GATA3, RORγt and FOXP3 in the uterus of *Chlamydia*–infected mice. In order to assess if the MC mediators that are normally bound to heparin in the secretory granules of MCs affect the Th1, Th2, Th17 and/or Treg responses in the FRT during *Chlamydia* infection, Ndst2-deficient mice and WT controls were treated with progesterone and infected intravaginally with *Chlamydia muridarum* (**Figure 3.1 D**). At 3dpi and 14dpi, the left uterus were collected for subsequent RNA extraction and reverse transcription. The levels of expression of the transcription factors Tbet, GATA3, RORγt and FOXP3 were measured by qPCR.

Although, at 14dpi, Ndst2-deficient mice and WT controls have similar levels of expression of those transcription factors in their uterus; at 3dpi, the expression of Tbet and RORγt is decreased in the uterus of *Chlamydia*-infected Ndst2-deficient mice compared to WT controls (**Figure 3.9**).

These results suggest that heparin-protease complexes and/or other factors affected by Ndst2 deficiency might mediate the induction of Th1 and Th17 responses during the early stages of *Chlamydia* infection. However, analysis of the expression of these factors specifically in T cells using intracellular staining for flow cytometry will be necessary to validate this observation.

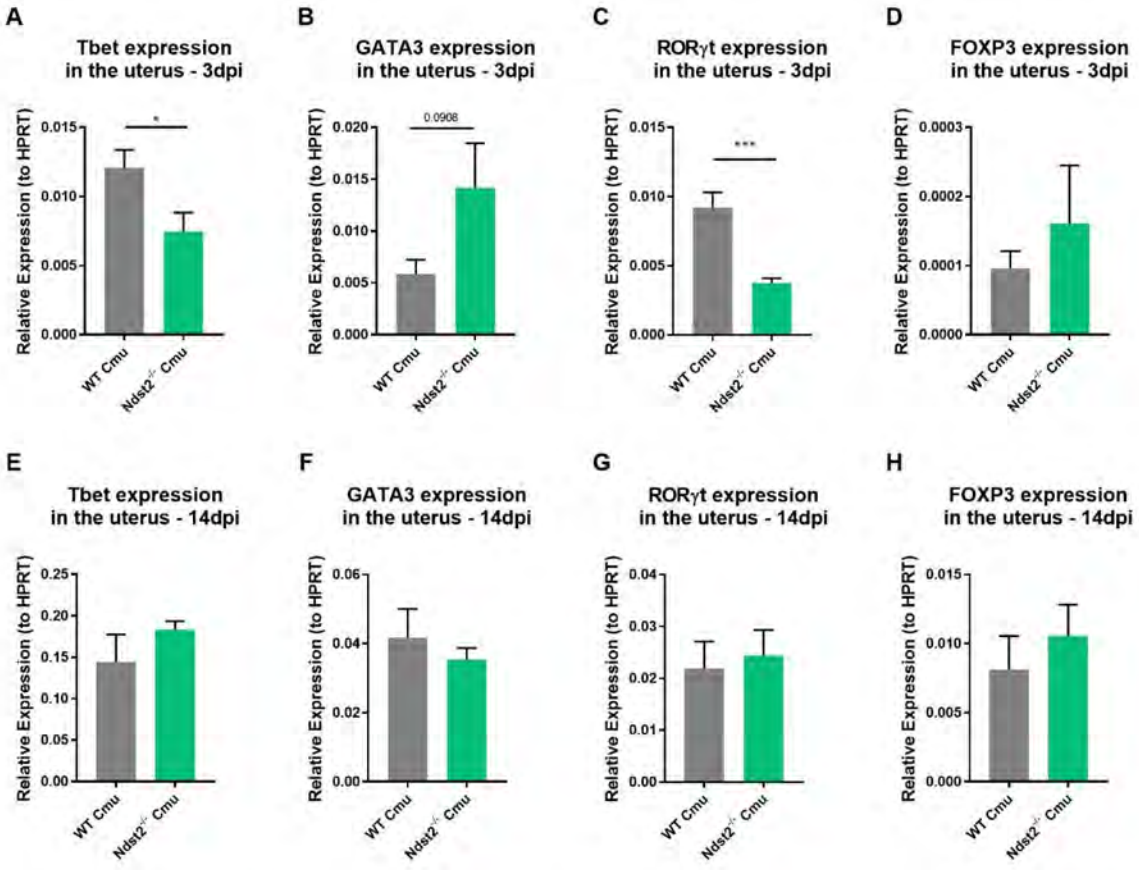


Figure 3.9 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice have reduced expression of the transcription factors Tbet and RAR-related orphan receptor (ROR)γt in their *Chlamydia*-infected uterus at 3 days post infection (dpi). Ndst2-deficient (*Ndst2*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at **(A-D)** 3dpi and **(E-H)** 14dpi. RNA was extracted from the uterus and the levels of expression of the transcription factors **(A, E)** Tbet, **(B, F)** GATA Binding Protein (GATA)3, **(C, G)** RAR-related orphan receptor (ROR)γt and **(D, H)** forkhead box P3 (FOXP3) were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and *** representing $p<0.001$.

3.4.8. Ndst2-deficient mice have altered expression of several immune mediators in their uterus during *Chlamydia* FRT infection

My previous data show that Ndst2-deficient mice are more susceptible to infection whilst being protected against *Chlamydia*-induced pathology. I also show that Ndst2-deficient mice have a strong reduction in the number of immune cells in their uterus during *Chlamydia* infection. In order to assess if the MC mediators that are normally bound to heparin in the secretory granules of MCs affect the expression of key immune mediators associated with susceptibility/clearance of *Chlamydia* infection and/or associated pathology, Ndst2-deficient mice and WT controls were treated with progesterone and infected intravaginally with *Chlamydia muridarum* (**Figure 3.1 D**). At 3dpi and 14dpi, the left uterus were collected for subsequent RNA extraction and reverse transcription. The levels of the key immune mediators associated with clearance of infection and *Chlamydia*-induced pathology were assessed using qPCR.

At 3dpi, whilst the expression of IFNγ, IL-4rα, IL-6, IL-10, IL-13, IL-17a, CXCL1, STAT1, STAT6, MMP9, TLR2 and PAR2 is similar in the uterus of *Chlamydia*-infected Ndst2-deficient mice and WT controls, the expression of TNFα (p=0.0847), IL-1β (p<0.05), CXCL15 (p<0.05) and GM-CSF (p=0.0852) is increased in Ndst2-deficient mice (**Figure 3.10**).

At 14dpi, whilst the expression of IFNγ, TNFα, IL-1β IL-6, IL-10, IL-17a, CXCL15, STAT1, STAT6, GM-CSF, MMP9, TLR2 and PAR2 is similar in the uterus of *Chlamydia*-infected Ndst2-deficient mice and WT controls, the expression of IL-4rα (p<0.05), IL-13 (p=0.0836) and CXCL1 (p<0.01) is increased in the infected uterus of Ndst2-deficient mice compared to WT mice (**Figure 3.11**).

These results suggest that heparin-protease complexes and/or other factors affected by Ndst2 deficiency might regulate the expression of some of the key mediators involved in the immune response to *Chlamydia* infection. In future studies, immunostaining and/or *in vitro* co-culture experiments will be realised to determine the cellular source of these mediators.

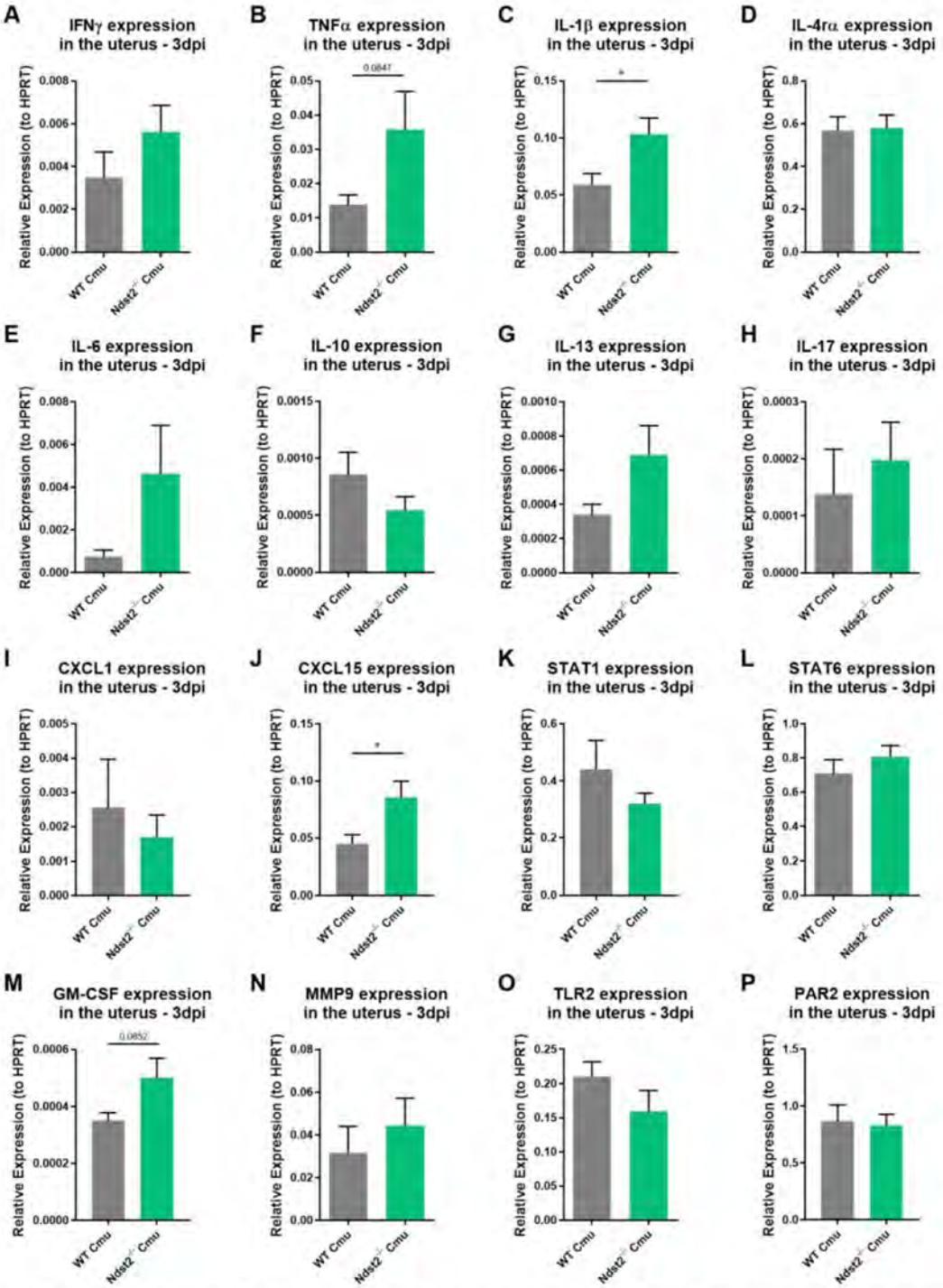


Figure 3.10 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice display enlarged expression of interleukin (IL)-1β and chemokine (C-X-C motif) ligand (CXCL)15 in their *Chlamydia*-infected uterus at 3 days post infection (dpi). Ndst2-deficient (*Ndst2*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 3dpi. RNA was extracted from the uterus and the expression of **(A)** interferon (IFN)γ, **(B)** tumor necrosis factor (TNF)α, **(C)** IL-1β, **(D)** IL-4rα, **(E)** IL-6, **(F)** IL-10, **(G)** IL-13, **(H)** IL-17, **(I)** CXCL1, **(J)** CXCL15, **(K)** signal transducer and activator of transcription (STAT)1, **(L)** STAT6 **(M)** granulocyte-macrophage colony-stimulating factor (GM-CSF), **(N)** matrix metallopeptidase (MMP)9, **(O)** toll-like receptor (TLR)2 and **(P)** protease-activated receptor (PAR)2 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$.

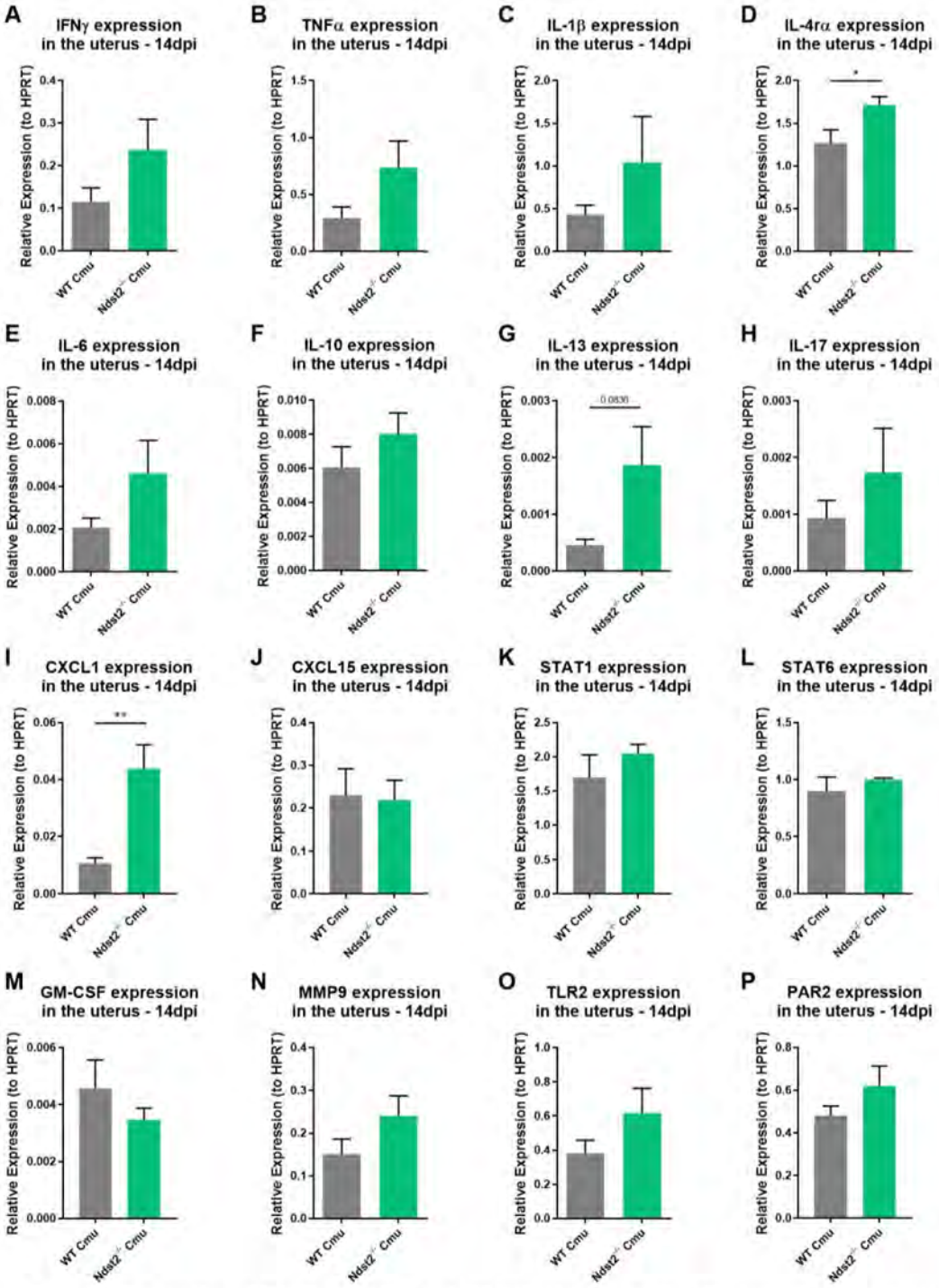


Figure 3.11 - *N*-Deacetylase *N*-Sulfotransferase 2 (Ndst2)-deficient mice display enlarged expression of IL-4rα and chemokine (C-X-C motif) ligand (CXCL)1 in their *Chlamydia*-infected uterus at 14 days post infection (dpi). Ndst2-deficient (*Ndst2*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14dpi. RNA was extracted from the uterus and the expression of **(A)** interferon (IFN)γ, **(B)** tumor necrosis factor (TNF)α, **(C)** IL-1β, **(D)** IL-4rα, **(E)** IL-6, **(F)** IL-10, **(G)** IL-13, **(H)** IL-17, **(I)** CXCL1, **(J)** CXCL15, **(K)** signal transducer and activator of transcription (STAT)1, **(L)** STAT6 **(M)** granulocyte-macrophage colony-stimulating factor (GM-CSF), **(N)** matrix metallopeptidase (MMP)9, **(O)** toll-like receptor (TLR)2 and **(P)** protease-activated receptor (PAR)2 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and ** representing $p<0.01$.

3.4.9. mMCP5-deficient mice are protected against *Chlamydia* FRT infection at early stages, but not at later stages

My previous results suggest that heparin-protease complexes might be protective for clearance of *Chlamydia* infection but detrimental for the development of associated pathology (**Figure 3.6**). Therefore, I decided to evaluate the individual roles of some MC proteases during *Chlamydia* FRT infection. In order to assess the roles of the chymase mMCP5 during *Chlamydia* FRT infection, mMCP5-deficient mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 3dpi and 14dpi (**Figure 3.1 D**). The levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

At 3dpi, the number of *Chlamydia* per vaginal lavage (**Figure 3.12 A**) and the *Chlamydia* burden in the uterus (**Figure 3.12 B**) were reduced in mMCP5-deficient mice compared to WT controls. However, at 14dpi, the number of *Chlamydia* per vaginal lavage (**Figure 3.12 C**), the *Chlamydia* burden in the uterus (**Figure 3.12 D**), and the levels of pathology in the oviducts (**Figure 3.12 E-F**) were similar between mMCP5-deficient mice and WT controls.

Therefore, my findings suggest that mMCP5 may play a detrimental role during the early stages of *Chlamydia* infection by favouring bacterial proliferation in the lower and upper FRT. However, mMCP5-deficient mice and WT controls appear to have similar levels of infection and pathology at 14dpi, which suggests that the detrimental effects of mMCP5 might not be maintained at later stages of infection.

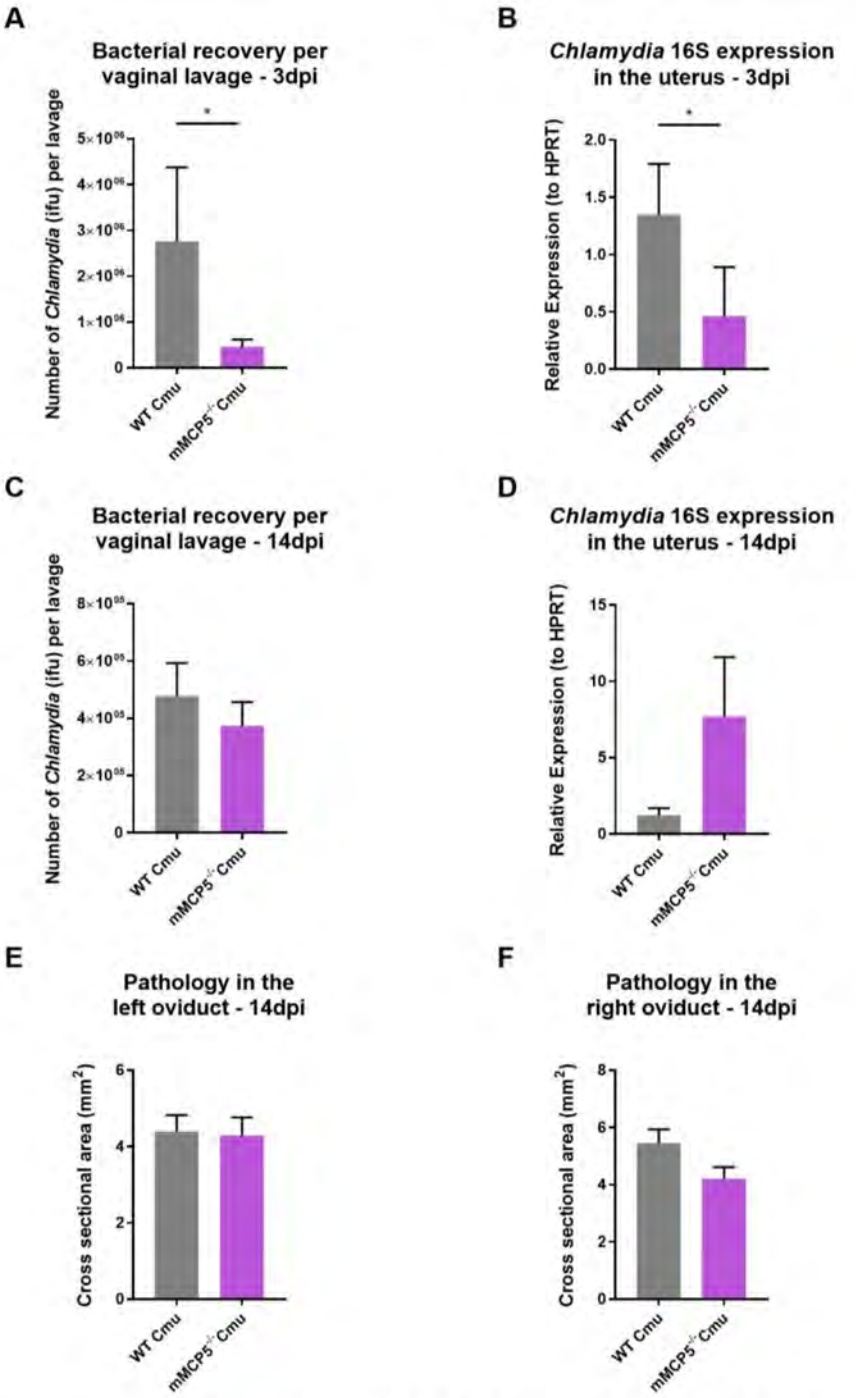


Figure 3.12 – Mouse mast cell protease 5 (mMCP5)-deficient mice have reduced *Chlamydia* burden at early stages of *Chlamydia* infection, but not at later stages. mMCP5-deficient (*mMCP5*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at **(A, B)** 3 days post infection (dpi) and **(C, D, E, F)** 14dpi. **(A, C)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per lavage. **(B, D)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross section area of the **(E)** left and **(F)** right oviducts of were measured using a calliper to evaluate the levels of *Chlamydia*-associated pathology. All data are presented as mean±SEM (n≥7) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$.

3.4.10. mMCP6-deficient/mMCP7-sufficient mice are partially protected against *Chlamydia* associated pathology

My previous results suggest that heparin-protease complexes might be protective for clearance of *Chlamydia* infection but detrimental for the development of associated pathology (**Figure 3.6**); and that the chymase mMCP5 might be detrimental during the early stages of *Chlamydia* infection (**Figure 3.12**). In order to assess the individual and collective roles of the tetramer-forming proteases mMCP6 and mMCP7 during *Chlamydia* infection, mMCP6-deficient mice (*mMCP6*$^{-/-}$ *mMCP7*$^{-/-}$), mMCP6-deficient/mMCP7-sufficient mice (*mMCP6*$^{-/-}$ *mMCP7*$^{+/+}$) and WT (*mMCP6*$^{+/+}$ *mMCP7*$^{-/-}$) controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 3dpi and 14dpi (**Figure 3.1 D**). The levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

At 3dpi, although the number of *Chlamydia* per vaginal lavage is slightly reduced in mMCP6-deficient mice and mMCP6-deficient/mMCP7-sufficient mice compared to WT mice (**Figure 3.13 A**; p=0.1222 and p=0.0606), the *Chlamydia* burden in the uterus is similar in all strains of mice (**Figure 3.13 B**). At 14dpi, both the number of *Chlamydia* per vaginal lavage (**Figure 3.13 C**) and the *Chlamydia* burden in the uterus (**Figure 3.13 D**) are similar in all strains of mice. High variability is present within each group, which is often the case in mouse models of *Chlamydia* infection. Increasing the number of mice would allow to definitely conclude on the effects of the tetramer-forming proteases on clearance of *Chlamydia* infection.

Whilst the cross sectional area of the oviducts were not affected by mMCP6 deficiency, the cross sectional area of the right oviducts was reduced in mMCP6-deficient/mMCP7-sufficient mice compared to WT controls and mMCP6-deficient mice (**Figure 3.13 F**, p<0.05 and p=0.0571). As mentioned previously, the reason why differences in phenotypes can be present between the two sides of the FRT are not clear, but these dissimilarities are likely to be caused by

differences in tissue architecture and/or in immune responses in each of the uterine horns/oviducts.

Overall, my results suggest that the presence of mMCP7, combined with the absence of mMCP6, is partially protective against *Chlamydia* infection and associated pathology. Ideally, mMCP6-sufficient/mMCP7-sufficient mice (*mMCP6*$^{+/+}$ *mMCP7*$^{+/+}$) would have been added to this study for full comparison of the differential effects of mMCP6 and mMCP7 during *Chlamydia* infection. However, this mouse strain was not available during my PhD studies.

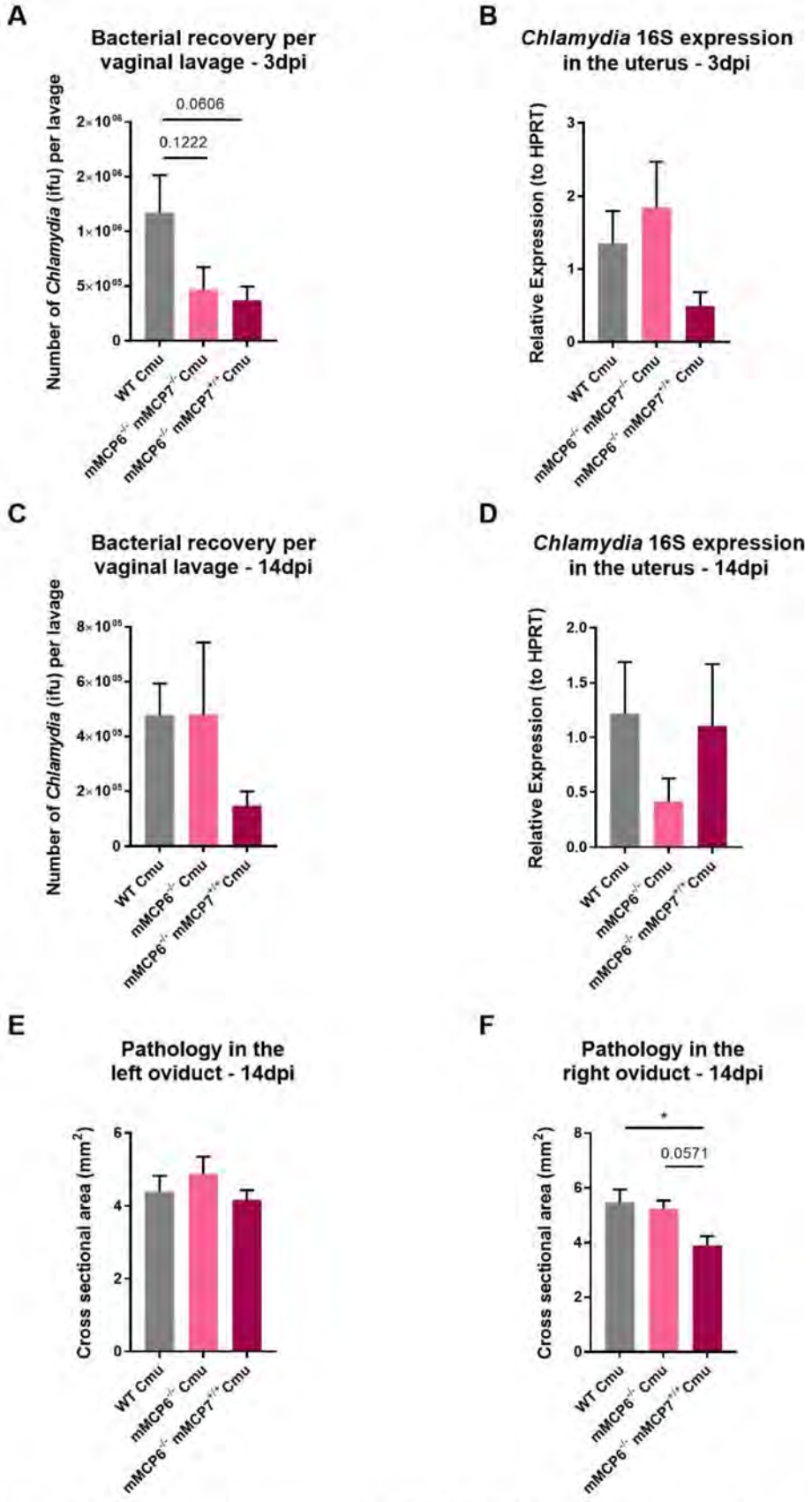


Figure 3.13 – Mouse mast cell protease 6 (mMCP6)-deficient mice have unaltered *Chlamydia* infection and associated pathology while mMCP6-deficient/mMCP7-sufficient mice are partially protected against *Chlamydia*-associated pathology. mMCP6-deficient (*mMCP6*$^{-/-}$ *mMCP7*$^{-/-}$), mMCP6-deficient/mMCP7-sufficient (*mMCP6*$^{-/-}$ *mMCP7*$^{+/+}$) mice and WT controls were treated with progesterone,

infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at **(A, B)** 3 days post infection (dpi) and **(C, D, E, F)** 14dpi. **(A, C)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per lavage. **(B, D)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross section area of the **(E)** left and **(F)** right oviducts of were measured using a calliper to evaluate the levels of *Chlamydia*-associated pathology. All data are presented as mean±SEM ($n \geq 6$) and, depending on the results of normality tests, ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons tests or Kruskal-Wallis tests with Dunn's multiple comparisons tests were performed, with * representing $p<0.05$.

3.5. Discussion

In **Chapter 2**, I demonstrate that MCs are present in the FRT and that their numbers fluctuate upon administration of female sex hormones. Moreover, I show that MC degranulation might increase the susceptibility to *Chlamydia* FRT infection during the early stages of infection. I also show that MCs contribute to the development of infection-induced pathology in the oviducts during the later stages of infection, possibly by mediating the recruitment of innate immune cells known to play a role in pathology, including neutrophils and macrophages. Since, MCs store a wide variety of mediators in their secretory granules that are rapidly released during degranulation and that could underpin the effects that I observed in **Chapter 2**, I next sought to examine the role of certain factors contained within MC granules in the pathogenesis of *Chlamydia* FRT infection and focused my PhD studies on MC proteases, as they are the major group of proteins stored in MC granules (199).

In **Chapter 2**, I showed that the number of uterine MCs is decreased by oestrogen treatment and/or oestrogen-induced oestrus (**Figure 2.4**). Interestingly, I show that the expression of the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, are increased in mice treated with oestrogen (**Figure 3.2**), which suggests that the phenotype of MCs regarding their production of these proteases, is altered by the changes in the levels of female sex hormones observed during different stages of the menstrual/oestrous cycle. In women, the expression of the β-tryptase, TPSAB1, and the α-chymase, CMA1, were shown to be unchanged in endometrial tissues obtained during different stages of the menstrual cycle (318). Therefore it is possible that the regulation of MC protease expression might be different between humans and mice. However, as the expression of TPSAB1 and CMA1 was measured only in endometrial tissues rather than in whole uterus, it is also possible that differences in the expression of MC proteases in the myometrium during different stages of the menstrual/oestrous cycle might be responsible for the differences in results between the previously mentioned study and mine (318). While another study showed that the expression of the chymases mMCP1 and mMCP5 remain unchanged in the uterus of mice treated with either progesterone or oestrogen (332), the dissimilarities in results regarding the expression

of mMCP5 between this study an mine may be caused by different experimental protocols used, with hormonal treatments administered once seven days before the endpoint in my study and administered for three consecutive days just prior to endpoint in the other study (332).

While the mechanism involved in my observations that oestrogen and/or oestrous stimulate the expression of the MC proteases, mMCP4, mMCP5, mMCP6 and mMCP7, remain to be clearly identified, oestrogen might affect the phenotype of MCs directly through binding to ERα and ERβ receptors present on MCs (318, 332-334). While the presence of such receptors on MCs in the FRT is not fully verified, oestrogen could also indirectly affect the phenotype of MCs, through hormone-induced changes in cytokine and chemokine production in the FRT (401-403). Indeed, MCs and their storage of MC proteases can be altered following exposure to cytokines such as IL-3 and IL-10 (202) and these factors have been shown to be increased in the uterus during oestrus compared to diestrus (459). Moreover, the *mi* transcription factor (MITF) and c-jun have been shown to synergistically participate in the transactivation of the *Mcpt6* and *Mcpt7* genes, hence enhancing their expression (460, 461). Given that oestrogen was shown to induce the expression and/or activity of c-jun in the FRT (462-464), oestrogen-signalling through c-jun may be responsible for the changes in the expression of MC proteases observed in the FRT following oestrogen treatment. Another explanation for the increase in expression of mMCP4, mMCP5, mMCP6 and Cpa3 following oestrogen treatment could be that these proteases are being expressed by other cells that infiltrate/proliferate within the FRT when oestrogen levels are increased and/or during oestrus. For instance, mMCP5 has been suggested to be expressed by uterine NK cells (465) and Cpa3 is expressed by basophils (237). However, uterine NK cell proliferation and/or recruitment appear to be largely stimulated by progesterone, with increased number of uterine NK cells detected in mid/late secretory stage of menstrual cycle (466-468). Therefore, the increase in expression of mMPC5 following oestrogen treatments is likely to not be caused by the potential expression of mMCP5 on NK cells. Moreover, since I show that the expression of mMCP4, mMCP5, mMCP6 and Cpa3 are all largely decreased in MC-deficient mice (**Figure 3.4**), I would argue that MCs are the major source of these proteases in the FRT.

To confirm this hypothesis, in future studies, immunostaining will be realised to identify the cellular source of mMCP4, mMCP5, mMCP6 and mMCP7 in the FRT.

As shown in Chapter 2 (**Figure 2.5**) and by others (368), MCs don't appear to be recruited to the FRT during *Chlamydia* infection. To support this observation, I show that the expression of MC proteases is not affected during the early stages of *Chlamydia* infection. However, at the later stages of infection, the expression of mMCP4, mMCP5, mMCP6 and Cpa3 is reduced in the uterus of *Chlamydia*-infected mice compared to non-infected controls (**Figure 3.3**). This decrease might be caused by the lower number of uterine MCs that I observed in the uterus during the later stages of infection (**Figure 2.5**). The drop in the expression of MC proteases may also be the result of *Chlamydia* infection and/or the associated host immune responses affecting MC phenotype in the FRT.

In the secretory granules of MCs, MC proteases are bound to serglycin proteoglycans that consist of heparin or chondroitin sulfate (244, 245, 445). Ndst2 deficiency results in altered storage of the MC mediators that are normally bound to heparin in MC granules, including histamine and the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3 (244, 245, 442). Although the number and phenotype of MCs appears to be regulated by female sex hormones and *Chlamydia* infection, I show that the expression of Ndst2 in the FRT remains stable in progesterone- or oestrogen-treated mice as well as during *Chlamydia*-infection (**Figure 3.5**). This agrees with findings that show that Ndst2 is constitutively expressed in most tissues, from embryonic life to adulthood (249). Interestingly, the expression of Ndst2 is not altered in MC-deficient mice, which suggests that Ndst2 is not restricted to MCs but possesses a broader expression pattern in different cell types (249, 469).

In Chapter 2, I showed that MC-deficient mice are protected against the development of pathology (**Figure 2.6**). In this chapter, I used Ndst2-deficient mice to assess the effect of the MC mediators that are normally bound to heparin in the secretory granules of MCs, including the heparin-protease complexes, in the pathogenesis of *Chlamydia* FRT infection. I show that Ndst2-deficient mice do not have altered levels of infection compared to WT controls during the early

stages of infection. However, I show that Ndst2-deficient mice have increased levels of infection in the uterus at later stages (**Figure 3.6**).

A potential limitation to those results is that some studies suggest that heparan sulfate on epithelial cells is required for attachment and infection by *Chlamydia* (81, 83, 470, 471). Even if Ndst2 is thought to be responsible for initiating sulfation of heparan sulfate only in MCs (244, 245, 247), and that Ndst2-deficient mice have been shown to have normal heparan sulfate in most tissues (246), it is possible that Ndst2-deficient mice have altered heparan sulfate on their epithelial cells in the FRT. This would alter the susceptibility of Ndst2-deficient mice to *Chlamydia* infection independently of MCs. While my data show no differences in susceptibility to *Chlamydia* infection in the vagina and in the uterus between Ndst2-deficient mice and WT controls during the early stages of infection, suggesting that Ndst2 might not alter epithelial cell infectivity, in future studies, uterine epithelial cells will be isolated from Ndst2-deficient mice and WT controls to investigate changes in infectivity due to possible Ndst2-mediated alteration of heparan sulfate on epithelial cells. While I have tried to realise those studies during my PhD, technical problems in efficiently isolating and growing uterine epithelial cells prevented the finalisation of those experiments in the timeframe of my PhD.

Interestingly, despite having an increase in *Chlamydia* infection compared to WT controls, I show that Ndst2-deficient mice are protected against hydrosalpinx at 14dpi (**Figure 3.6**). This paradoxical observation of increased *Chlamydia* burden and protection against pathology at 14dpi is not necessarily surprising given that many of the immune responses that are often induced during *Chlamydia* FRT infection in order to help clear infection are also responsible for infection-induced pathology (472). My data demonstrate that the presence of Ndst2 is necessary for both inducing responses that help clear infection but also drive *Chlamydia*-induced pathology. Therefore, despite being present in small proportion in the FRT compared to MCs containing chondroitin sulfate (**Figure 2.3 G-M**), MCs containing heparin might have strong effects on *Chlamydia* infection through the requirement of Ndst2 to store some MC proteases in the secretory granules of those MCs. To supplement my findings, future studies are required to assess the roles of MCs containing chondroitin sulfate in mediating clearance of *Chlamydia* infection

and/or development of *Chlamydia*-induced pathology, to fully define the roles of the different MC types during *Chlamydia* FRT infection.

To assess how Ndst2 deficiency may result in increased susceptibility to infection but decreased pathology, I assessed immune cell responses in the upper FRT of *Chlamydia*-infected Ndst2-deficient and WT controls using flow cytometry. Ndst2-deficient mice have a large reduction in the total cells, as well as in the immune cells present in their uterus during *Chlamydia* infection suggesting that heparin-protease complexes and/or other factors affected by Ndst2 deficiency are important for immune cell recruitment to the *Chlamydia*-infected uterus. More specifically, I show a decline in the number of neutrophils and eosinophils in Ndst2-deficient mice, which suggests a possible role for heparin-protease complexes in inducing granulocyte recruitment to the uterus (**Figure 3.7**). While the role of eosinophils during *Chlamydia* infection is not clear, neutrophils are well known for their role in the induction of immunopathology (127, 133). Therefore, the reduction in neutrophils could account for the decrease in pathology in Ndst2-deficient mice. The number of antigen presenting cells, including inflammatory monocytes/macrophages as well as myeloid dendritic cells, are also decreased in the uterus of Ndst2-deficient mice (**Figure 3.7**). The drop in infiltrating macrophages may also explain the reduction in pathology in the Ndst2-deficient mice as IL-1β, which is produced predominantly by macrophages during *Chlamydia* FRT infection, has been associated with development of hydrosalpinx (149). Many studies have identified a role for MCs, as well as histamine and cytokines (IL-6 and TNFα) released by MCs, in the induction of DC maturation and tracking to the FRT-draining lymph nodes for naïve T cell activation, thereby contributing to the induction of both $CD4^+$ and $CD8^+$ T cell responses (260, 281, 282, 284, 285, 473). Importantly, I show that myeloid DCs are reduced in the upper FRT of Ndst2-deficient mice during *Chlamydia* infection. The reduction in myeloid DCs in the uterus could suggest a diminution in the ability to induce *Chlamydia*-specific adaptive T cell responses with Ndst2 deficiency, which may explain the increased susceptibility to infection (154)

While MC deficiency did not alter T cell number and phenotype, Ndst2-deficient mice have a strong decrease in the number of T cells in their uterus, with both $CD4^+$ and $CD8^+$ T cells

reduced (**Figure 3.7**). The decrease in T cell responses in Ndst2-deficient mice might be caused by the reduction in myeloid DCs mentioned above, or could result from a direct role of some of the factors affected by Ndst2 deficiency, such as histamine or heparin-protease complexes, in the induction of *Chlamydia*-specific T cell responses. The reduction in the number of $CD8^+$ T cells could account for the diminished pathology in Ndst2-deficient mice as $CD8^+$ T cells have been associated with increased immunopathology through their secretion of TNFα and IL-13 and their capacity to enhance neutrophil accumulation in the upper FRT (182, 183, 474). On the contrary, $CD4^+$ T cells are recognised as being vitally important for protecting against *Chlamydia* FRT infection (95, 96, 161). Therefore, the reduction in these cells in Ndst2-deficient mice may account for the increased infection.

I next sought to examine whether these changes in $CD4^+$ T cell number were associated with a change in Th phenotype. While the polarisation of $CD4^+$ T cells in response to *Chlamydia* infection have been shown to be heterogeneous, Th1 cells are thought to be the dominant phenotype induced in response to infection and offer the most robust protection against infection through their secretion of IFNγ (95, 96, 161, 475). In contrast, Th17 cell responses, which are also induced in response to *Chlamydia* FRT infection, are associated with increased pathology (95, 476, 477). During the early stages of *Chlamydia* infection, Ndst2-deficient mice display reduced expression of the transcription factors Tbet and RORγt, with slight rise in GATA3 expression, however, those differences were not maintained at later stages (**Figure 3.9**). The expression of the main cytokines associated with Th1 and Th17 responses are not changed between Ndst2-deficient and WT controls, but a slight increase in the Th2 factors IL-4rα and IL-13 is present in the uterus of Ndst2-deficient at 14dpi (**Figures 3.10 and 3.11**). Evaluating the protein levels of some of these key factors would allow to verify if the alteration in expression of transcription factors lead to changes in cytokines profile in the uterus. Overall, my data suggest that heparin-protease complexes might play an important role for the induction of type 1 and type 17 responses during the early stages of infection. My findings agree with previous data that show that MCs and/or the mediators that they release promote type 1 and type 17 immunity under a number of conditions (260, 288, 478-480). However, studies also suggest that MCs and/or MC

mediators induce type 2 responses (284-286, 444). Taken together, these studies highlight the complex regulatory functions of MCs and MC mediators in mediating immune responses. Future studies are required to help understand how MC protease storage and release affect $CD4^+$ T cell polarisation responses in the uterus and in the FRT-draining lymph nodes during *Chlamydia* FRT infection to better understand how Ndst2 deficiency results in increased susceptibility to infection with decreased pathology.

I next examined whether Ndst2 deficiency was associated with changes in the expression of key immune factors associated with protection/susceptibility to infection and/or *Chlamydia*-induced pathology. I show that the expression of TNFα, IL-1β, CXCL15 and GM-CSF at 3dpi, and of IL-4rα, IL-13 and CXCL1 at 14dpi, are slightly increased in the uterus of Ndst2-deficient mice compared to WT controls during *Chlamydia* FRT infection (**Figures 3.10 and 3.11**). These results appear to contradict my previously described results, as they suggest an enhancement of pro-inflammatory responses in Ndst2-deficient mice that should be associated with enhanced recruitment of immune cell to the uterus, increased clearance of infection and amplified development of *Chlamydia*-induced pathology (149, 481, 482). It is possible that other cytokines not investigated in this study are decreased in Ndst2-deficient mice or that the increased expression of anti-inflammatory factors observed at 14dpi may reduce inflammation in the Ndst2-deficient mice. As MC factors have been shown to mediate the concentration and activity of various immune factors at the protein level, it is possible that Ndst2 deficiency results in pronounced changes to proteins that cannot be ascertained by examining mRNA expression alone. Therefore, evaluating the protein levels of key immune factors will be necessary to verify if Ndst2 deficiency is associated with changes in immune factors associated with protection and/or susceptibility to infection and/or *Chlamydia*-induced pathology in future studies. In complement, immunostaining and/or *in vitro* co-culture experiments will be realised to determine the cellular source of some of the key immune mediators identified.

Overall, the phenotype observed in Ndst2-deficient mice appears to partially replicate what observed during infection in MC-deficient mice, with both strains of mouse protected against pathology. However, in contrast to MC-deficient mice, Ndst2-deficient mice exhibit increased

susceptibility to infection in the uterus and have a much more robust decrease in immune cell infiltration into their uterus. I propose that the reduction in pathology in the two strains may arise from the decrease in granulocytes and infiltrating macrophages observed. The increase in infection observed in Ndst2-deficient mice may be explained by the reduction in the protective $CD4^+$ T cells. While I show strong effects of Ndst2 deficiency during *Chlamydia* infection, additional studies are required to characterise the mechanisms that underpin the effects of Ndst2 deficiency in mediating immune cell recruitment to the uterus, but such studies were not able to be performed in the timeframe of my thesis.

Since I show such strong effects on susceptibility to *Chlamydia* infection and associated pathology in Ndst2-deficient mice, I next sought to determine the roles played by some of the individual proteases that require heparin for appropriate storage in the secretory granules of MCs. To do this, I subjected mice deficient in the α-chymase mMCP5 (which also experience secondary loss of the metalloprotease Cpa3) (239, 296, 457), mice deficient in the tryptase mMCP6 (274, 442, 458, 483) and mice deficient in the tryptase mMCP6 but with a functioning tryptase mMCP7 (275, 442, 458, 483) to our murine model of *Chlamydia* FRT infection.

Whilst no difference in clearance of *Chlamydia* infection and in infection-induced pathology is detectable between mMCP5-deficient mice and WT controls at 14dpi, mMCP5-deficient mice appear to be protected against lower and upper FRT infection during the early stages of infection (**Figure 3.12**). During pulmonary infections, mMCP5-deficient mice have been shown to have enhanced clearance of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, with similar levels of histopathology compared to WT controls. Whilst the mechanisms involved were not fully characterised in this study, mMCP5 deficiency was associated with altered airway inflammation affecting the number of macrophages and neutrophils, as well as the expression of cytokines and chemokines, in the lungs (302). Therefore, taken together, these data suggest a potential detrimental role of mMCP5 and/or Cpa3 during the early stages of bacterial infection. Although preliminary results do not allow me to conclude on the mechanism that underpins the function of mMCP5 during *Chlamydia* infection, several hypotheses can be made. Firstly, mMCP5 has been shown to activate MMP9 (239) and, whilst MMP9 has been shown to play a

role in the induction of *Chlamydia*-induced pathology (135, 136), MMP9-deficient mice have also been shown to have reduced infection (136), suggesting that mMCP5-mediated activation of MMP9 might result in early changes in host inflammatory responses leading to increased susceptibility at 3dpi. Moreover, mMCP5 has been shown to participate in the disruption of the epithelium through dysregulation of epithelial tight junctions (296, 297). Therefore, mMCP5 might disturb the epithelium in the FRT, hence amplifying susceptibility to *Chlamydia* infection. Indeed, Sun *et al*., suggest that disruption of tight junctions in the epithelium might facilitate the cellular rearrangement of infected epithelial cells that are required for *Chlamydia* infectious cycle and/or cause infected epithelial cells to detach into the lumen, hence favouring dissemination of the *Chlamydia* infection, while simultaneously protecting the intracellular bacterium from host immune response (484).

I also evaluated the roles of the tetramer-forming tryptases mMCP6 and mMCP7 during *Chlamydia* infection. I show that mMCP6-deficient mice have a relatively similar course of infection compared to WT controls, suggesting that mMCP6 might not play a major role during *Chlamydia* infection (**Figure 3.13**). mMCP6 has been shown to be protective during *Kelbsiella pneumoniae* infection by contributing in the induction of neutrophil recruitment (274, 275). In contrast, mMCP6-deficient mice have been shown to have increased clearance of infection, with no change in pathology nor in airway inflammation during *Streptococcus pneumoniae* infection (302). Therefore, my results and that of others show that the role of mMCP6 in immune responses to bacterial infection appear to be highly dependent on the infected organ and on the pathogen involved. I also show that mMCP6-deficient/mMCP7-sufficient mice are slightly protected against infection in the lower FRT during the early stages of infection, and are protected against *Chlamydia*-induced pathology in the later stages. This suggests that mMCP7 might play a protective during *Chlamydia* infection. However, analysis of the outcome of *Chlamydia* infection in mMCP6-sufficient/mMCP7-sufficient mice ($mMCP6^{+/+}$ $mMCP7^{+/+}$) is necessary to fully conclude on the role of mMCP7 and to study the individual *versus* synergistic effects of mMCP6 and mMCP7. Unfortunately, this strain of mouse was not available for my PhD studies.

The differential roles of the two tetramer-forming tryptases during *Chlamydia* infection might be caused by a different location of enzymatic activities between the two tryptases following degranulation. Indeed, mMCP7 separates from serglycin proteoglycans upon MC degranulation, disseminate throughout the extracellular environment and can reach circulation, whilst mMCP6 only acts at in the immediate surroundings of the degranulating MC (221). The *in vivo* activity of the tryptase mMCP7 has been less studied than that of mMCP6. Similar to most MC proteases, mMCP7 can cleave fibrinogen (222, 303), thereby potentially mediating pro-inflammatory signalling and recruitment of immune cells (454, 455). In support of this hypothesis, unpublished data from Miyasaki *et al.*, described in a review by Stevens and Adarchi, suggest a role for mMCP7-mediated cleavage of fibronectin in the induction of IL-6-mediated neutrophils and eosinophils infiltration into the conjunctiva (202).

Overall, none of the MC proteases, mMCP5/ Cpa3, mMCP6 and/or mMCP7, appear to be individually responsible for the development of *Chlamydia*-induced pathology observed in Ndst2-deficient and MC-deficient mice. This observation could be caused by redundant activities of MC proteases, with absence of one protease compensated by another. Hence, the combined absence of several proteases is required to display protection against *Chlamydia*-induced pathology. Supporting this hypothesis, a study showed that the individual absences of mMCP4, mMCP6 or Cpa3 does not affect melanoma cell establishment in the lung, but concomitant deficiency of those proteases is detrimental for disease progression (485). It is also possible that another MC factor might be contributing to the development of *Chlamydia*-induced pathology. The β-chymase mMCP4 could be a potential candidate as it has been shown to activate MMP9 (315), with MMP9 is shown to be required for the development of *Chlamydia*-induced pathology (135, 136). In addition, mMCP4 has been shown to mediate pro- and anti-inflammatory responses (309-312, 486), affect tight junctions (296, 297) and degrade component of the ECM such as fibronectin, laminin and collagen (233, 305, 306, 487). During group B *Streptococcus* vaginal infection, mMCP4-mediated fibrinogen cleavage has been shown to be protective, though hindering adherence of the bacteria, which reduces severity of infections (305). Therefore, with a wide range of activities *in vivo*, mMCP4 might be involved in mediating immune responses to *Chlamydia*

infection. Histamine might also be involved in host responses to *Chlamydia* infection, due to its capacity to increase blood vessel dilatation, vascular permeability and pro-inflammatory responses (268, 488, 489). Therefore, the reduction in histamine in Ndst2-deficient mice might be responsible for the reduced infiltration of immune cells into the uterus during *Chlamydia* FRT infection. In future studies, treatments with anti-histamine, siRNAs and/or recombinant protease treatments in WT and genetically modified mice will be important to identify the MC factors involved during *Chlamydia* FRT infection and in infection-induced pathology.

3.6. Conclusion

In this chapter, I show that MC mediators, including heparin-protease complexes, promote clearance of *Chlamydia* infection, whilst contributing to the induction of *Chlamydia*-associated pathology. The robust decrease in immune cell infiltration seen in Ndst2-deficient mice is likely to account for the increased susceptibility of these mice to infection as well as their reduced pathology. Individually, the deficiency in the MC proteases, mMCP5/Cpa3, mMCP6 and/or mMCP7, did not recapitulate the phenotype observed in Ndst2-deficient and MC-deficient mice, suggesting that another MC factor might be important for mediating immune response to *Chlamydia* infection or that the individual functions of the MC proteases during *Chlamydia* infection might be redundant. Even if the mechanism that underpins the effects of MC proteases during *Chlamydia* infection are not yet fully understood due the complex regulatory functions of MC and MC factors in mediating immune responses, my study reveals, for the first time, that MC proteases and/or MC factors contribute to immune responses to *Chlamydia* FRT infection. The next chapter of my thesis will focus on determining the roles of a unique MC protease, the membrane-anchored γ-tryptase Prss31, during *Chlamydia* FRT infection.

Chapter four: The role of Prss31 during *Chlamydia* female reproductive tract infection

4.1. Abstract

Chlamydia trachomatis is the most common bacterial STI worldwide. Some women develop severe complications following *Chlamydia* infection, such as ectopic pregnancy and tubal factor infertility. Greater understanding of the mechanisms that underpin the clearance of infection *versus* those that mediate pathology are required to inform novel prevention and/or treatment strategies. While the roles of many key immune cells for protection against *Chlamydia* infection have been extensively studied, the roles of other cells such as MCs and eosinophils remain globally unclear.

In **Chapter 2**, I identified a detrimental role for MCs in the induction of *Chlamydia*-associated pathology. In **Chapter 3**, I show an important role for MC proteases and/or MC mediators stored in the secretory granules of MCs in both the clearance of infection and in the development of pathology. Specifically, I show that Ndst2-deficient mice, which lack the ability to store the MC proteases mMCP4, mMCP5, mMCP6 and CPA3, are protected against *Chlamydia*-induced pathology and this protection is associated with a decrease in immune cell infiltration to the FRT. Interestingly I also show that different MC proteases have different functions in mediating immune responses to *Chlamydia* infection. For example, I show that mMCP5 may increase susceptibility during the early stages of infection, that mMCP6 does not have much of an influence on the course of infection or pathology and that mMCP7 might protect against pathology.

In this chapter, I extend upon my findings investigating the effects of MC proteases by characterising the effects of Prss31 during *Chlamydia* FRT infection and infection-induced disease. Prss31 is a tryptase that has key differences when compared to other MC proteases. The substrate binding cleft of Prss31 is different compared to other tryptases, suggesting different substrate specificity. Furthermore, and perhaps more importantly, Prss31 is characterised by the

presence of a hydrophobic peptide membrane anchor that acts as a membrane-spanning domain and bind Prss31 to the plasma membrane of MCs following degranulation, instead of being released into the extracellular environment like other proteases.

In this chapter, I show that, contrary to the expression of mMCP4, mMCP5, mMCP6 and CPA3, the expression of Prss31 is not affected by female sex hormones or *Chlamydia* infection. In addition, I show that the expression of Prss31 is not decreased in the uterus of MC-deficient mice, suggesting that Prss31 might not be specific to MCs in the FRT. While I tried to identify the cellular source of Prss31 using immunohistochemistry, unspecific binding visible in Pss31-deficient mice prevented me to make definite conclusion on the cellular source and tissue location of Prss31 in the FRT. By subjecting Prss31-deficient mice (and WT controls) as well as WT mice treated with recombinant Prss31 (and vehicle-treated controls) to our *Chlamydia muridarum* mouse model, I identify a protective role for Prss31 during *Chlamydia* FRT infection through promoting clearance of infection and/or protecting against infection-associated pathology. The mechanisms that underpin those effects are not fully elucidated in my study, but Prss31 might be involved in mediating pro-inflammatory responses, as Prss31-deficient mice have a decrease in the number of immune cells present in their uterus during *Chlamydia* infection.

Elucidating the cellular source and the mechanism underpinning the protective role of Prss31 during *Chlamydia* infection may be important for the development of new therapeutic target for the prevention/treatment of *Chlamydia* infection.

4.2. Introduction

As outlined in previous chapters, despite decades of research dedicated to understanding mechanisms of pathogenesis of *Chlamydia* FRT infection, many of the cells, signalling molecules and pathways that mediate clearance of infection *versus* those that drive pathology are yet to be described. In **Chapters 2 and 3** of my thesis, I have outlined the studies that I conducted during my PhD using a mouse model of *Chlamydia* FRT infection, cromolyn treatments and a suite of transgenic mice. These studies highlight novel and unique roles for MCs, MC degranulation and MC proteases in the pathogenesis of *Chlamydia* FRT infection and/or infection-induced pathology. Specifically, in **Chapter 2**, using a combination of MC deficient and cromolyn-treated mice, I show that MC degranulation might increase susceptibility to infection during the early stages of a *Chlamydia* FRT infection and that MCs and MC degranulation also play an important role in driving hydrosalpinx in the later stages of infection. In **Chapter 3**, I extended upon these findings to show important, yet differential, roles for different MC proteases in mediating MC-dependent effects on *Chlamydia* infection. Using Ndst2 deficient mice, which do not correctly store the proteases mMCP4, mMCP5, mMCP6 and CPA3 in the secretory granules of MCs, I show an important role for these proteases in the development of *Chlamydia*-induced hydrosalpinx. Interestingly, using mMCP5- and mMCP6-deficient mice and mMCP7 knock-in mice I show that different proteases have differential effects on both infection and/or pathology, thus highlighting the importance of investigating the role(s) of individual MC factors in the pathogenesis of *Chlamydia* infection and infection-induced disease. In this Chapter, I outline the final series of studies that were conducted during my PhD studies, which examined the role of another MC protease, the membrane-anchored tryptase, Prss31 in the pathogenesis of *Chlamydia* FRT infection.

Similarly to other MC tryptases, Prss31 is encoded on the chromosomes 16p13.3 in humans and 17A3.3 in mice (202, 212). However, whilst most the other MC tryptases appear to be closely related, for instance 90% homology exists between α- and β-tryptases (217), Prss31 is quite unique (447). One of the major differences between Prss31 and the other MC proteases is

the presence of a hydrophobic peptide membrane anchor in the C-terminal region of Prss31. This additional peptide acts as a membrane-spanning domain, which results in Prss31 being the only membrane-anchored tryptase (212, 226). Upon MC degranulation, when all the other MC proteases are released (freely or in association with serglycin proteoglycans) into the extracellular environment, Prss31 is proposed to remain bound to the plasma membrane of MCs. Therefore, the enzymatic activity of Prss31 is confined to the immediate environment that surrounds the MC following degranulation (226). Theoretically, it is possible that Prss31 could be cleaved from its membrane anchor by another protease and be shed as a soluble tryptase, however, no evidence of this process has been identified *in vivo* to date. Another key difference is that, unlike other MC proteases where the pro-peptide is normally removed upon activation, the activation pro-peptide of Prss31 remains attached to the catalytic domain upon activation of Prss31 (226). Prss31 also differs from the main MC tryptase (β-tryptase in human and mMCP6 in mouse), in its susceptibility to the serpin-class inhibitors. This is potentially due to the capacity of β-tryptase/mMCP6 to form tetramers protected by serglycin proteoglycans, whereas Prss31 lacks the Trp- and Pro- rich domains necessary for tetramer formation (202, 212, 226). Whilst, in the skin and large intestine, the expression of Prss31 has been shown to be restricted to MCs (212), it remains unknown if the storage of Prss31 in the secretory granules of MCs is dependent on heparin proteoglycans, as with the other MC proteases, mMCP4, mMCP5, mMCP6 and CPA3 (244, 245). Similar to mMCP7, Prss31 is expressed in a strain-dependent manner in mice, with expression detected in C57BL/6 and W/W^v mice but not in BALB/c mice (212).

Analyses of the loops forming the S1-pocket and the substrate-binding cleft of both mouse and human Prss31 suggest normal tryptase-like activity of this unique protease (226). However, six of the seven loops constituting the substrate-binding clefts of Prss31/γ-tryptases are different from βI-tryptases, suggesting differences in substrate specificity (226). Importantly, most of the studies investigating the substrate specificity of Prss31, have been conducted using recombinant Prss31 proteins, which do not hold the membrane anchor on the C-terminal region and are, therefore, soluble. This could potentially change the activity of Prss31 and/or the nature of the responses it produces within the extracellular environment in tissues (225, 226).

The function of Prss31 *in vivo* has not been extensively studied. Prss31 has been shown to play roles in the induction of airways hyper-responsiveness and the development of chronic obstructive pulmonary disease in mice (226, 490, 491). Specifically, the tracheal administration of recombinant Prss31 was shown to activate the IL-13/IL-4rα/STAT6 pathway and result in bronchial hyper-responsiveness in mice (226). Furthermore, Prss31-deficient mice, was well as WT mice treated with anti-Prss31 antibodies, have been shown to have reduced inflammation, small airways fibrosis and/or alveolar enlargement (emphysema) in murine models of cigarette smoke-induced chronic obstructive pulmonary disease (490, 491). During pulmonary infections, Prss31 appears to play different roles according to the pathogen, with Prss31-deficient mice being more susceptible to Influenza, while being protected against *Streptococcus pneumonia* (302). In an acute colitis model, Prss31-deficient mice were protected against colitis-associated weight loss and gastrointestinal tract pathology, in association with a decrease in the expression of CXCL2 and IL-6 in the colon (491).

Together, the limited number of studies that have investigated the role of Prss31 *in vivo* demonstrate that this unique MC protease can have either protective or detrimental roles depending on the context of disease (226, 302, 490, 491). Given that the role of Prss31 in *Chlamydia* FRT infection and infection-induced pathology is yet to be explored, this chapter will characterise the expression of Prss31 in the FRT and investigate the roles of Prss31 during infection by subjecting Prss31-deficient mice (and WT controls), as well as WT mice treated with recombinant Prss31 (and vehicle-treated controls) to a *Chlamydia muridarum* mouse model.

4.3. Material and methods

4.3.1. Ethics statement

All the animals and procedures used in this study were approved by the Animal Care and Ethics Committee at the University of Newcastle (Callaghan, NSW, Australia).

4.3.2. Mouse strains used to investigate the roles of Prss31 during *Chlamydia* FRT infection

WT C57BL/6 female mice were obtained from the ABR facility (Moss Vale) in order to [1] evaluate the effects of female sex hormones and *Chlamydia* FRT infection on the expression of Prss31, [2] assess the cellular source of Prss31 in the FRT, and [3] determine the effect of intravaginal treatments with recombinant Prss31 during *Chlamydia muridarum* FRT infection (226) (**Table 4.1**).

MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) female mice and their appropriate controls (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) were obtained from the UniSA animal facility (Adelaide) to assess if the expression of Prss31 is MC-dependent (**Table 4.1**) (237).

Prss31-deficient (*Prss31*$^{-/-}$) female mice and WT controls were obtained from the ABR facility (Moss Vale) to assess the roles of Prss31 during *Chlamydia* FRT infection (**Table 4.1**) (491).

Upon delivery, mice were housed in SPF conditions in the Bioresources facility at the HMRI (New Lambton Heights), with *ad libitum* access to food and water under a 12 hours light-dark cycle.

Transgenic mouse line	Mouse background	Facility of origin	Description of the genetic modification
WT	C57BL/6	ABR	-
WT *Cpa3-Cre; Mcl-1*$^{WT/WT}$	C57BL/6	UniSA	-
Cpa3-Cre; Mcl-1$^{fl/fl}$	C57BL/6	UniSA	Deficiency in the intracellular anti-apoptotic factor myeloid cell leukemia-1 (Mcl-1) required for MC survival
WT	C57BL/6	ABR	-
Prss31$^{-/-}$	C57BL/6	ABR	Deficiency in protease serine member S31 (Prss31)
WT	C57BL/6	ABR	-

Table 4.1 - Description of the wild type and transgenic mouse strains used to investigate the roles of Prss31 during *Chlamydia* infection. *WT: wild type, MC: mast cell, ABR: Australia BioResources, UniSA: University of South Australia, CPA3: carboxypeptidase A3, Prss31: protease serine member S31.*

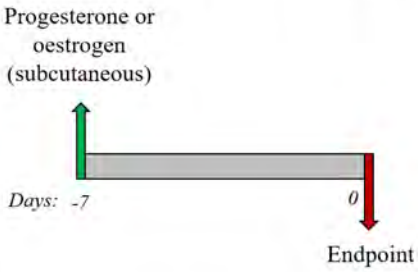
As explained in **Chapter 2** (**Section 2.3.2**), in order to assess the effects of female sex hormones on the expression of Prss31 in the FRT, WT mice (10-11 weeks old) were subcutaneously administrated with either medroxyprogesterone acetate (Depo-Provera) or 17β-oestradiol (Sigma-Aldrich) to synchronise their oestrus cycle and induce respectively diestrus or oestrus, respectively (332, 388). Seven days later, mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb) and FRT tissues were collected for analyses (**Figure 4.1 A**).

As explained in **Chapter 2** (**Section 2.3.2**), in order to assess the effect of *Chlamydia* infection on the expression of Prss31 in the FRT (**Figure 4.1 B**), to verify if the expression of Prss31 if MC-dependent (**Figure 4.1 C**) and to characterise the roles of Prss31 during *Chlamydia* infection (**Figure 4.1 D**), MC-deficient mice, Prss31-deficient mice and appropriate WT controls (7-18 weeks old) were subcutaneously administrated with medroxyprogesterone acetate (Depo-Provera). Seven days later, mice were infected intravaginally with $5x10^4$ ifu of *Chlamydia muridarum* (ATCC VR-123) in 10µl SPG or sham-infected with 10µl SPG alone under ketamine:xylazine anaesthesia (Ilium Ketamil® and Ilium Xylazil-20®) (177, 389). For infections in the MC-deficient mice and their associated WT controls, ketamine:xylazine

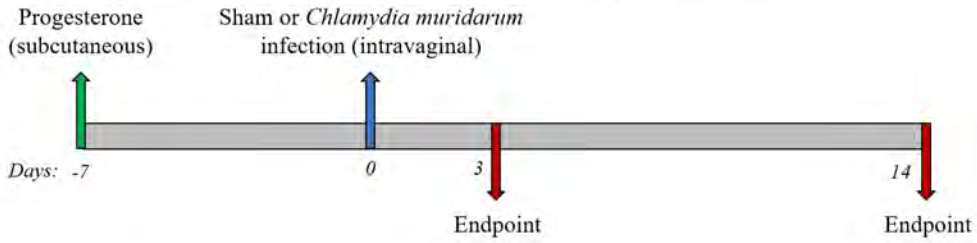
anaesthesia was replaced by ketamine:medetomidine anaesthesia (with atipamezole reversal; Ilium Ketamil®, Ilium Medetomidine® and Ilium Atipamezole®) in order to conform with best practices*. At 3 and/or 14dpi, mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb) and FRT tissues were collected for analyses.

* All the other infections were approved and/or conducted prior to an update in the Animal Care and Ethics Committee (ACEC) guidelines for best practice for anaesthesia.

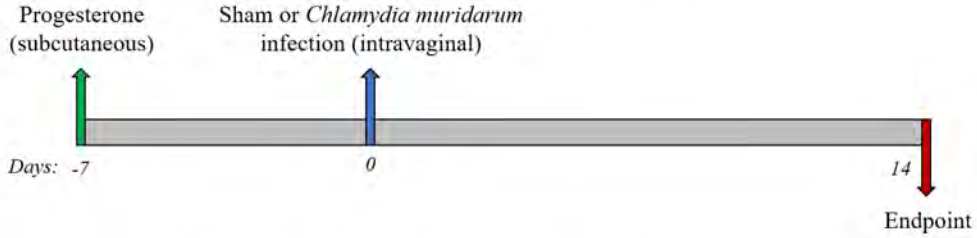
(A) Effect of female sex hormones on the expression of Prss31 (WT mice)



(B) Effect of *Chlamydia* infection on the expression of Prss31 (WT mice)



(C) Effect of MC deficiency on the expression of Prss31 (*Cpa3-Cre;Mcl-1$^{WT/WT}$* and *Cpa3-Cre;Mcl-1$^{fl/fl}$* mice)



(D) Role of Prss31 during *Chlamydia* infection (WT and *Prss31$^{-/-}$* mice)

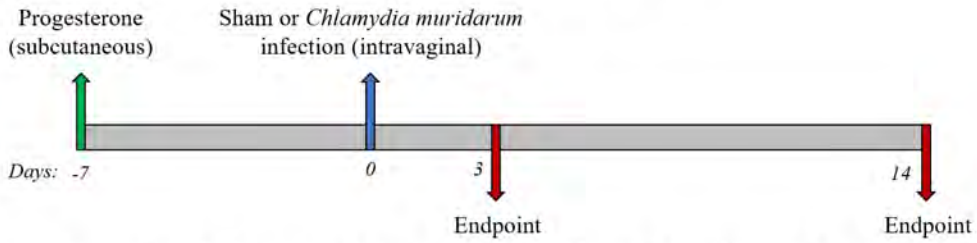


Figure 4.1 - Mouse model of hormonal treatment and *Chlamydia muridarum* female reproductive tract (FRT) infections. (A) Progesterone or oestradiol was administrated subcutaneously to wild type (WT) female C57BL/6 mice. Seven days later, tissues were collected for analyses of the expression of Prss31 in the FRT [Chapter 2]. **(B)** WT C57BL/6 female mice were administrated progesterone subcutaneously seven days prior to intravaginal infection with $5x10^4$ inclusion forming units of *Chlamydia muridarum* or sham-infection. Tissues were collected at 3 and 14 days post infection (dpi) for subsequent analyses of the expression of Prss31 in the FRT [Chapter 2]. **(C)** MC-deficient (*Cpa3-Cre;Mcl-1$^{fl/fl}$*) female mice and WT (*Cpa3-Cre;Mcl-1$^{WT/WT}$*) controls were administrated progesterone subcutaneously seven days prior to intravaginal infection with $5x10^4$ inclusion forming units (ifu) of *Chlamydia muridarum*. Tissues were collected at 14dpi for analyses of the expression of Prss31 in MC-deficient mice [Chapter 2]. **(D)** Prss31-deficient (*Prss31$^{-/-}$*) female mice and WT controls were administrated progesterone subcutaneously seven days prior to intravaginal infection with $5x10^4$ ifu of *Chlamydia muridarum*. Tissues were collected at 3dpi and 14dpi for subsequent assessment of the roles of Prss31 during *Chlamydia* FRT infection.

4.3.3. Intravaginal treatments with recombinant Prss31 during *Chlamydia* FRT infection

To assess the effect of intravaginal treatments with recombinant Prss31 during *Chlamydia* FRT infection, WT C57BL/6 female mice were subjected to the previously described mouse model of *Chlamydia* FRT infection and, in addition, treated daily intravaginally with 1.2µg of recombinant Prss31 (rPrss31) in 20µL Tris Hydrochloride (Tris-HCl) or with vehicle alone starting 1dbi until 2pi, under isofluorane gas anaesthesia (**Figure 4.2**) (226). Mice were sacrificed at 3dpi and 14dpi by intraperitoneal injection of an overdose of sodium pentobarbitone (Lethabarb) and FRT tissues were collected for analyses.

The recombinant Prss31, kindly provided by Professor Richard Stevens, was bioengineered as an isoform of the human Prss31 tryptase and has previously been used to determine the effect of Prss31 in the induction of airway hyperresponsiveness in Balb/c mice, which are naturally deficient in Prss31 (212, 226). In order to prevent unspecific binding of recombinant Prss31 to the membrane of wrong type of cells, the C-terminal membrane-spanning domain of Prss31 was removed in the recombinant Prss31 (226).

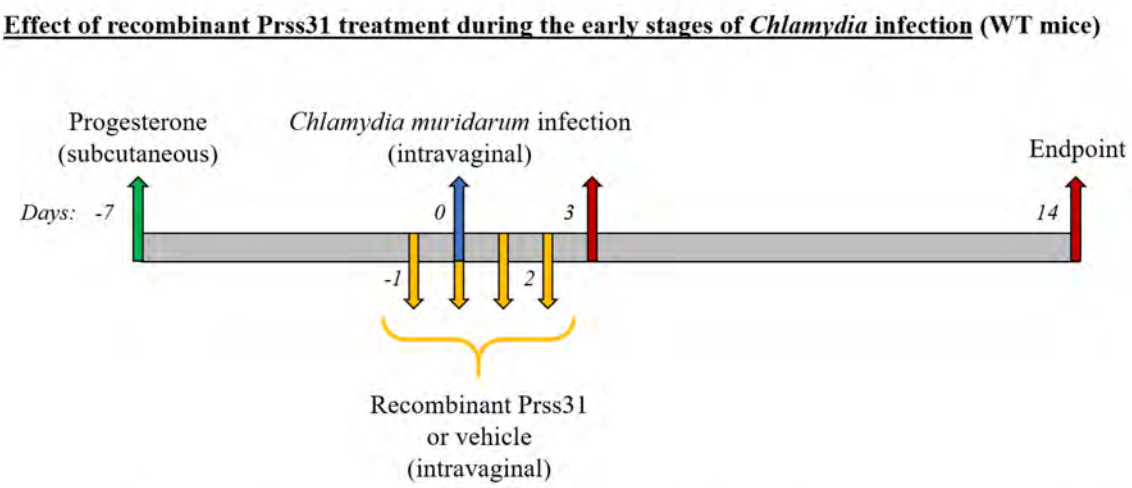


Figure 4.2 - *In vivo* treatments with recombinant protease serine member S31 (Prss31) during a murine model of *Chlamydia muridarum* reproductive tract infection. Wild type female C57BL/6 mice were administrated progesterone subcutaneously seven days prior intravaginal infection with $5x10^6$ inclusion forming units of *Chlamydia muridarum*. Mice were treated with recombinant Prss31 or vehicle control intravaginally daily from 1 day before infection to 2 days post infection (dpi). Tissues were collected at 3dpi and 14dpi to assess the effect of rPrss31 treatments during *Chlamydia* infections.

4.3.4. RNA extractions from FRT tissues

As explained in **Chapter 2** (**Section 2.3.5**), the left ovaries, oviducts and uterine horns were harvested at endpoint, snap frozen and stored at -80°C. Total RNA extractions were performed using the TRIzol® method according to the manufacturer's instruction.

4.3.5. Reverse transcription of RNA from reproductive tract tissues

As explained in **Chapter 2** (**Section 2.3.6**), the purity, quality and concentration of the RNA samples were measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The RNA samples were then treated with DNase I and reversed transcribed into cDNA using M-MLV reverse transcriptase enzyme (Life Technologies) according to the manufacturer's instructions.

4.3.6. DNA extraction from vaginal lavage

As explained in **Chapter 2** (**Section 2.3.7**), vaginal lavages were realised at endpoint to evaluate the *Chlamydia muridarum* burden in the lower FRT. The GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies Sdn. Bhd.) was used according to the manufacturer's instruction in order to extract the bacterial DNA from the vaginal lavage fluids.

4.3.7. Real-time quantitative polymerase chain reaction

4.3.7.1. RNA expression analyses

As explained in **Chapter 2** (**Section 2.3.8.1**), qPCR were realised on the cDNA samples using custom designed primers (IDT; **Appendix, Table 6.1**) and iTaq™ Universal SYBR® Green Supermix (Bio-Rad), cycling conditions of 50°C for 2 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds and 55-65°C for 1 minute in a Mastercycler® ep Realplex2 Real-time PCR System (Eppendorf), a CFX96 or a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Dissociation analyses were performed to verify the specificity of each primer pairs and the relative expression of each target gene was determined by comparison to HPRT.

The expression profile of various genes coding for cytokines, chemokines and immune mediators associated with susceptibility/clearance of *Chlamydia* infection and/or associated pathology and/or host immune responses (IFNγ, TNFα, IL-1β, IL-6, IL-10, IL-13, IL-13rα, IL-17, CXCL1, CXCL15, STAT1, STAT6, MMP9, TLR2, PAR2 and arginase-1) were determined using specific primers (IDT; **Appendix, Table 6.1**) to investigate the changes in inflammation status in the *Chlamydia*-infected uterus of Prss31-deficient and WT controls.

The gene expression of Prss31 was measured in FRT tissues using specific primers (IDT; **Appendix, Table 6.1**) to evaluate if the expression of Prss31 is affected by female sex hormones, *Chlamydia* FRT infection and/or the absence of MCs.

The *Chlamydia* burden in the uterus was evaluated using primers targeting the *Chlamydia muridarum* 16S rRNA (IDT; **Appendix, Table 6.1**) in order to evaluate the levels of ascending infection in Prss31-deficient and WT controls (177, 389).

4.3.7.2. DNA expression analyses

As explained in **Chapter 2** (**Section 2.3.8.2**), primers targeting the gene encoding for *Chlamydia* MOMP (IDT; **Appendix, Table 6.1**) were used to estimate the *Chlamydia* burden in the lower FRT. The presence of the *Chlamydia* MOMP gene in each sample was then compared to DNA standards extracted from known concentrations of *Chlamydia muridarum* to evaluate the number of *Chlamydia* in each vaginal lavage fluid sample of Prss31-deficient and WT controls.

4.3.8. Evaluation of *Chlamydia*-associated pathology

As explained in **Chapter 2** (**Section 2.3.9**), the levels of hydrosalpinx in the oviducts of *Chlamydia*-infected mice at 14dpi were evaluated by measuring the size of their oviducts in two planes using a digital calliper (Sontax) in order to estimate the cross sectional area of each oviduct as a representation of oviduct swelling caused by *Chlamydia* infection.

4.3.9. Characterisation of the immune cell numbers in the uterus during *Chlamydia* FRT infection using flow cytometry

Flow cytometry was used to characterise the immune cells in the *Chlamydia*- infected uterus of Prss31-deficient mice and WT controls. Briefly, as explained in details in **Chapter 2** (**Section 2.3.10**), uterine horns were dissociated and digested 30 minutes at 37°C with 40U/mL of DNase I and 2mg/mL of collagenase D (Roche) at 37°C. After further dissociation, samples were passed through a 70µm nylon cell strainer, treated with red blood cell lysis buffer and the number of living cells was enumerated using trypan blue exclusion and a Countess™ automated cell counter (Invitrogen). The single cell suspensions were blocked at 4°C for 15 minutes with 10ng/mL of anti-mouse CD16/32 (InVivoMAb).

For staining, the single cell suspensions were incubated at 4°C for 20 minutes with a cocktail of antibodies specific for surface markers conjugated with either a flurorochrome or biotin. Then, they were incubated at 4°C for 20 minutes with a streptavidin antibody conjugated with a BV605 fluorochrome (**Appendix, Table 6.2**). An unstained control was generated as a negative control, and single fluorochrome controls for each antibody used in the panel were produced in order to set the voltages and the compensation for removal of signal overlap between fluorochromes.

Finally, the stained cells were fixed overnight at 4°C in 4% PFA and the fluorescence of each sample was measured using a BD LSRFortessa™ X-20 cell analyzer and examined with the BD FACSDiva™ Software (BD Biosciences) to determine the percentages and total numbers of innate and adaptive immune cells in the *Chlamydia*-infected uterus of Prss31-deficient mice and WT controls, with the gating strategy based on the specific forward scatter, side scatter and surface markers of each immune cell type (**Table 4.2** and **Appendix, Figure 6.1**).

Cell types	Surface marker expressions
Immune cells	$CD45^+$
Neutrophils	$CD45^+$ $CD11b^+$ $Ly6G^+$
Eosinophils	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^+$
Myeloid dendritic cells	$CD45^+$ $CD11b^+$ $CD11c^+$ $Ly6C^-$ $PDCA^-$
Plasmacytoid dendritic cells	$CD45^+$, $CD11b^-$ $CD11c^+$ $Ly6C^+$, $PDCA^+$
Resident monocytes/macrophages	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^-$ $F4/80^+$ $Ly6C^-$
Infiltrating monocytes/macrophages	$CD45^+$ $CD11b^+$ $Ly6G^{-/low}$ $SiglecF^-$ $F4/80^+$ $Ly6C^+$
T cells	$CD45^+$ $CD3^+$
$CD4^+$ T cells	$CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$
$CD8^+$ T cells	$CD45^+$ $CD3^+$ $CD4^-$ $CD8^+$

Table 4.2 – Surface markers used for identifying immune cells in the uterine tissue of *Chlamydia*-infected Prss31-deficient mice and WT controls.

4.3.10. Investigation of the cellular source of Prss31 in the FRT by immunohistochemistry

Mice were sacrificed as described above and the right side of the FRT (right ovary, right oviduct, right uterine horn) as well as cervix and vagina were isolated and fixed in formalin for histological analyses to investigate the cellular source of Prss31 in the FRT. Samples were sent to the John Curtin School of Medical Research (Australian National University) for processing. Briefly, tissues were embedded in paraffin and cut in longitudinal sections of 5µm thickness.

For immunohistochemistry staining of Prss31, sections were first deparaffinised using xylene and rehydrated using absolute alcohol gradients. Antigen retrieval was performed by incubation in 0.05% citraconic anhydride buffer (pH=7.4) for 40 minutes at 80°C. After washes in PBS-Tween (PBS-T; 0.05% Tween-20), the sections were blocked 30 minutes at room temperature with Blocker™ Casein in PBS (Thermo Fisher Scientific) to prevent unspecific staining.

The sections were then stained overnight at 4°C with a rabbit polyclonal primary antibody anti-Prss31 (ab201296, Abcam, San Francisco, USA) immunogen for the amino acids 136 to 181 of human TPSG1/Prss31. As a negative control, a section was incubated with PBS-T without antibody for visualisation of potential unspecific binding of the primary antibody. After all traces of the primary antibody were removed by performing PBS-T washes, the sections were incubated at room temperature for 75 minutes with a donkey anti-rabbit IgG H&L (Alexa Fluor® 647)

preadsorbed secondary antibody (ab150063; Abcam). After further PBS-T washes, the sections were stained with Hoechst 33342 staining dye solution (ab228551; Abcam) for 5 minutes at room temperature to stain DNA and identify the nuclei of the cells. Finally, after further PBS-T washes, the sections were mounted using FluorSave™ reagent (345789; Calbiochem® Merck Millipore, Bayswater, VIC, Australia) and sealed using clear nail polish. Slides were visualised using the 20X magnification of a Zeiss Axio Imager M2 microscope (Carl Zeiss Microscopy) and a ZEN 2.3 software (Blue edition, Carl Zeiss Microscopy).

4.3.11. Statistical analyses

Data are presented as mean ± SEM. All statistical tests were performed using the GraphPad Prism software (version 7.0; GraphPad Software). Grubbs outlier tests were realised followed by normality testing using the D'Agostino & Pearson normality test for analysis of 8 or more samples or the KS normality test for analysis of less than 8 samples. Then, as appropriate, Student's t-tests or Mann-Whitney tests were performed, with a $p<0.05$ considered as statistically significant.

4.4. Results

4.4.1. The expression of Prss31 is not altered by female sex hormones nor by *Chlamydia* FRT infection

In **Chapter 3**, female sex hormones and *Chlamydia* infection were shown to affect the expression of the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, in the FRT. In order to examine the effects of progesterone and oestrogen on the expression of Prss31, female WT mice were treated with progesterone (diestrus) or oestrogen (oestrous) subcutaneously and sacrificed seven days later (**Figure 4.1 A**). In order to examine if *Chlamydia* infection affects the expression of Prss31, female WT mice were treated with progesterone, infected intravaginally with *Chlamydia muridarum* or sham-infected, and sacrificed at 3dpi and 14dpi (**Figure 4.1 B**). At endpoints, the left ovaries, oviducts and/or uterus were collected for subsequent RNA extraction, reverse transcription and analysis of the expression of Prss31 were measured by qPCR.

The expression of Prss31 remains unchanged in the ovary, oviduct and uterus of WT mice treated with either progesterone or oestrogen (**Figure 4.3 A-C**). Similarly, despite a slight reduction in expression at 3dpi (**Figure 4.3 E**, p=0.0701), the expression of Prss31 in the uterus is not significantly modified in *Chlamydia*-infected mice compared to WT controls (**Figure 4.3 E-F**).

Overall my results show the expression of Prss31 is not modified by female sex hormones nor by *Chlamydia* infection, contrary to the expression of mMCP4, mMCP5, mMCP6 and CPA3.

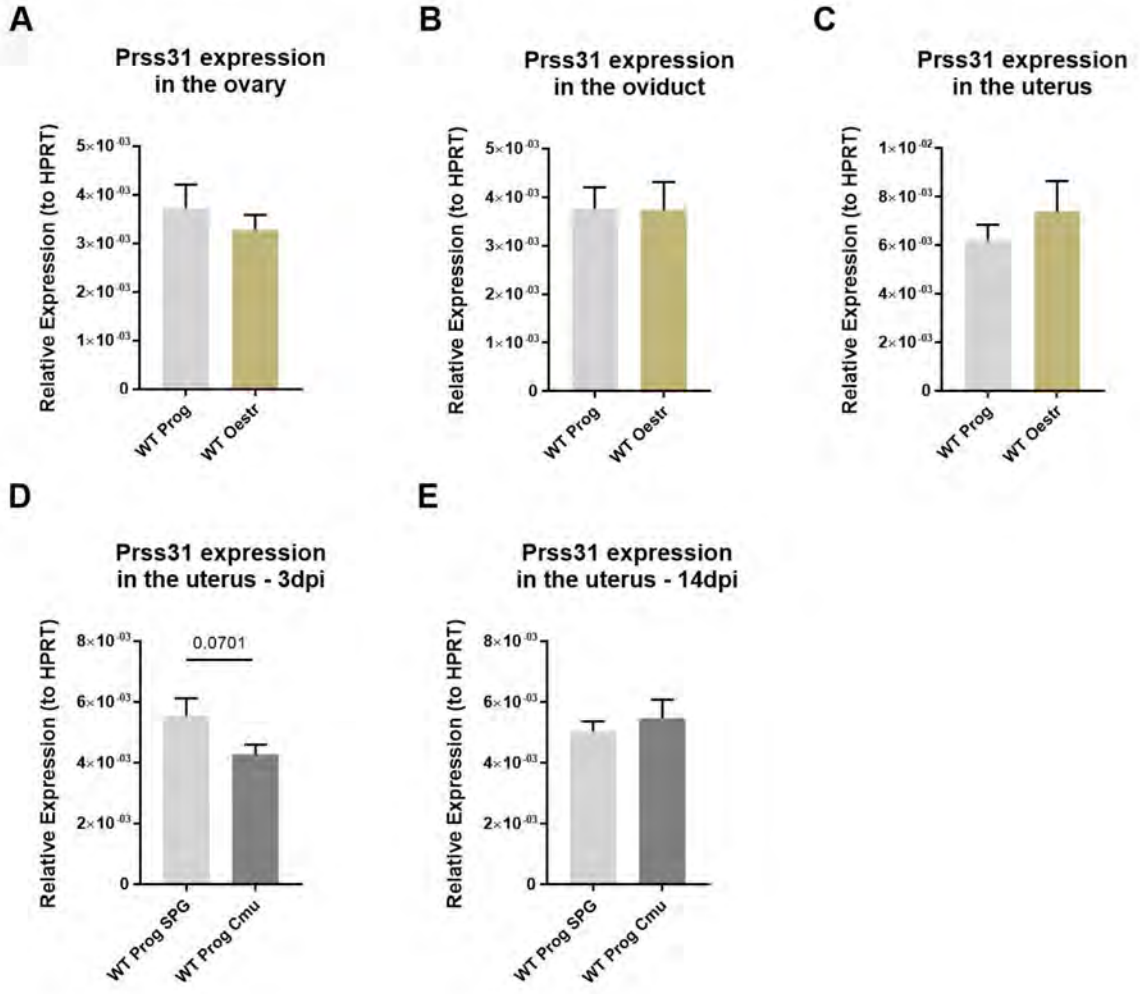


Figure 4.3 - The expression of protease serine member S31 (Prss31) in the female reproductive tract (FRT) is not significantly altered by progesterone or oestrogen treatments nor by *Chlamydia*-infection. (A-C) Female C57BL/6 wild-type (WT) mice were treated with either progesterone (Prog) or oestrogen (Oestr) and sacrificed 7 days later. **(D, E)** Female C57BL/6 WT mice were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) or sham-infected with sucrose-phosphate-glutamate buffer (SPG). Mice were sacrificed at **(D)** 3 days post infection (dpi) and **(E)** 14dpi. RNA was extracted from the left **(A)** ovary, **(B)** oviduct and **(C-E)** uterus, and the levels of expression of Prss31 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed.

4.4.2. Prss31 might not be specifically expressed by MCs

In **Chapter 3**, MC-deficient mice were shown to have highly reduced expression of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3, in their uterus. In order to verify if the expression of Prss31 is also affected by MC deficiency, MC-deficient mice (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls were treated with progesterone and infected with *Chlamydia muridarum* (**Figure 4.1 C**). At 14dpi, the left uterus were collected for subsequent RNA extraction and reverse transcription. The levels of expression of Prss31 were assessed by qPCR.

My result show that MC-deficient mice have similar levels of expression of Prss31 in their uterus as WT controls (**Figure 4.4**), suggesting that Prss31 might not be specifically expressed by MCs in the FRT.

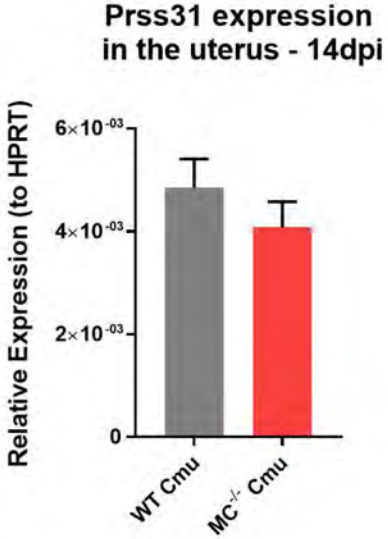


Figure 4.4 - The expression of protease serine member S31 (Prss31) in the uterus is not altered in mast cell (MC)-deficient mice compared to WT controls. MC-deficient (*MC*$^{-/-}$, *Cpa3-Cre;Mcl-1*$^{fl/fl}$) mice and their associated WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) controls and were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection (dpi). RNA was extracted from the uterus and the levels of expression of Prss31 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and Mann-Whitney tests were performed.

To assess the cellular source of Prss31 in the FRT and verify if Prss31 is specific to MCs in the FRT, Prss31-deficient mice and WT controls were treated with progesterone, infected with *Chlamydia muridarum* and sacrificed at 3dpi (**Figure 4.1 D**). The FRT tissues were fixed in formalin, embedded in paraffin and longitudinal sections were cut, deparaffinised and rehydrated. After antigen retrieval and blocking, the sections were stained with a primary antibody anti-Prss31 and a secondary antibody combined to Alexa Fluor® 647 for detection of Prss31; and with the Hoechst 33342 staining dye for nuclear staining. Fluorescence was visualised using the 20X magnification of a Zeiss Axio Imager M2 microscope with Prss31 positive staining represented in yellow and nuclear staining in blue (**Figure 4.5**).

Surprisingly, large areas of FRT tissues of WT mice were positively stained for Prss31 (**Figure 4.5, WT Cmu**), including the epithelium of the oviduct, the endocervical epithelium and lumen as well as the uterine epithelium (**Figure 4.5**; red arrows). In addition, large cells in the endometrial and myometrial tissues of the uterus were positively stained for Prss31 (**Figure 4.5**; green arrows).

These results could indicate that epithelial cells and uterine cells might be expressing Prss31 in the FRT. Some of the uterine cells positive for Prss31 could be MCs, given the relatively large size of those cells and their location, while other appear smaller and could be structural cells or resident/infiltrating immune cells.

However, when similar staining was done in Prss31-deficient mice as a negative control, similar staining profile with relatively similar intensity was present (**Figure 4.5, *Prss31*$^{-/-}$ Cmu**). This observation suggests that unspecific staining might be present, even if the no primary antibody control did not reveal any positive staining for Prss31 (**Figure 4.5, Negative control**) and that different concentration of antibodies have been used and showed similar staining (data not shown). To verify the cellular source of Prss31, Professor Richard Stevens kindly provided us with a specific antibody for mouse Prss31 generated in his laboratory, but, due to time restraints, it was not possible to perform this experiment in the timeframe of my PhD.

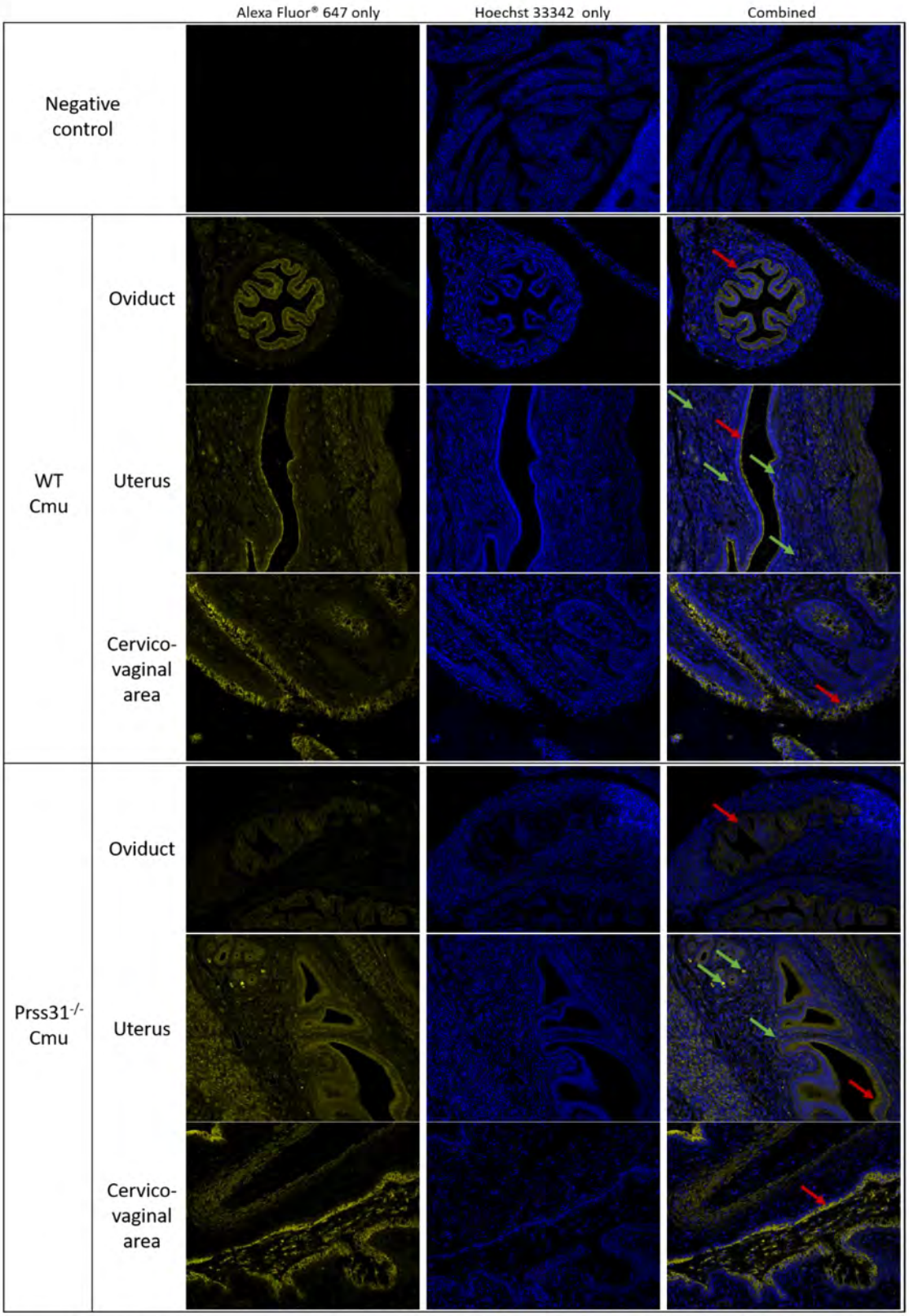


Figure 4.5 – Staining for protease serine member S31 (Prss31) in uterine cells and in the epithelium of the FRT of wild-type (WT) and Prss31-deficient mice. Prss31-deficient (*Prss31*$^{-/-}$) and WT mice were given progesterone, infected with *Chlamydia muridarum* (Cmu) and sacrificed at 3dpi. Longitudinal sections of the FRT were stained for Prss31. The fluorescence for Hoechst 33342 (nuclear stain; blue) and/or Alexa Fluor® 647 (Prss31 stain; yellow) was visualised (20X). *Red arrows: Prss31 staining on the epithelium. Green arrows: Prss31 staining on uterine cells. Negative control: no anti-Prss31 antibody.*

4.4.3. Prss31-deficient mice have increased susceptibility to *Chlamydia* FRT infection

In **Chapter 3**, I show that MC proteases have diverse effects during *Chlamydia* infection. In order to investigate the role of Prss31 during *Chlamydia* FRT infection, Prss31-deficient mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 3dpi and 14dpi (**Figure 4.1 D**). The levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

At 3dpi, the number of *Chlamydia* per vaginal lavage (**Figure 4.6 A**) is significantly increased in *Chlamydia*-infected Prss31-deficient mice compared to WT controls. Similarly, the relative expression of *Chlamydia* 16S is slightly higher in the uterus of Prss31-deficient mice compared to WT controls (**Figure 4.6 B**, not significant). At 14dpi, the relative expression of *Chlamydia* 16S in the uterus is higher in the uterus of *Chlamydia*-infected Prss31-deficient mice compared to WT controls (**Figure 4.6 D**, not significant), while the number of *Chlamydia* per vaginal lavage is similar between Prss31-deficient mice and WT controls (**Figure 4.6 C**). Overall, high variability is present, which, as mentioned previously, is often the case in mouse models of *Chlamydia* infection. In future studies, increasing the number of mice would allow to definitely conclude on the effect of Prss31 on clearance of *Chlamydia* infection in the lower and upper FRT, at 3dpi and 14dpi.

The cross sectional area of the left and right oviducts is similar between Prss31-deficient mice and WT controls, suggesting that Prss31 might not affect *Chlamydia*-associated pathology. (**Figure 4.6 E-F**).

Therefore, my findings suggest that Prss31 may play a protective role during the early stages of *Chlamydia* infection by favouring clearance of infection. However, Prss31-deficient mice and WT controls appear to have relatively similar levels of infection and pathology at 14dpi, which suggests that the protective effects of Prss31 might not be maintained at later stages of infection.

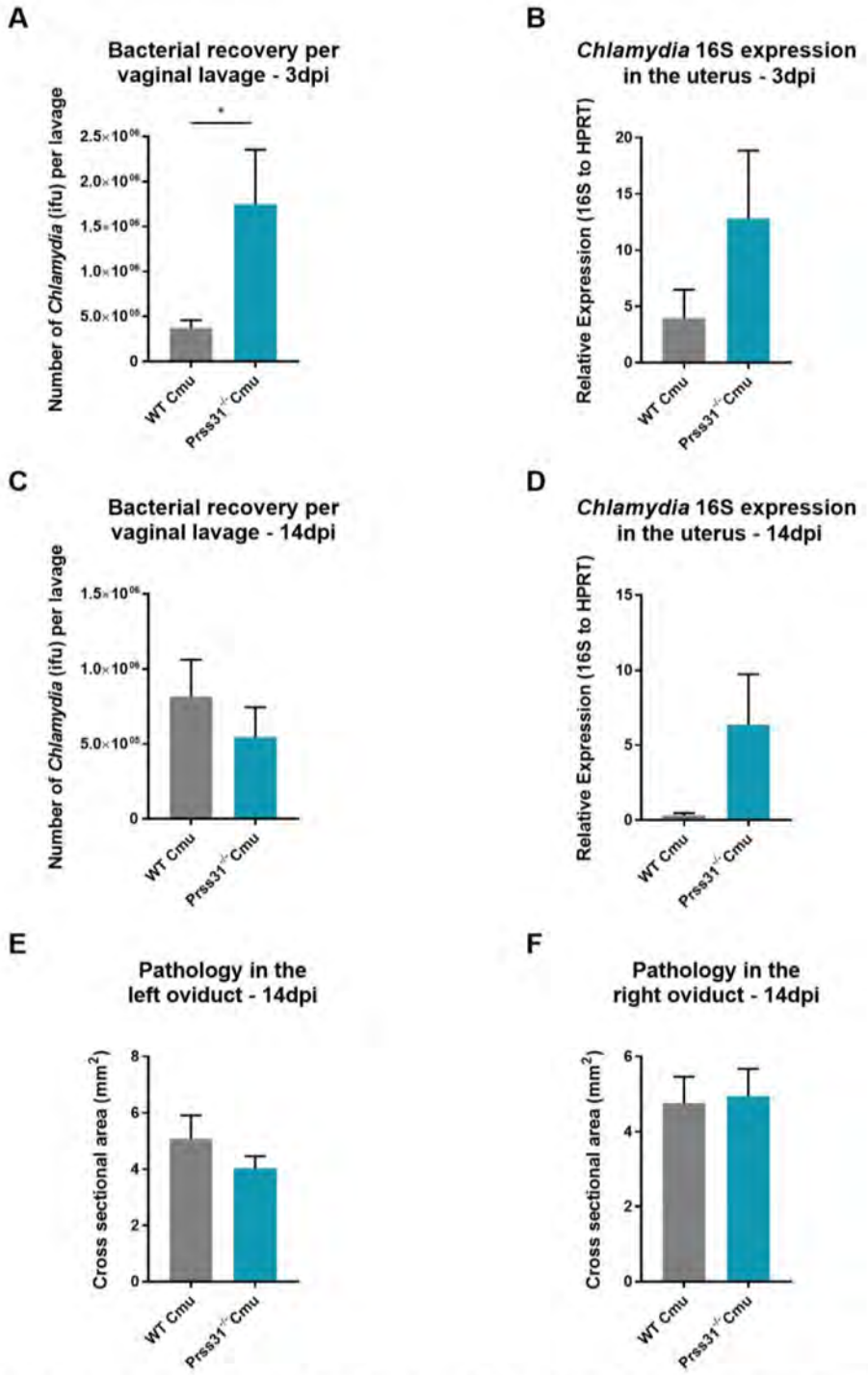


Figure 4.6 – Protease serine member S31 (Prss31)-deficient mice are more susceptible to *Chlamydia* infection at early stages. Prss31-deficient (*Prss31*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at **(A, B)** 3 days post infection (dpi) and **(C, D, E, F)** 14dpi. **(A, C)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per lavage. **(B, D)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross section area of the **(E)** left and **(F)** right oviducts of were measured using a calliper to evaluate the levels of *Chlamydia*-associated pathology. All data are presented as mean±SEM ($n \geq 6$) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$.

4.4.4. Early intravaginal treatments with recombinant Prss31 improve clearance of *Chlamydia* FRT infection early and protect against *Chlamydia*-associated pathology at later stages

To verify the potential protective role of Prss31 during the early stages of *Chlamydia* infection, the effects of intravaginal treatments with recombinant Prss31 on *Chlamydia* infection were assessed. Starting 1 day before infection until 2dpi, WT mice were treated intravaginally each day with recombinant Prss31 or vehicle alone, subjected to a murine model of *Chlamydia muridarum* FRT infection and sacrificed at 3dpi and 14dpi (**Figure 4.2**). The levels of *Chlamydia* was measured in vaginal lavage using qPCR targeting the *Chlamydia* MOMP DNA. Ascending infection was evaluated by extracting the RNA from the uterus and measuring the expression of *Chlamydia* 16S by qPCR. Moreover, *Chlamydia*-induced pathology was evaluated by measuring the size of the oviducts and estimating the cross sectional area as a representation of hydrosalpinx.

Mice treated with recombinant Prss31 are protected against *Chlamydia* infection at 3dpi, with a strong decrease in both the number of *Chlamydia* per vaginal lavage and the relative expression of *Chlamydia* 16S in their uterus (**Figure 4.7 A-B**). Although the levels of infection in the vagina and in the uterus are similar between vehicle or recombinant Prss31 treated mice at 14dpi (**Figure 4.7 C-D**), the cross sectional area of the left and right oviducts of recombinant Prss31 treated mice are strongly lower compared to those of vehicle treated mice (**Figure 4.7 E-F**).

Therefore, my results validate the protective role of Prss31 in the early stages of *Chlamydia* infection and suggest that early recombinant Prss31 treatments are protective against *Chlamydia*-associated pathology in the later stages of infection.

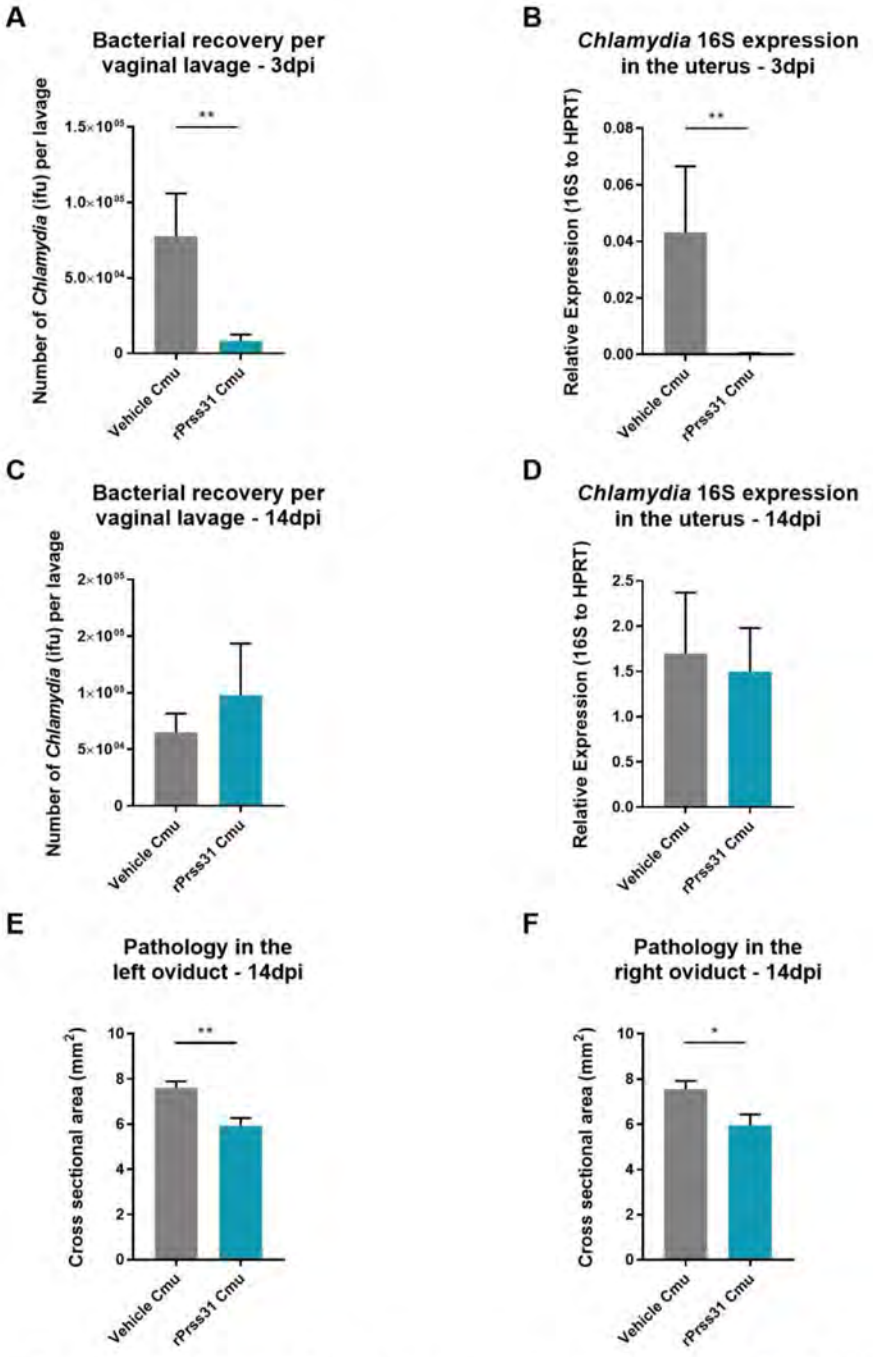


Figure 4.7 – Treatments with recombinant protease serine member S31 (rPrss31) are protective against *Chlamydia* infection at early stages and against *Chlamydia*-associated pathology at later stages. Wild-type C57BL/6 mice were treated with progesterone and infected intravaginally with *Chlamydia muridarum* (Cmu). From 1 day before infection to 2 days post infection (dpi), mice were treated daily intravaginally with either 1.2µg of recombinant Prss31 or vehicle alone. Mice were sacrificed at **(A, B)** 3dpi and **(C, D, E, F)** 14dpi. **(A, C)** DNA was extracted from vaginal lavages and the expression of the *Chlamydia* major outer membrane protein (MOMP) was determined by comparison to standards of known concentration to evaluate the number of *Chlamydia* infection forming units (ifu) per lavage. **(B, D)** RNA was extracted from the uterus and the levels of expression of *Chlamydia* 16S were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) to evaluate the levels of infection in the uterus. The cross section area of the **(E)** left and **(F)** right oviducts of were measured using a calliper to evaluate the levels of *Chlamydia*-associated pathology. All data are presented as mean±SEM (n≥8) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and ** representing $p<0.01$.

4.4.5. Prss31-deficient mice exhibit decreased immune cell recruitment to the uterus following *Chlamydia* FRT infection

My previous results suggest a protective role of Prss31 during *Chlamydia* infection. In order to assess if Prss31 mediates immune cell recruitment to the FRT during *Chlamydia* infection, Prss31-deficient mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 4.1 D**). The uteri were collected and digested into a single cell suspension and the total number of cells were enumerated. Single cell suspensions were then stained for extracellular markers and key immune cells were identified by flow cytometry. The number of cells in the uterine tissue was expressed both as the total number of each immune cell type and as a percentage of each immune cell type among all viable cells.

Globally the number of cells in the uterus of Prss31-deficient mice is reduced compared to the uterus of WT controls (**Figure 4.8 A**). This global decrease in the number of cells in the uterus of Prss31-deficient mice is concomitant with a decrease in the number of immune cells (**Figure 4.8 B**). However, the proportion of immune cells is only slightly reduced in Prss31-deficient mice (**Figure 4.9 B**, p=0.0702).

The total number of eosinophils (**Figure 4.8 D**), myeloid DCs (**Figure 4.8 E**), plasmacytoid DCs (**Figure 4.8 F**), resident and infiltrating monocytes and macrophages (**Figure 4.8 G-H**), T cells (**Figure 4.8 I**), $CD4^+$ T cells (**Figure 4.8 J**) and $CD8^+$ T cells (**Figure 4.8 K**) is significantly reduced in Prss31-deficient mice compared to WT controls. However, the proportion of all these immune cells is unchanged between Prss31-deficient mice and WT controls (**Figure 4.9 C-J**). Whilst the total number of neutrophils in the uterus remains unchanged between Prss31-deficient mice and WT controls (**Figure 4.8 C**), the proportion of neutrophils was increased in Prss31-deficient mice (**Figure 4.9 B**).

Those results suggest that Prss31-deficient mice might have a reduced recruitment of immune cells to their uterus during *Chlamydia* infection.

Due to restrictions in the breeding of genetically modified mice, the number of mice available was insufficient to include sham-infected control mice in this study. In future experiments, the

numbers and percentages of each immune cell type in the uterus of sham-infected Prss31-deficient mice (and WT controls) will be determined in order to establish if the differences observed in this study are caused by Prss31 deficiency alone or if they are mediated by *Chlamydia* infection. In complement, additional studies will be conducted to determine the numbers and percentages of each immune cell type in the uterus of sham-infected and *Chlamydia*-infected WT mice treated with recombinant Prss31 (and vehicle-treated controls) to determine the effects of early intravaginal treatments with recombinant Prss31 on immune cell response and identify the mechanisms that underpin the protective effects of this treatment.

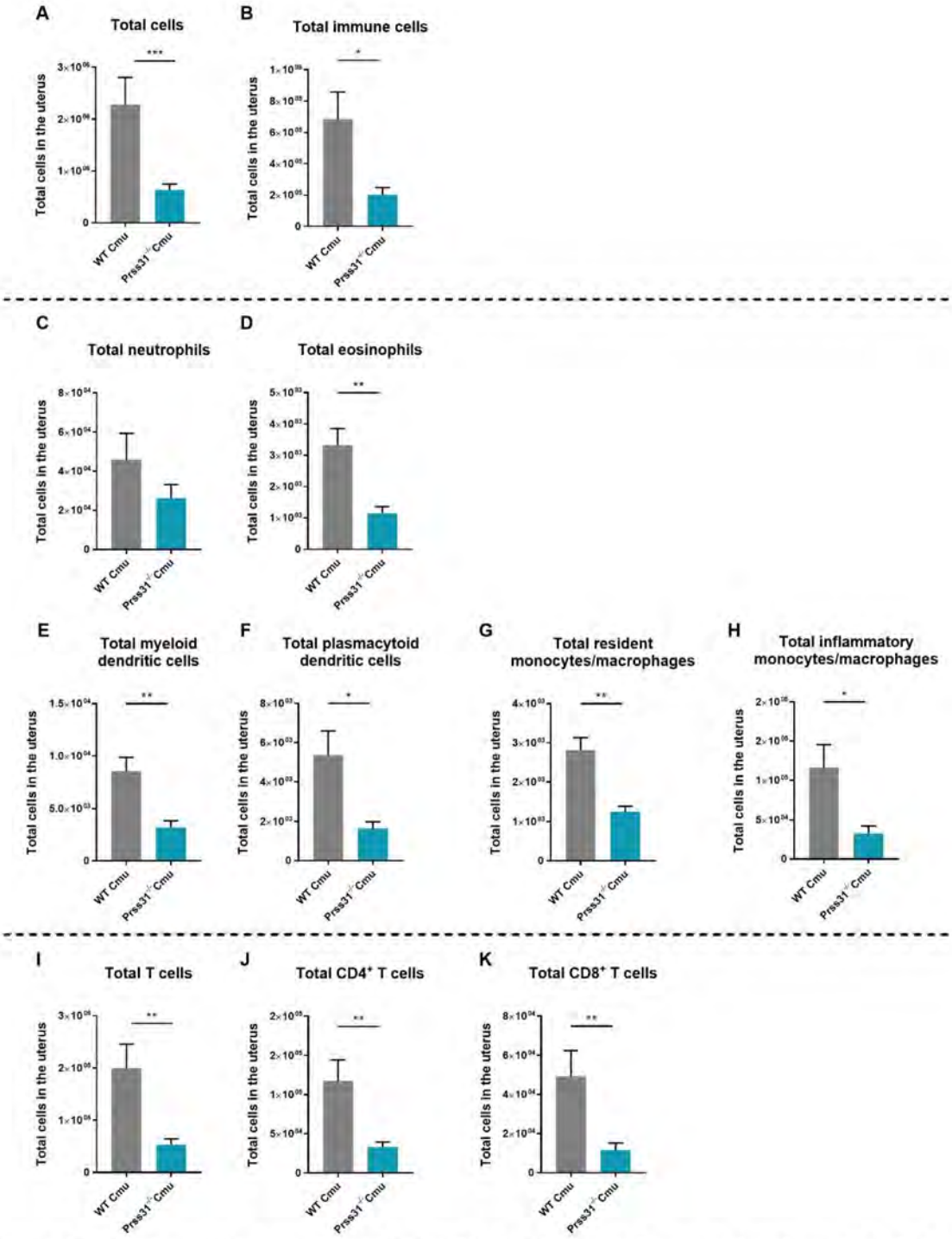


Figure 4.8 - Protease serine member S31 (Prss31)-deficient mice have a reduced number of viable cells, immune cells, eosinophils, myeloid dendritic cells, plasmacytoid dendritic cells, monocytes and macrophages, CD4$^+$ T cells and CD8$^+$ T cells in their uterus during *Chlamydia* infection. Prss31-deficient (*Prss31*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. **(A)** Single cell suspensions from the uterus were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the total number of **(B)** immune cells, **(C)** neutrophils, **(D)** eosinophils, **(E)** myeloid and **(F)** plasmacytoid dendritic cells, **(G)** resident and **(H)** inflammatory monocytes and macrophages, **(I)** T cells, **(J)** CD4$^+$ T cells and **(K)** CD8$^+$ T cells in the uterus. All data are presented as mean±SEM (n≥6) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and *** representing $p<0.001$.

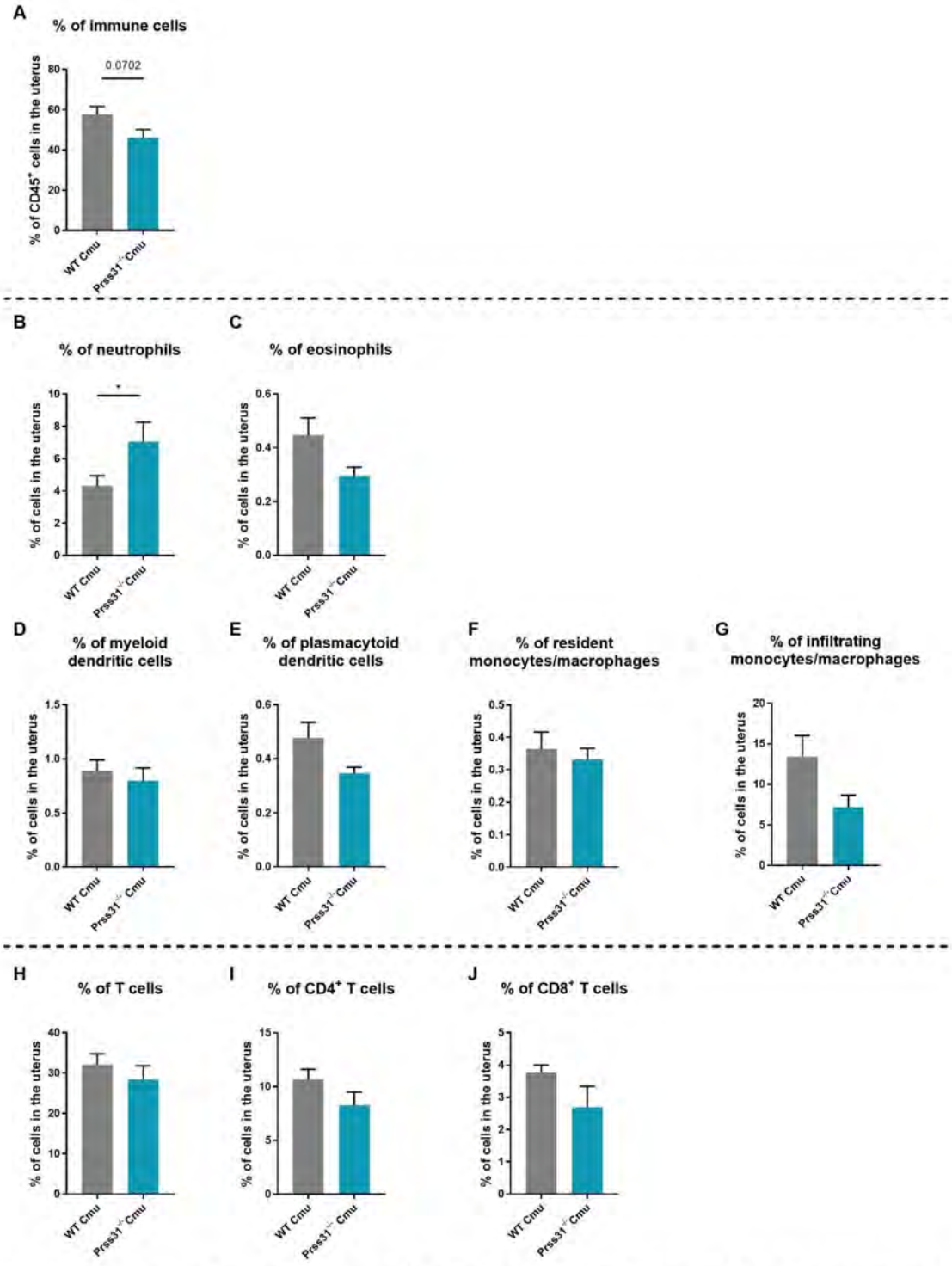


Figure 4.9 - Protease serine member S31 (Prss31)-deficient mice have a higher proportion of neutrophils in their uterus compared to wild type (WT) controls during *Chlamydia* infection. Prss31-deficient (*Prss31*$^{-/-}$) female mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Single cell suspensions from the uterus were obtained and counted. Samples were then blocked, stained, fixed and fluorescence was measured to determine the percentages of **(A)** immune cells, **(B)** neutrophils, **(C)** eosinophils, **(D)** myeloid and **(E)** plasmacytoid dendritic cells, **(F)** resident and **(G)** inflammatory monocytes and macrophages, **(H)** T cells, **(I)** CD4^{+} T cells and **(J)** CD8^{+} T cells among total viable uterine cells. All data are presented as mean±SEM (n≥6) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$.

4.4.6. Prss31-deficient mice have increased expression of IL-13 and IL-13rα during the early stages of *Chlamydia* FRT infection

Prss31 has been shown to affect the activation of the IL-13/IL-13rα/STAT6 pathway and/or the polarisation of macrophages to the M2 phenotype in the lungs (226, 490). In order to determine if Prss31 mediates IL-13/IL-13rα/STAT6 pathway and/or M2 polarisation during *Chlamydia* infection, Prss31-deficient mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* and sacrificed at 14dpi (**Figure 4.1 C**). The left uteri were collected for subsequent RNA extraction and reverse transcription. The levels of expression of the cytokines IL-13, IL-13rα, STAT6 and arginase-1 were measured by qPCR. Arginase-1 was chosen as a marker of M2 phenotype given that it is one of the main enzyme associated with M2 polarisation in macrophages (492)

The expression of the transcription factor STAT6 remains similar in the uterus of *Chlamydia*-infected Prss31-deficient mice compared WT controls at both 3pi and 14dpi (**Figure 4.10 C-G**). Whilst the expression of IL-13 and IL-13rα is increased in the uterus of *Chlamydia*-infected Prss31-deficient mice compared WT controls at 3dpi (**Figure 4.10 A-B**), those differences are not present at later stages (**Figure 4.10 E-F**).

The expression of arginase-1 remains similar between Prss31-deficient mice and WT controls at 3pi and 14dpi (**Figure 4.10 D-H**), indicating that arginase-1 is produced at similar levels in the uterus of Prss31-deficient mice and WT controls. However, for full characterisation of the M1/M2 phenotype of macrophages in the uterus of Prss31-deficient mice and WT controls during *Chlamydia* infection, flow cytometry will need to be done using antibodies specific for surface markers of macrophages (CD45 CD11b Ly6G F4/80 Ly6C) of the M1 phenotype (CD40, CD80, CD86 and MHC-II) and M2 phenotype (CD206) (493, 494). Whilst I did run this experiment during my PhD, high levels of auto-fluorescence compromised the analysis of the data and the results were not able to be appropriately interpreted (data not shown). Therefore, in future studies, optimisation of this staining should allow to characterise if Prss31 contribute in the induction of M1/M2 macrophages during *Chlamydia* infection.

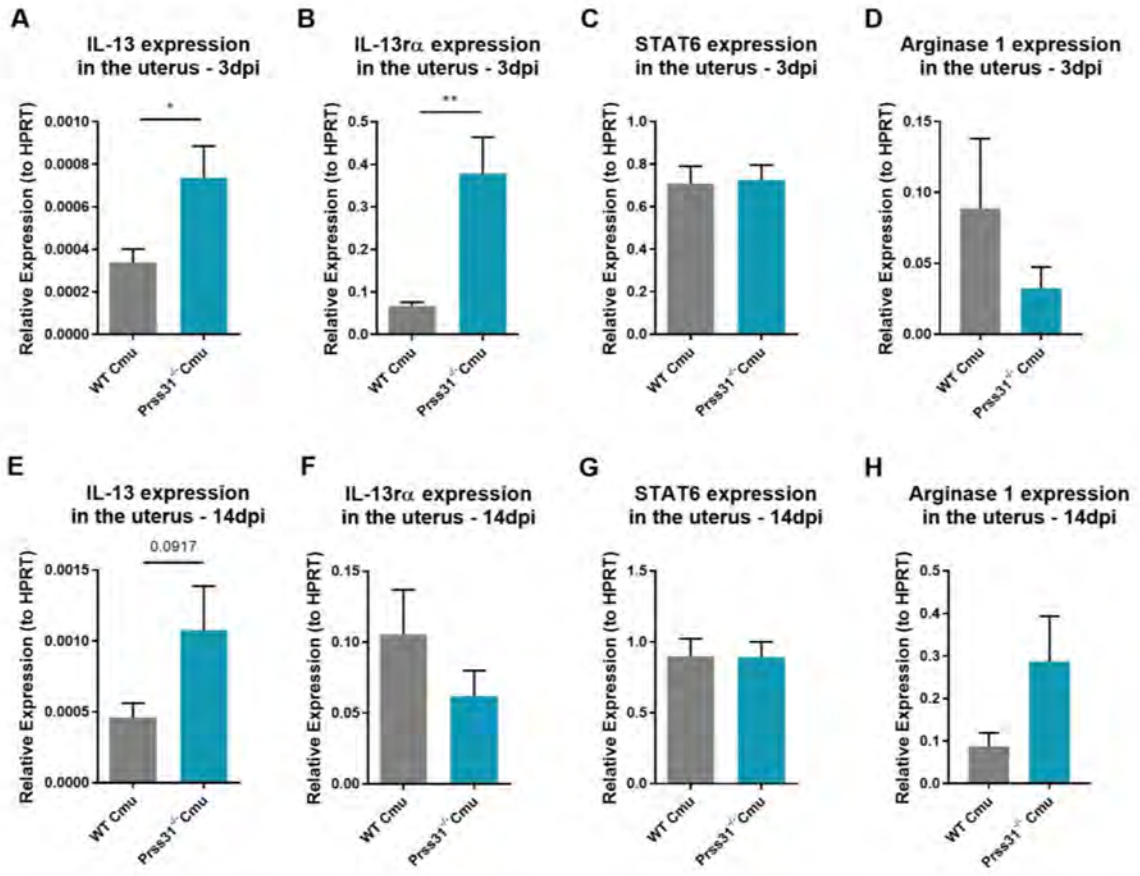


Figure 4.10 - Protease serine member S31 (Prss31)-deficient mice have enhanced expression of interleukin (IL)-13 and IL-13rα in their *Chlamydia*-infected uterus at 3 days post infection (dpi). Prss31-deficient (*Prss31*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at **(A-D)** 3dpi and **(E-H)** 14dpi. RNA was extracted from the uterus and the levels of expression of **(A, E)** IL-13, **(B, F)** IL-13rα, **(C, G)** signal transducer and activator of transcription (STAT)6 and **(D, H)** arginase-1 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$ and ** representing $p<0.01$.

4.4.7. Prss31-deficient mice have increased expression of pro-inflammatory mediators in the uterus during *Chlamydia* FRT infection

My previous data show that Prss31-deficient mice are more susceptible to *Chlamydia* FRT infection and that they have a reduced number of immune cells in their uterus during *Chlamydia* infection. In order to assess if Prss31 affect the level of expression of key immune mediators of *Chlamydia* infection, Prss31-deficient mice and WT controls were given progesterone subcutaneously, infected with $5x10^4$ ifu *Chlamydia muridarum* intravaginally and sacrificed at 3dpi and 14dpi. At endpoints, the left uterus were collected for subsequent RNA extraction and reverse transcription. The levels of expression of key immune mediators associated with susceptibility/clearance of *Chlamydia* infection and/or associated pathology in the uterus using qPCR were measured by qPCR.

During the early stages of infection, the expression of STAT1 ($p<0.05$), IL-10 ($p<0.05$), CXCL1 ($p<0.05$) and CXCL15 ($p<0.05$) is s increased in the uterus of *Chlamydia*-infected Prss31-deficient mice compared to WT controls, but the expression of IFNγ, IL-17, TNFα, IL-1β, IL-6, TLR2, MMP9 and PAR2 remains unchanged (**Figure 4.11**).

At later stages of infection, whilst the expression of STAT1, IFNγ, IL-17, IL-6, CXCL1 and PAR2 was unchanged in the uterus of *Chlamydia*-infected Prss31-deficient mice compared to WT controls, the expression of TNFα ($p=0.1111$), IL-1β ($p<0.05$), IL-10 ($p=0.0734$), CXCL1 ($p=0.0734$), TLR2 ($p<0.05$) and MMP9 ($p=0.0667$) is slightly increased in the uterus of Prss31-deficient mice (**Figure 4.12**).

Overall these data suggest an induction of the expression of pro-inflammatory mediators in the uterus of Prss31-deficient mice during *Chlamydia* infection. In future studies, immunostaining and/or *in vitro* co-culture experiments will be realised to determine the cellular source of these mediators

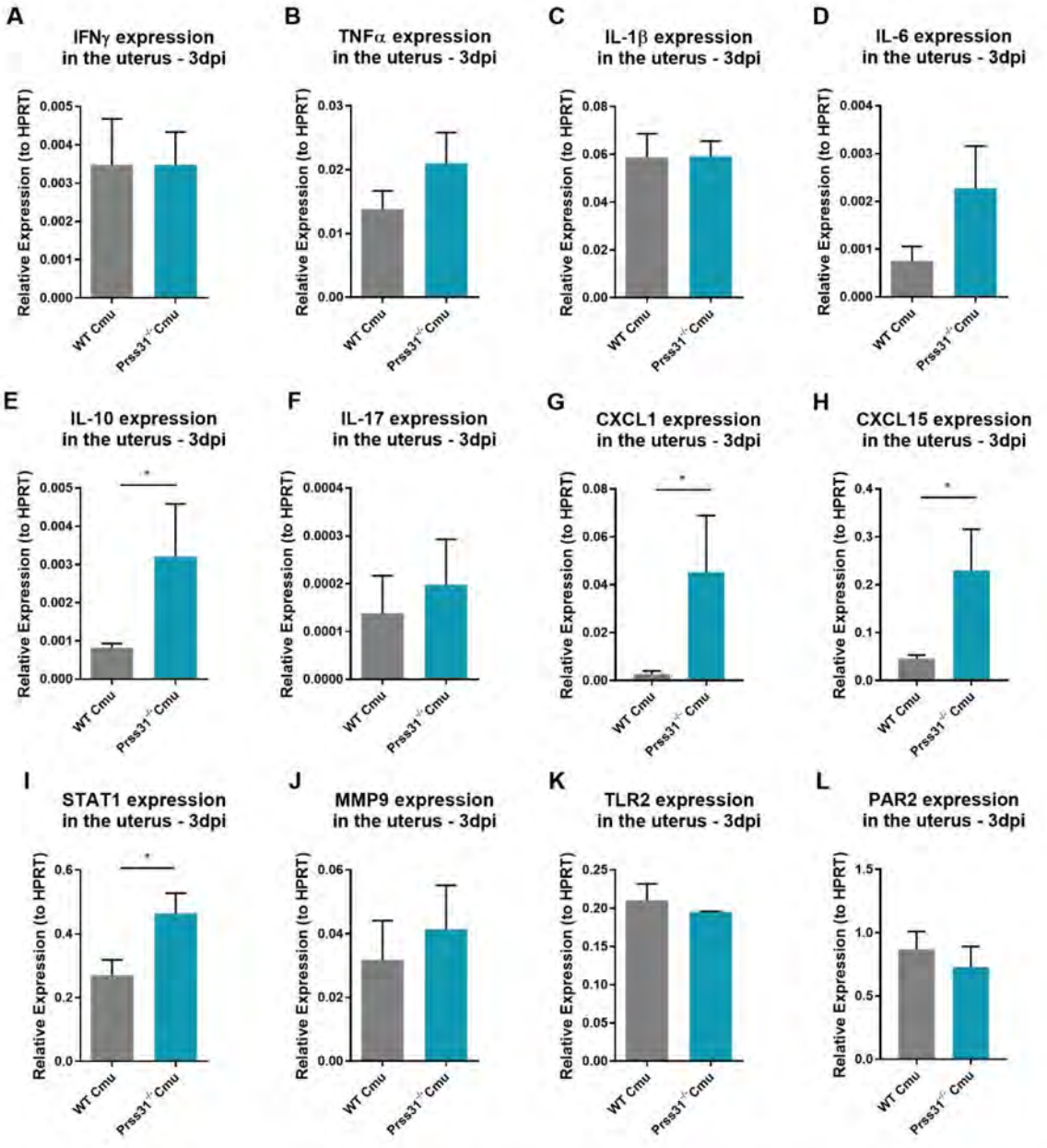


Figure 4.11 – Protease serine member S31 (Prss31)-deficient mice have enhanced expression of the signal transducer and activator of transcription (STAT)1, interleukin (IL)-10, chemokine (C-X-C motif) ligand (CXCL)1 and CXCL15 in their *Chlamydia*-infected uterus at 3 days post infection (dpi). Prss31-deficient (*Prss31*$^{-/-}$) mice and WT controls were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 3dpi. RNA was extracted from the uterus and the levels of expression of **(A)** interferon (IFN)γ, **(B)** tumor necrosis factor (TNF)α, **(C)** IL-1β, **(D)** IL-6, **(E)** IL-10, **(F)** IL-17, **(G)** CXCL1, **(H)** CXCL15, **(I)** STAT1, **(J)** matrix metallopeptidase (MMP)9, **(K)** toll-like receptor (TLR)2 and **(L)** protease activated receptor (PAR)2 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$.

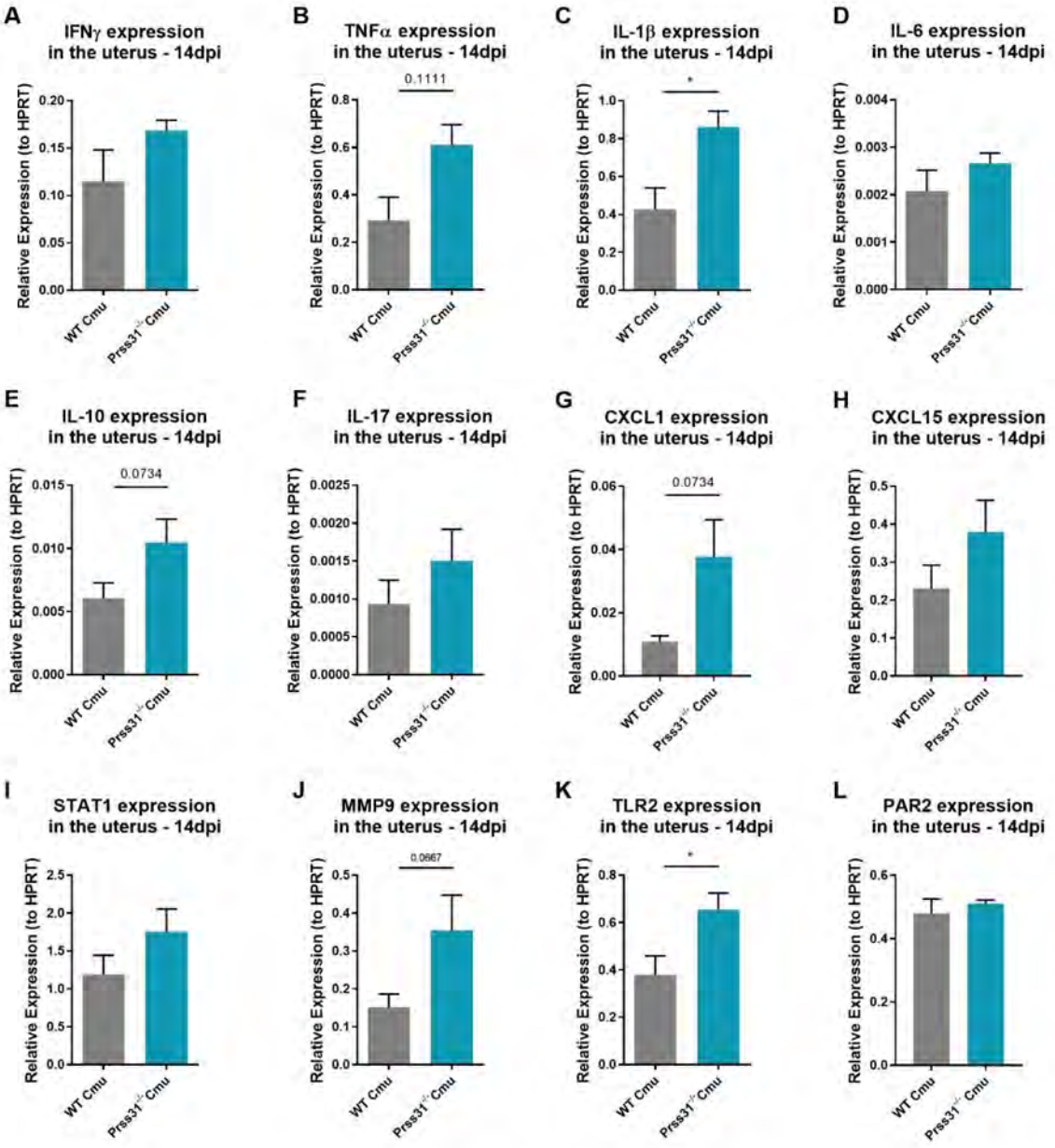


Figure 4.12 - Protease serine member S31 (Prss31) deficient mice have enhanced expression of the interleukin (IL)-1β and toll-like receptor in their *Chlamydia*-infected uterus at 14 days post infection (dpi). Prss31-deficient (*Prss31*$^{-/-}$) mice and WT controls were were treated with progesterone, infected intravaginally with *Chlamydia muridarum* (Cmu) and sacrificed at 14dpi. RNA was extracted from the uterus and the levels of expression of **(A)** interferon (IFN)γ, **(B)** tumor necrosis factor (TNF)α, **(C)** IL-1β, **(D)** IL-6, **(E)** IL-10, **(F)** IL-17, **(G)** CXCL1, **(H)** CXCL15, **(I)** STAT1, **(J)** matrix metallopeptidase (MMP)9, **(K)** toll-like receptor (TLR)2 and **(L)** protease activated receptor (PAR)2 were quantified by qPCR and normalised using the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). All data are presented as mean±SEM (n≥5) and, depending on the results of normality tests, Student's t-tests or Mann-Whitney tests were performed, with * representing $p<0.05$.

4.5. Discussion

In previous chapters, I showed that the number of MCs, and the expression of MC proteases, fluctuates upon administration of female sex hormones and during *Chlamydia* infection. I also show that MC degranulation might increase susceptibility to *Chlamydia* FRT infection during the early stages of infection. Moreover, I also show that MCs, early MC degranulation and heparin-protease complexes (and/or other factors affected by Ndst2 deficiency) contribute to the development of *Chlamydia*-associated pathology at later stages of infection, possibly by mediating the recruitment of immune cells associated with the development of hydrosalpinx. In addition, I also show that heparin-protease complexes (and/or other factors affected by Ndst2 deficiency) might be important for the induction of adaptive responses through $CD4^+$ and $CD8^+$ T cell mobilisation to the *Chlamydia*-infected uterus. I also show differential roles for MC proteases, mMCP5/Cpa3, mMCP6 and mMCP7, during *Chlamydia* infection, highlighting the importance of investigating the role(s) of individual MC factors for clearance of *Chlamydia* FRT infection and/or development of associated disease. In this Chapter, I outline the final series of studies conducted during my PhD, which examined the role of the membrane-anchored tryptase, Prss31, in the pathogenesis of *Chlamydia* FRT infection.

While the MC proteases investigated in my previous chapter are soluble and released into the extracellular environment upon MC degranulation, Prss31 is believed to stay bound to the plasma membrane of MCs. Hence, it is proposed that Prss31 only acts in the immediate environment surrounding the degranulated MC (212). In **Chapter 3**, I show that the expression of the soluble MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, is [1] increased in the ovary, oviduct and uterus of oestrogen-treated mice compared to progesterone-treated mice and [2] decreased at late stages of *Chlamydia* infection (**Figures 3.2 and 3.3**). Interestingly, I show that the expression of the membrane-anchored tryptase Prss31 is not affected by female sex hormone treatments nor by *Chlamydia* infection (**Figure 4.3**). Therefore, oestrous/menstrual cycle and/or infection status do not appear to regulate the expression of Prss31 in the FRT. Treatments with cigarette smoke extract have been shown to induce a dose-dependent increase in the expression of the Prss31

transcript as well as protein production in BMMCs (490), suggesting that the expression of Prss31 in MCs is not constitutive and can be modulated by environmental stimuli. The difference in profile of expression between Prss31 and mMCP4/mMCP5/mMCP6/CPA3 might be due to different pathways regulating their expression. However, surprisingly, I also show that the expression of Prss31 is similar in the uterus of *Chlamydia*-infected, MC-deficient mice and WT controls (**Figure 4.4**). These findings suggest that the expression of Prss31 might not be specific to MCs in the FRT. Compared to other MC proteases, Prss31 has been relatively less studied and, therefore, its pattern of expression has not been fully characterised. While Prss31 protein was shown to be specific to MCs in the human large intestine and skin (212), to the best of my knowledge, no one has studied Prss31 responses in the FRT. Therefore, in order to investigate the cellular source of Prss31 and advise its specificity to MCs in the FRT, I performed immunofluorescence staining using anti-Prss31 antibody (**Figure 4.3**).

I show positive staining for Prss31 in the uterine cells as well as in the apical epithelium of the oviduct, uterus and cervico-vaginal regions of the FRT in WT mice. While this finding could be interpreted as validating the hypothesis that Prss31 is not specific to MCs in the FRT, relatively similar positive staining is present in the FRT of Prss31-deficient mice. These results are questionable, given that Prss31-deficient mice were created by removal of the first exon of the *Prss31* gene containing the initiation site for the translation of *Prss31*. Therefore, Prss31-deficient mice should act as a negative control, with only limited transcripts being present, coding for a non-functional protein with levels largely reduced compared to WT mice. Therefore, my staining for Prss31 in the FRT of WT mice might not be reliable and, at this stage, I cannot definitively conclude on the cellular source of Prss31. Sections with no primary antibody added were included during staining to verify that the secondary antibody is not responsible for the nonspecific staining. Another possibility is that the primary anti-Prss31 antibody used is not specific to Prss31. Using BLAST, I investigated the specificity of the amino acid sequence used and, while γ-tryptase/Prss31 was the primary substrate, other tryptases showed relatively high identity of sequence, including the mouse tryptase 5 (mT5) or mMCP6. Some parts of the amino acid sequences of Prss31 display 100% identity to other tryptases, including mMCP6 and mMCP7 but

also with other tryptases whom genes are encoded at the tryptase locus, including mT5 (212, 447). Therefore, it is possible that the antibody that I used targeted a variety of tryptases, hence explaining the presence of positive staining in Prss31-deficient mice.

A rabbit anti-Prss31 antibody, specific for a shorter amino acid sequence (residues 147-163) of the mouse Prss31, as opposed to the antibody used in this study, has been generated in Professor Richard Stevens's laboratory (212, 447). This antibody, believed to not have high cross-reactivity with other tryptases, has been made available to us, courtesy of Professor Richard Stevens, to determine the cellular source of Prss31 in the FRT. However, due to time limitation, additional experiments with these antibodies could not be completed within the timeframe of my studies.

While the cellular source of Prss31 in the FRT remains unclear, the Human Protein Atlas suggest that the human endometrium, along with the gastrointestinal tract, is one of the tissues where Prss31 has the highest level of expression (495, 496). To assess a possible role of Prss31 during *Chlamydia* FRT infection, Prss31-deficient mice were subjected to our murine model of *Chlamydia* FRT infection. I show that Prss31-deficient mice have significantly higher levels of *Chlamydia* in their vagina at 3dpi and have slightly higher levels of infection in their uterus at 3dpi and 14dpi. Despite their apparent increased susceptibility to infection, Prss31-deficient mice have similar levels of hydrosalpinx compared to WT controls (**Figure 4.6**). These results suggest that Prss31 plays a protective role during *Chlamydia* infection, especially in early stages of infection. The high variability between samples in the same experimental group, a phenomenon often seen in the context of *Chlamydia* FRT infection, might have prevented the differences in infection in the uterus reaching statistical significance (140, 497, 498). In future studies, increasing the number of mice per group will be required in order to definitively conclude on the effect of Prss31 in ascending *Chlamydia* infection.

In order to validate the potential protective role of Prss31 during the early stages of *Chlamydia* infection, the effects of early intravaginal treatments with recombinant Prss31 during *Chlamydia* infection were also assessed. Interestingly, recombinant Prss31 treatments were highly protective against *Chlamydia* infection in the vagina and uterus at 3dpi (**Figure 4.7 A-B**), and

against the development of *Chlamydia*-associated pathology at 14dpi (**Figure 4.7 E-F**). While my results indicate that Prss31 is protective against the early stage of *Chlamydia* infection, its effect on pathology appear to differ between the study using Prss31-deficient mice and the study using recombinant treatments. Those differences may be caused by the fact that intravaginal treatments cannot reach the upper FRT and suggest that Prss31 may have differential effects in the lower *versus* upper FRT. Moreover, recombinant Prss31 does not possess the membrane anchor domain characteristic of Prss31, which could potentially change its location and/or activity compared to endogenous Prss31 (225, 226). Since recombinant Prss31 treatments were given only during the early stages of infection, while Prss31 deficiency occurred throughout the entire time course of infection, the contrasting effects observed between the two studies may also highlight differential effects of Prss31 during different stages of infection. Taken together the results from my gain- and loss-of-function of Prss31 studies show a protective role for Prss31 in mediating clearance of *Chlamydia* infection and/or development of infection-induced pathology. As mentioned previously, I did not identify the cellular source of Prss31 in the FRT. If it is specific to MCs and bound to the plasma membrane of degranulated MCs, Prss31 is likely to mediate its effects in the tissues surrounding MCs, probably through regulating immune responses *via* cleavage of local cytokines and chemokines or *via* interaction with neighbouring immune cells (226). If Prss31 is expressed within the mucosal surfaces of the FRT epithelium, my results suggest that this γ-tryptase might have powerful anti-bacterial effects that act directly on *Chlamydia* and/or induce protective responses in epithelial cells to help protect against infection. Future *in vitro* studies will assess this hypothesis by evaluating potential changes in [1] *Chlamydia muridarum* infectivity between epithelial cells isolated from the uterus and vagina of Prss31-deficient mice and WT controls, and [2] *Chlamydia trachomatis* infectivity in primary human vaginal, endometrial and fallopian epithelial cells treated with recombinant Prss31. Due to technical issues in isolating and growing primary epithelial cells from FRT, these experiments could not be finalised in the timeframe of my studies.

To assess if the protective effect of Prss31 during *Chlamydia* infection is caused by Prss31-mediated changes in immune cell recruitment, flow cytometry was performed to assess immune

cell numbers in the upper FRT of *Chlamydia*-infected Prss31-deficient mice and WT controls (**Figures 4.8 and 4.9**). Prss31-deficient mice have a significant decrease in the total cells as well as the number of immune cells in their uterus during infection compare to WT controls, suggesting a role for Prss31 in mediating immune cell recruitment. More specifically, the number of eosinophils, myeloid DCs, plasmacytoid DCs, resident and infiltrating monocytes and macrophages and $CD4^+$ and $CD8^+$ T cells were reduced in the uterus of *Chlamydia*-infected Prss31-deficient mice suggesting a role for Prss31 in mediating both innate and adaptive immune cell responses (**Figure 4.8**). While the number of neutrophils appeared to be unchanged between Prss31-deficient and WT controls, their proportion among total uterine cells is increased in the uterus of Prss31-deficient mice (**Figures 4.8 C and 4.9 B**), suggesting that neutrophils might be relatively more frequent in the uterus of Prss31-deficient mice, despite no change in total number. Previous studies have shown that the number of macrophages and/or neutrophils present in the bronchoavleolar lavage fluid or the colon of Prss31-deficient mice was decreased compared to WT mice in chronic obstructive pulmonary disease and colitis, respectively, confirming a pro-inflammatory capability of Prss31 (491). As discussed in **Chapter 3** with regards to Ndst2-deficient mice, the decrease in protective immune cells, such as myeloid DCs and $CD4^+$ T cells, in the uterus of *Chlamydia*-infected Prss31-deficient mice could explain why these mice have higher levels of infection (95, 96, 154, 161). However, contrary to Ndst2-deficient mice, Prss31-deficient mice are not protected against *Chlamydia*-associated pathology. This could be explained by the similar number, and higher proportion, of neutrophils in the uterus of *Chlamydia*-infected Prss31-deficient mice compared to WT controls (127, 133). It is possible that the levels of activation of the neutrophils might be higher in Prss31-deficient mice, that results in a compensation for the low immune cells presence, which lead to a similar level of hydrosalpinx as observed in WT mice.

During chronic obstructive pulmonary disease, the expression of Prss31 has been correlated with the recruitment of macrophages and their polarisation to the M2 phenotype (490, 491). Interestingly, the number of monocytes/macrophages is reduced in the uterus of *Chlamydia*-infected Prss31-deficient mice compared to WT controls (**Figure 4.8 G-H**) and, during

Chlamydia FRT infection, M2 macrophages have been shown to result in increased *Chlamydia* growth (146). The expression of arginase-1, a marker of M2 polarisation (492, 499), was measured in my studies. Unfortunately, I did not show any differences in expression of arginase-1 between Prss31-deficient mice and WT controls at 3dpi and 14dpi (**Figure 4.10 D, H**). While this observation appears to contradict the results of the study associating Prss31 with M2 polarisation (490), I measured the expression of arginase-1 in whole tissue and not specifically in macrophages, which is not considered sufficient to draw conclusions. Therefore, in future studies, characterising the M1/M2 polarisation of macrophages by flow cytometry will be necessary to conclude on a potential role of Prss31 in polarising macrophages towards the M2 phenotype during *Chlamydia* FRT infection. While I made an attempt to profile the M1/M2 polarisation of macrophages in the uterus of *Chlamydia*-infected Prss31-deficient mice and WT controls by flow cytometry using antibodies specific for surface markers of M1 macrophages (CD40, CD80, CD86 and MHC-II) and M2 macrophages, (CD206) (493, 494), high levels of auto-fluorescence compromised the analysis of the data and the results were not able to be appropriately interpreted (data not shown).

Recombinant Prss31 has been shown to induce the activation of the IL-13/IL-13rα/STAT6 pathway in macrophages and T cells *in vitro* and in the lung *in vivo* (226, 490). Therefore, I next examined the effects of Prss31 deficiency on changes in the expression of factors from the IL-13/IL-13rα/STAT6 pathway during *Chlamydia* FRT infection. Surprisingly, I show that the expression of IL-13 and IL-13rα is increased in Prss31-deficient mice at 3dpi. The opposite effect of Prss31 in my study might be due to differences between *in vitro* and *in vivo* studies, between organ/disease and/or to the use of recombinant Prss31/Prss31-deficient mice between the studies (226, 490). IL-13 has been shown to be detrimental during *Chlamydia* FRT infection, with IL-13-deficient mice displaying decreased bacterial burden in their vagina (177). Therefore, during *Chlamydia* FRT infection, Prss31 might be involved in limiting the expression of IL-13, hence mediating protective immune responses.

In the context of other infections and diseases, Prss31 deficiency has been associated with changes in expression of cytokines and chemokines such as IL-1β, IL-6 or IL-10 (302, 490, 491).

Therefore, I examined whether Prss31 deficiency was associated with changes in the expression of a number of key immune factors associated with protection and/or susceptibility to *Chlamydia* infection and/or *Chlamydia*-induced pathology. Surprisingly, I show an increase in expression of IL-1β, IL-10 and MMP9 in Prss31 deficient mice compared to WT controls. All of these factors associated with development of *Chlamydia*-induced pathology (136, 149, 181). I also show that pro-inflammatory mediators, such as STAT1, CXCL1, CXCL15 and TLR2, which largely protect against infection but may also cause pathology, are increased in the uterus of *Chlamydia*-infected Prss31-deficient mice compared to WT controls. These results seem to contradict my findings which show similar levels of oviduct pathology and decreased immune cell numbers in the uterus of Prss31-deficient mice. While it is possible that the increase expression of anti-inflammatory factors, such as IL-10, may account for the reduced inflammation in Prss31-deficient mice (181), as discussed previsouly in **Chapters 2 and 3,** it is possible that the changes in mRNA expression of the factors that I have investigated are not indicative of changes at the protein level. Similarly, while the expression of PAR2 is similar between Prss31-deficient mice and WT controls, Prss31 is likely to change PAR2 activation rather than its expression, and, therefore, assessment at protein levels will be necessary to conclude on a possible Prss31-mediated activation of PAR2 leading to changes in inflammation following *Chlamydia* infection. Therefore, in future studies, evaluating the protein levels of some of these key immune factors will be necessary to determine the mechanism underpinning Prss31 protection against *Chlamydia* infection. In complement, immunostaining and/or *in vitro* co-culture experiments will be realised to determine the cellular source of the key immune mediators identified.

4.6. Conclusion

In this chapter, I show that the expression of Prss31 is not altered by female sex hormones or *Chlamydia* infection, unlike what is observed with the MC proteases mMCP4, mMCP5, mMCP6 and CPA3. While the cellular source of Prss31 in the FRT remains unclear, my results strongly suggest that the expression of Prss31 might not be specific to MCs in the FRT (since Prss31 expression is not decreased in MC-deficient mice compared to WT controls). Importantly, by

using Prss31-deficient mice and recombinant Prss31 treatments, I reveal, for the first time, a strongly protective role for Prss31 in clearance of *Chlamydia* FRT infection and that the effects of Prss31 on the early stages of infection can be harnessed to not only protect against infection, but also the development of infection-induced pathology. Even if the mechanisms that underpin these effects were not fully elucidated in my studies, the decrease in the number of immune cell in the uterus of Prss31-deficient mice during *Chlamydia* infection suggest a potential role of Prss31 in mediating immune responses to *Chlamydia* infection in the FRT. The final chapter of my thesis will focus on discussing the results of my PhD studies and will outline the future directions for this research program.

Chapter five: General discussion and conclusions

5.1. Significance of the research

Chlamydia trachomatis is the most important bacterial STI worldwide, accounting for approximately 130 million cases of infection annually (1). Whilst diagnosis and antibiotic treatments are relatively effective, a large proportion of infections are asymptomatic and, therefore, frequently go undiagnosed and untreated. In some women, *Chlamydia trachomatis* can ascend to the upper FRT tissues resulting in the development of a range of diseases including pelvic inflammatory disease, salpingitis, tubal factor pregnancy and ectopic pregnancy (23). Many factors, affect both the course of *Chlamydia* infection and the development of infection-induced pathology. These include the serovar of *Chlamydia*, hormonal/reproductive status during infection, immune responses triggered by infection, whether the infection is a primary or secondary infection as well as coinfections with another STIs (500). In most cases, the complications of infection are caused by host immune responses rather than by *Chlamydia* itself.

While the function of some immune mediators have been broadly studied and shown to play protective (E.g. type 1 immune responses, $CD4^+$ T cells *etc.*) or detrimental (E.g. IL-1β, neutrophils *etc.*) roles during infection and in infection-induced pathology (96, 133, 161), the function of other immune cells that infiltrate and/or reside in the FRT remain unclear. In my PhD studies I set out to characterise the roles of MCs and their proteases during *Chlamydia* FRT infection. I have made a number of novel discoveries throughout my PhD that expand upon the known mechanisms of pathogenesis of *Chlamydia* FRT infection.

5.2. Female sex hormones regulate the number and phenotype of MCs in the FRT

I show that MCs are widespread and can present with a number of phenotypes in terms of serglycin proteoglycan content in the different tissues of the FRT. I also show that female sex hormones and/or the oestrous cycle stage appear to affect both the number and phenotype of MCs. Indeed, I show that oestrogen-treated mice have reduced numbers of uterine MCs, but increased expression of the main soluble MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, in their ovary, oviduct and uterus compared to progesterone-treated mice. However, whether oestrogen-induced oestrus prevents the survival of uterine MCs or progesterone-induced diestrus triggers MC proliferation and/or recruitment to the FRT cannot be resolved in my study (501). Similarly, the mechanism underpinning the changes in expression of mMCP4, mMCP5, mMCP6 and Cpa3 mediated by female sex hormones remain to be elucidated. It is possible that oestrogen stimulates the maturation of MCs, hence favouring the expression of those MC proteases, or that oestrogen mediates the activation of transcription factors, such as c-jun, that might, in turn, stimulate the expression of MC proteases in the FRT (460). As discussed in previous chapters, while some of my results might appear dissimilar to those of other studies, differences in animal/experimental procedures might have caused any disparities and additional studies are required to validate all findings (318, 324, 327, 332, 335).

Overall, my study reveals that female sex hormones might alter the number and phenotype of MCs in the FRT, agreeing with the premise that MCs mediate physiological processes during different stages of the menstrual cycle, such as menstruation and tissue remodelling, as well as pregnancy (329, 339, 502). Moreover, given that pregnancy has been associated to alteration in the outcome of MC-mediated diseases such as asthma, psoriasis, atopic dermatitis and mastocytosis (503), it is possible that female sex hormones and/or reproductive state regulate MC numbers and/or activation beyond the FRT tissues. Therefore, taking into account the menstrual/oestrous stage might be critical for research that focussed on MC-mediated responses during infection and/or in disease in other organs.

5.3. Effect of *Chlamydia* FRT infection on MC numbers and phenotype in the FRT

While MCs are relatively widespread in the FRT tissues and have been studied for their role in mediating physiological and pathophysiological processes, their role in immune responses to infection in the FRT remain relatively unknown. In other organs, MCs have been shown to be recruited to the infectious sites to mediate protective responses (376, 504, 505). However, my results, in consensus with results from another study, show that MCs are not recruited to the FRT tissues following *Chlamydia* infection (368). Although it will need to be confirmed by flow cytometry, the slight decrease in the number of MCs at 14dpi could suggest that adaptive immune responses and/or other effects induced by *Chlamydia* infection may affect MC tissue homeostasis in the longer term following infection (501). I show that these potential changes in MC numbers are associated with a profound decrease in the expression of the MC proteases, mMCP4, mMCP5, mMCP6 and Cpa3, suggesting that *Chlamydia* infection might not only alter the number of MCs but also their phenotypes.

5.4. MCs mediate immune responses to *Chlamydia* FRT infection

5.4.1. MCs contribute to the development of oviduct pathology during *Chlamydia* FRT infection

Perhaps most importantly, my studies reveal that MC-deficient mice are protected against the development of *Chlamydia*-induced hydrosalpinx. This suggests that MCs play an important role in the development of *Chlamydia*-induced diseases. The reduction in neutrophils, eosinophils and inflammatory monocytes and macrophages observed in the *Chlamydia*-infected uterine tissue of these mice might account for the protection against *Chlamydia*-induced pathology, given the association between these cells and development of hydrosalpinx (127, 133, 134, 149). Despite the reduction in innate immune cells, MC-deficient mice do not display profound changes in *Chlamydia* burden in the vagina and uterus compared to WT controls, probably due to similar number of protective $CD4^+$ T cells and myeloid DCs (96, 125, 152, 154, 159, 161).

Despite a decrease in the neutrophil chemoattractant CXCL15 in the uterus and alteration of the profile of the immune cell present in the bone marrow of *Chlamydia*-infected MC-deficient mice, the mechanism that might explain the decreased cellular infiltration and pathology observed MC-deficient mice cannot be not elucidated from my studies and will require additional experiments. The decrease in the number of B cells in the bone marrow of MC-deficient mice, suggest that MC-mediated responses may play a protective role in the induction of humoral immunity that is important in protecting against *Chlamydia* in the long term, especially during reinfection (160, 162, 185). Further studies are required to determine if MCs mediate B cell-mediated protection against reinfection, which would be of particular importance given the high rates of reinfection (E.g. 19%-26% within 6–12 months (506, 507)) of *Chlamydia trachomatis* in some cohorts of women.

5.4.2. Inhibition of MC degranulation during the early stages of *Chlamydia* FRT infection promotes clearance of infection early and reduces associated pathology later

Given the wide variety of factors that are produced by MCs, and their complex functions on mediating immune responses, my studies also aimed to identify the specific role(s) of MC degranulation and MC proteases during infection. I show that inhibition of MC degranulation through intravaginal administration of cromolyn, protects against *Chlamydia* infection during the early stages of infection, however, I also show that the continued administration of cromolyn is detrimental for infection during the later stages. Moreover, inhibition of MC degranulation during the early stages of infection confers slight protection against *Chlamydia*-induced pathology. Therefore, my findings suggest that MC degranulation might play differential roles during different stages of infection, with degranulation being particularly detrimental during the early stages of infection. Furthermore, I show that therapeutic targeting of MC degranulation may be harnessed therapeutically for the protection against *Chlamydia*-induced pathology. However, further studies are required to determine the mechanisms of action to elucidate and optimise the most ideal treatment strategies.

Although my results also show that degranulation of MCs was not significantly altered by *Chlamydia* infection at 3dpi and 14dpi, it is possible that degranulation was altered at different stages of infection, potentially in the very early stages following *Chlamydia* infection. MCs act as tissue sentinels and degranulate almost immediately following activation through detection of environmental stimuli. I hypothesise that *Chlamydia* components and/or infection-induced changes in the immune environment of the FRT induced by *Chlamydia* infection prompt rapid MC degranulation (190). Further *in vitro* and *in vivo* studies are required to investigate the mechanisms by which *Chlamydia* induces MC degranulation, what factors are released by MCs during infection and the effects that these factors have on infection, host immune responses and infection-induced diseases.

Interestingly, the inhibition of MC degranulation did not recapitulate the phenotype observed in MC-deficient mice, which strongly suggests that factors released by degranulation, *versus* those that are secreted through other pathways, play differential roles during *Chlamydia* infection. This lead to my investigations into the role of MC proteases, which constitute the majority of the proteins stored in secretory granules that are released during degranulation, in the pathogenesis of disease.

5.4.3. MC factors stored in secretory granules through binding with heparin contribute to *Chlamydia* FRT infection and the development of *Chlamydia*-induced oviduct pathology

Given that I show a strong effect of MCs and MC degranulation during *Chlamydia* infection, that the numerous factors released by MCs have range of differential effects and that a large amount of MC granular content consists of MC proteases, I next chose to focus my studies on determining the role of MC proteases during *Chlamydia* infection. For my studies, I used Ndst2-deficient mice, in which the storage of the MC factors that are normally bound to heparin, including histamine, mMCP4, mMCP5, mMCP6 and Cpa3, is almost completely deficient.

Similar to MC-deficient mice, Ndst2-deficient mice are protected against *Chlamydia*-associated pathology. This protection is associated with a reduction in cells linked with the

development of hydrosalpinx, including neutrophils, macrophages and $CD8^+$ T cells (127, 133, 134, 149, 182, 183, 474). However, Ndst2-deficient mice also have increased *Chlamydia* burden in their uterus. This can potentially be explained by the decline in the protective $CD4^+$ T cells in their uterus (95, 96, 161). My study also reveals a potential role for histamine and/or MC proteases stored in secretory granules through heparin-protease complexes in $CD4^+$ T cell polarising responses in the uterus, with a decrease in the expression of the transcription factors, Tbet and RORγt, associated with Th1 (protective for clearance of *Chlamydia* infection) and Th17 (detrimental for development of *Chlamydia*-associated pathology) responses observed in the *Chlamydia*-infected uterine tissue of Ndst2-deficient mice at 3dpi. While this finding is supported by the fact that MCs have been shown to promote type 1 and type 17 immunity during other infections (260), further studies are required to analyse the tissue expression of cytokines associated with Th1 (IFNγ) and Th17 (IL-17) at the protein level and to fully characterise the number, activation status, phenotype (Tbet/RORγt and IFNγ/IL-17 intracellular staining) of $CD4^+$ T cells in the uterus and FRT-draining lymph nodes of Ndst2-deficient mice during *Chlamydia* FRT infection.

Overall, my results show that factors affected by Ndst2 deficiency, including histamine and/or heparin-protease complexes, contribute to the induction of both innate and adaptive immune responses in the FRT during *Chlamydia* infection, thereby promoting immune responses associated with both clearance of infection as well as the development of pathology.

5.4.4. Different MC proteases stored in heparin-protease complexes differentially affect the pathogenesis of *Chlamydia* FRT infection

Given that my study suggests a critical role for heparin-protease complexes for the clearance of *Chlamydia* infection and development of *Chlamydia*-induced pathology, I next focused my studies on investigating the individual role of some of the MC proteases stored as heparin-proteases complexes in MCs. For this, I used a suite of genetically modified mice and recombinant protein.

My data show that the α-chymase mMCP5 and/or the metalloprotease Cpa3 appear to be detrimental during the early stages of *Chlamydia* infection. Although the mechanism that underpins this observation was not elucidated in my studies, it is possible that mMCP5 might affect tissue remodelling through cleavage of fibronectin, activation of MMP9 and/or disruption of the tight junctions of the epithelium of the FRT thereby favouring *Chlamydia* infection (239, 296, 297, 306, 315).

While mMCP6 does not appear to strongly affect the course of *Chlamydia* infection, the other tetramer-forming tryptase, mMCP7, appears to offer protection against *Chlamydia* infection and associated pathology. This tryptase has been shown to have pro-inflammatory functions and to induce the recruitment of neutrophils and eosinophils (202, 275). However, to fully conclude on the role of mMCP6 and mMCP7, investigating the outcome of *Chlamydia* infection in mice expressing the two tetramer-forming tryptases, mMCP6 and mMCP7, is necessary.

By using Prss31-deficient mice and recombinant protein treatments in WT animals, my studies reveal that the membrane-anchored tryptase, Prss31, potently protects against *Chlamydia* infection and/or associated pathology. Again, while the exact mechanisms involved were not fully realised during the course of my studies, my results suggest that Prss31 promotes immune cell recruitment to the *Chlamydia*-infected uterus. Interestingly, I also show that, unlike other MC proteases, the expression of Prss31 is not affected by female sex hormone treatment, *Chlamydia* infection nor by MC deficiency. While these preliminary results suggest that Prss31 might not be specific to MCs in the FRT but instead might be constitutively expression in FRT tissues, I was not able to conclusively identify the cellular source of Prss31 owing to potential deficiencies in the antibody used to conduct Prss31-specific staining of the FRT tissues.

Overall, I show that gain- or loss-of-function of individual MC proteases does not replicate the protective effects observed in Ndst2-deficient or MC-deficient mice. Therefore, my results highlight potentially redundant functions for the individual MC proteases that I focussed on during *Chlamydia* infection. My studies might suggest that it is the combined absence of several MC proteases that is required to show protection against *Chlamydia*-induced pathology. It is also possible that other MC factors, not investigated in my studies, contribute to *Chlamydia*-associated

pathology. Possible candidates include histamine, which has been shown to increase vascular permeability and immune cell infiltration (268, 508, 509) or mMCP4, which has been shown to mediate a range of functions *in vivo* and to be protective against bacterial infections in the FRT (305, 309, 310, 312, 486). It is highly likely that a multitude of MC factors act simultaneously and at different stages of *Chlamydia* infection and display opposite and/or redundant functions that mediate the pathogenesis of infection and the development of *Chlamydia*-associated pathology. Further studies are required to tease out the roles of individual factors at different time points of infection in future studies.

Nevertheless, my studies highlight a previously underappreciated role for MCs and MC proteases in mediating host immune responses to *Chlamydia* FRT infection and infection-induced disease.

5.5. Potential role of eosinophils during *Chlamydia* FRT infection

My studies reveal a decrease in the number of eosinophils in the uterus of *Chlamydia*-infected MC-, Ndst2- and Prss31-deficient mice. This observation is congruent with the well-characterised bidirectional interactions between MCs and eosinophils, with mediators released by one cell type, such as histamine or prostaglandin D_2 from MCs and eosinophil cationic protein or major basic protein from eosinophils, able to change the migration, function and activation of the other cell type (426). Therefore, it is possible that MCs and/or MC proteases act in concert with eosinophils during *Chlamydia* FRT infection to mediate pathogenesis. The role of eosinophils during *Chlamydia* FRT infection remains largely unknown, although one study has shown that the production of IL-4 by eosinophils may protect against *Chlamydia*-induced endometrial damage, through Il-4rα/STAT6 signalling, endometrial cell proliferation and enhancement of tissue repair (140).

Therefore, future research is required to characterise the role of eosinophils during *Chlamydia* infection and to investigate the potential interaction between eosinophils and MCs in mediating immune responses to *Chlamydia* FRT infection.

5.6. Ramifications of my research for other STIs and STI-associated *sequelae*

Whilst the roles of MCs and MC mediators in influencing host immune responses to STIs has not been extensively studied, there are a number of studies that support roles for MCs in other infections of the FRT. An increase in the number of MCs has been observed in the cervix of HIV-infected women and a role for HIV as reservoir for HIV during latent infections has been proposed (360-362). Moreover, MCs appear to offer protection against group B *Streptococcus* infections through regulation of pro-inflammatory responses, induction of neutrophil migration and mMCP4-mediated fibronectin cleavage (305, 367). MCs appear to also play a role in immune response to *Trichomonas vaginalis* infection, possibly through regulation of inflammatory responses, including by inducing neutrophil migration (363-365).

Given that I, and others, show roles for MCs in a small number of FRT infections, MCs might be participating in the host immune responses to numerous STIs, particularly the other major bacterial STI, *Neisseria gonorrhoeae*. Little is known regarding the immune responses that are associated with the pathogenesis of *Neisseria gonorrhoea* infections due the lack of a reliable mouse model. However, the few studies that have been conducted show that *Neisseria gonorrhoea* infections induce Th17 responses that prompt a large influx of neutrophils that release MMP9, which restrain the induction of adaptive immune responses (510). It is possible that MC and/or the factors stored in their secretory granules, might have roles in mediating these immune responses to *Neisseria gonorrhoeae* as I have seen with *Chlamydia*. Investigations that focus on the role of MCs and their proteases in the pathogenesis of *Neisseria gonorrhoeae* infection may lead to the discovery of new MC targeting/harnessing treatment strategies that may be able to counter the escalating antibiotic resistance observed in this bacteria (511). For example, recombinant Prss31 or cromolyn treatments may show similar protective effects for *Neisseria gonorrhoeae* as I observed with *Chlamydia*.

Besides their potential role in mediating the clearance and direct FRT damage observed with *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, MCs might also be associated with the development of pelvic pain that is sometime associated with these infections (23, 510). Indeed,

MCs are often located at close proximity of nerves where they modulate pain through nociceptor interactions. MCs and nerve receptors are believed to form a positive feedback activation loop. The release of MC mediators, such as histamine, tryptases and bradykinins, during degranulation activates nerve receptors, results in the release of substance P and other neuropeptides from the adjacent nerve endings, which consequently triggers further MC activation and the stimulation of pain pathways (353, 512, 513). MCs have been associated with development of pain in several diseases, including inflammatory bowel disease, endometritis, interstitial cystitis or vulvodynia, a chronic vulvar pain. Furthermore, treatments with the MC stabilisers, cromolyn or ketotifen, have been shown to improve some of these diseases (353, 419, 512, 514, 515). Therefore, it is possible that, in symptomatic women, MCs accumulate and/or degranulate at higher frequency, which could instigate and/or amplify the development of pelvic pain during *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections.

5.7. Limitations of my studies

5.7.1. Lack of sham-infected controls

By using a mouse model of *Chlamydia muridarum* and a range of genetically modified mice, my studies reveal a role for MC and MC proteases in mediating *Chlamydia* FRT infection. However, various limitations need to be considered. Firstly, one of the limitations of my studies include the lack of sham-infected mice that would allow to fully conclude on the differences in immune responses observed between WT and genetically modified mice. Due to the logistical restrictions associated with breeding of genetically modified mice, insufficient numbers of mice were available for including sham-infected controls in my studies. I decided to infect all available mice with *Chlamydia* in order to allow a full characterisation of the roles of MCs and their proteases during *Chlamydia* infection. The results obtained from my studies, nevertheless show effects of MCs on immune responses during infection and highlight the need for future experiments to elucidate the mechanisms involved.

5.7.2. Use of a mouse model of *Chlamydia* FRT infection

Another limitation of my studies is the differences that exist between mice and humans in terms of genetics and anatomy and in menstrual/oestrous cycles, which make drawing conclusions from my *in vivo* studies to *in vivo* responses in women difficult (92, 516). Whilst advances are being made with human *ex vivo* primary cell culture, and/or whole FRT tissue infection, these models have their disadvantages in that they do not represent the complex interactions that occur between different cells and tissues in the whole organism and appropriate amounts of tissues required to fully characterise effects are difficult to collect in great quantities. Given these issues, the fact that the immune responses in mice are well-characterised and that a large suite of genetically modified animals are available, murine models of *in vivo Chlamydia* FRT infection are still considered useful tools for understanding the mechanisms of pathogenesis of infection and for testing new therapies (90).

Another possible limitation is the choice of *Chlamydia muridarum* over *Chlamydia trachomatis* as a pathogen. As mentioned in **Chapter 1**, there are various differences between the two strains of *Chlamydia* (100, 517), but because intravaginal infection by *Chlamydia trachomatis* does not readily cause a productive infection nor pathology in mice, *Chlamydia muridarum*, which does cause both ascending infection and upper FRT pathology similar to that observed in women, was chosen for my studies.

Many of the results that assessed levels of *Chlamydia* burden in my studies were affected by high variability within the experimental groups. In many studies, high variability is often seen in the context of *Chlamydia* FRT infection (140, 371, 497, 498). This is likely caused by the fact that a complex variety of parameters affect the course of *Chlamydia* infection *in vivo*. For example, *Chlamydia* needs to pass through the cervical barrier to reach the uterus and the endometrial/uterine tissue itself shows quite a high level of variability between mice (even within the same experimental group). Therefore, the architecture of the FRT and the dynamic changes in morphology that I observe are likely responsible for the relatively high variability infection detected in the upper FRT.

Despite these limitations, the mouse model of *Chlamydia muridarum* FRT infection used in my study remains an important tool for investigating the complex immune responses responsible for clearance/susceptibility to *Chlamydia* infection and/or development of associated pathology and has been used in my studies to show important new roles for MCs in disease.

5.7.3. Use of genetically modified mice

While the use of genetically modified mouse strains allow the determination of roles of single genes (and the proteins that they code for) in disease, their use is not without limitation. For instance, all the genetically modified mouse strains used in my studies have a constitutive deletion/addition of targeted gene(s). Therefore, the loss/gain of a protein in these mice is present from conception and throughout their development. Such absences might have unforeseen effects on immunology and organs development, even before mice are subjected to *Chlamydia* infection that may indirectly affect outcomes. The use of inducible conditional knockout (or knock in) mice, such as the Mcpt5-Cre; iDTR or the Mas-TRECK, that display MC deficiency inducible by diphtheria toxin treatments, would allow for targeted loss of a protein during a specific length of time, which would limit possible off-target effects (518, 519). Moreover, the use of small interfering RNA (siRNA) and/or recombinant proteins can also be used to allow for loss- and gain-of-function of specific factors during specific stages of infection and disease (520).

5.7.4. Differences between human and mouse MC proteases

Another major limitation of my studies lies in the differences between mouse and human MC proteases. Firstly, some tryptases are expressed in a strain-dependent manner in mice. For instance, C57BL/6 mice lack mMCP7, while BALB/c and 129/Sv mice lack Prss31 (226, 227). More importantly, while humans possess a single gene coding for the α-chymase, Cma1, the mouse locus contains 14 genes coding for various chymases including the α-chymase, mMCP5, and the β-chymases, mMCP1, mMCP2, mMCP4 and mMCP9 (230, 231). The α-chymase, mMCP5, has a mutation in its active site, which changes its enzymatic activity from chymotrypsin-like to elastase-like (210). Therefore, mMCP5 is expected to display

different activities to the human chymase *in vivo* (210). In order to translate my results to humans, investigating the role of mMCP4, which is considered the functional homolog of CMA1, during *Chlamydia* infection is required (200, 233, 234).

Although, limits exist, the transgenic mice used in my studies still represent ideal tools for understanding the complex *in vivo* roles of MC proteases in diseases, including those associated with *Chlamydia* FRT infection.

5.8. Future directions

5.8.1. Further characterisation of the role of MCs during *Chlamydia* FRT infection

The results of this study suggest that female sex hormones and *Chlamydia* infection affect the number of uterine MCs and their expression of MC proteases. In future studies, the number of MCs, as well as their phenotype, will be fully characterised in the different sections of the FRT including the vagina, cervix, oviducts and ovaries. To fully characterise the effect of oestrous cycle, vaginal smears will be collected at all endpoints, to verify the oestrous stage of mice. To characterise whether MC degranulation is induced following *Chlamydia* infection, infected mice will be assessed at earlier time points (E.g. within hours to 1 day post infection). In addition, future studies will focus on optimising flow cytometric approaches for the validation of the MC count data that I performed on histology sections, as well as to investigate potential changes in activation markers in MCs that occur in response female sex hormone treatment and/or *Chlamydia* infection (262).

The experiments that I performed in MC-deficient mice and WT controls infected by *Chlamydia* will need to be repeated with a higher number of *Chlamydia*-infected animals as well as with sham-infected mice. This is required to validate the changes that I observed in MC-deficient mice, especially regarding bacterial burden and neutrophil, eosinophil and infiltrating monocyte/macrophage numbers that did not reach statistical significance. Such studies will also be designed to identify the mechanism(s) involved. In addition, the activation status of the innate immune cells, as well as the number of B cells will be investigated. If these future studies reveal

a reduction in the number of B cells in the uterus of *Chlamydia*-infected MC-deficient mice, a role for MCs in mediating protection against reinfection will be assessed. Moreover, an additional early time point is required in order to characterise the function of MCs during the early stages of *Chlamydia* infection. Protein levels and/or activation status of key immune factors associated with clearance/susceptibility to *Chlamydia* infection and/or development of associated *sequelae* will also be assessed. Bone marrow transplantation from WT to MC-deficient mice will be done to verify if the original phenotype of WT mice can be restored in MC-deficient mice by restoring MCs.

To complement these *in vivo* studies, *in vitro* studies will also be performed to investigate mechanisms and validate findings. BMMC and the human LAD2 MC line will be incubated with, *Chlamydia muridarum* and *Chlamydia trachomatis*, respectively, to confirm if *Chlamydia* directly activates MC degranulation and/or induces cytokine/chemokine release from MCs. In order to assess if the MC factors that are stored in the secretory granules of MCs affect epithelial cell infectivity, the specific MC activator compound 48/80 and/or *Chlamydia muridarum*/*Chlamydia trachomatis* will be used to induce degranulation of BMMC/LAD2 cells. The supernatants from these stimulated cells, which contain the compounds released by MCs, will be used to treat primary murine or human vaginal, endometrial and fallopian epithelial cells that will subsequently be infected with *Chlamydia muridarum* or *Chlamydia trachomatis*.

While all of the above mentioned *in vitro* experiments were initiated during my PhD studies, technical issues in isolating and growing uterine mouse epithelial cells prevented me from collecting any meaningful data during the timeframe of my PhD.

5.8.2. Further characterisation of the roles of MC proteases during *Chlamydia* FRT infection

Whilst I show that Ndst2-deficient mice have an increased susceptibility to *Chlamydia* infection and protection against infection-induced pathology, additional studies are required to characterise the mechanisms that underpin this phenotype. Firstly, identifying the number, activation and phenotype of DCs and T cells in the uterus and the FRT-draining lymph nodes at

early and later time points is be necessary to determine the roles of histamine and/or heparin-protease complexes that are stored in MC granules in the induction of specific adaptive immune responses to *Chlamydia* infection. $CD4^+$ T cell polarisation will be determined using intracellular staining for key transcription factors and/or cytokines associated with Th1 (Tbet/IFNγ), Th2 (GATA3/IL-13), Th17 (RORγt/IL-17) and Treg (FOXP3/transforming growth factor beta [TGFβ]). Furthermore, the protein levels and/or activation status of key immune factors associated with clearance/susceptibility to *Chlamydia* infection and/or development of associated *sequelae* will be assessed. Moreover, to investigate the possibility that Ndst2 deficiency alters heparan sulfate on the epithelium of the FRT, thereby affecting susceptibility to *Chlamydia* independently of MCs, primary uterine epithelial cells from Ndst2-deficient mice and WT controls will be isolated and infected with *Chlamydia muridarum*. In addition, bone marrow transplantation from WT to Ndst2-deficient mice will be done to verify if the original phenotype of WT mice can be restored in Ndst2-deficient mice by restoring MCs.

The cellular source of the MC proteases mMCP4, mMCP5, mMCP6 and Cpa3, will be established using immunostaining in order to verify that they are specifically expressed by MCs. Furthermore, to investigate the mechanisms underlying the detrimental role(s) of mMCP5 or Cpa3 during the early stage of infection, the immune cells present in the uterus of mMCP5-deficient mice will be characterised by flow cytometry. Moreover, the activation of MMP9 will be analysed, and the levels of claudins measured to investigate potential mMCP5-mediated disruption of the FRT epithelium during *Chlamydia* infection. To delineate between the effect of mMCP5 and Cpa3, recombinant intravaginal and/or intrauterine treatments with mMCP5 or Cpa3 will be performed in mMCP5-deficient mice and WT controls.

Whilst my studies show that the absence of mMCP6 does not appear to affect the course of *Chlamydia* infection, I show that the presence of mMCP7 resulted in partial protection in the absence of mMCP6. As mentioned previously, to fully validate the role of mMCP7 *versus* mMCP6, the effect of *Chlamydia* infection on mMCP6-sufficient/mMCP7-sufficient mice ($mMCP6^{+/+}$ $mMCP7^{+/+}$) will need to be evaluated.

My results raise the possibility that Prss31 might not be specific to MCs in the FRT. In future studies, a new antibody, that is specific to the mouse Prss31 protein, will be used in immunofluorescence staining to identify the cellular source of Prss31 in the different tissues of the FRT. In order to determine the mechanism(s) that underpin the protective role of Prss31 during *Chlamydia* infection, flow cytometry will be performed at early time point to identify a possible deficit in innate immune cell mobilisation. In addition, the polarisation of macrophages towards M1/M2 phenotypes will be assessed at early and late time points (using markers such as CD40, CD80, CD86 and MHC-II *versus* CD206) (226, 490). Although this experiment has been performed already, high levels of auto-fluorescence prevented the reliable analysis of the data. Henceforward, a live and dead stains will be added to the antibody panel of all flow cytometry experiment to limit auto-fluorescence. The protein levels of IL-13, IL-13rα and STAT6, as well as of key immune factors associated with clearance/susceptibility to *Chlamydia* infection and/or development of associated *sequelae* will also be assessed. Moreover, a series of *in vitro* studies will be performed to determine the nature of expression of Prss31 in the FRT epithelium and its effects on epithelial cell infectivity. Infectivity assays will be performed on primary vaginal, uterine and oviduct epithelial cells from the FRT of Prss31 mice and WT controls. If Prss31 is indeed expressed by the epithelium and has a direct effect on epithelial cell infectivity, increased infectivity should be observed in Prss31-deficient cells. Similarly, the infectivity will be evaluated in human and mouse epithelial cells following treatment with recombinant Prss31 to assess whether exogenous Prss31 can protect the epithelium from infection. Complementary studies will also be performed by infecting un-treated epithelial cells with *Chlamydia muridarum* or *Chlamydia trachomatis* that has been incubated with recombinant Prss31 to identify whether Prss31 has direct bactericidal activity.

Because none of the individual MC proteases investigated reproduced phenotypes similar to Ndst2-deficient or MC-deficient mice, it is possible that the MC proteases investigated have redundant functions during *Chlamydia* infection. To test this hypothesis, recombinant treatments with mMCP4, mMCP5 mMCP6 and/or Cpa3 in Ndst2-deficient may help to delineate the role of each factor. Moreover, a multi-deficient mice lacking mMCP4, mMCP5 mMCP6 and Cpa3 could

be subjected to *Chlamydia* infection (485). The role of mMCP4 and histamine could be investigated by using mMCP4–deficient mice or histamine receptor-deficient mice, respectively (297, 313, 521, 522).

5.9. Concluding remarks

MCs are key immune cells of haematopoietic lineage that have been conserved through millions of years of evolution. In addition to their well-recognized role in allergic diseases, MCs play important roles in a plethora of physiological processes and in mediating immune responses to pathogens. To date, their role in mediating immune responses to STIs has largely been ignored. My PhD studies show that MCs play a detrimental role during *Chlamydia* FRT infections by contributing to the development of pathology. Moreover, the mediators stored in the granules of MCs, particularly heparin proteoglycans and MC proteases, appear to affect the host immune responses and the outcome of *Chlamydia* infection. Due to the complexity of the MC responses, which involve a wide variety of mediators that can have cumulative and/or redundant effect on infection, the mechanisms involved were not fully characterised in my studies. It is important to extend upon my body of research to gain an even greater understanding of how MCs mediate *Chlamydia* FRT infection and/or the development of pathology in women, in order to help highlight novel prevention and treatment strategies for infection and infection-associated *sequelae*. It is hoped that the results from my studies will encourage further *in vivo* and *in vitro* studies, to help better understand the roles of MCs and individual MC proteases in the host immune response to *Chlamydia* FRT infection with the overarching goal of identifying improved therapeutic strategies.

Importantly, this work could potentially be broadened to investigate the role and therapeutic manipulation of MCs and MC proteases in the context of other bacterial STIs, especially *Neisseria gonnorrhoeae*, which is of great concern given the level of antibiotic resistance being reported in this bacterium.

Appendix

6.1. Nucleotide sequences of the primers used for analyses of gene expression

Target gene	Primers nucleotide sequence (5' to 3')	
***Chlamydia 16S* rRNA**	Forward	GCGGCAGAAATGTCGTTTT
	Reverse	CGCTCGTTGCGGGACTTA
Chlamydia MOMP	Forward	GCCGTTTTGGGTTCTGCTT
	Reverse	CGTCAATCATAAGGCTTGGTTCA
Mouse *Arginase-1*	Forward	GGCAGAGGTCCAGAAGAATG
	Reverse	GTGAGCATCCACCCAAATG
Mouse *Cpa3*	Forward	ACATCTACGGCCCAATAGCA
	Reverse	ACATGTTGGCTTTATCCGGG
Mouse *CXCL1*	Forward	GCTGGGATTCACCTCAAGAA
	Reverse	CTTGGGGACACCTTTTAGCA
Mouse *CXCL15*	Forward	AAGGAAGTGATAGCAGTCCCAAA
	Reverse	GCCAACAGTAGCCTTCACCC
Mouse *CXCR2*	Forward	GCAGAGCCCCCAGAGTTTAG
	Reverse	GAAGGGAGCAGACAGCCAAT
Mouse *FOXP3*	Forward	GGCGAAAGTGGCAGAGAGGTATT
	Reverse	GGCATTGGGTTCTTGTCAGAGG
Mouse *GATA3*	Forward	AGAACCGGCCCCTTATGAA
	Reverse	AGTTCGCGCAGGATGTCC
Mouse *GM-CSF*	Forward	ATGCCTGTCACGTTGAATGAAGAGG
	Reverse	AGGCGGGTCTGCACACATGTTA
Mouse *HPRT*	Forward	AGGCCAGACTTTGTTGGATTTGAA
	Reverse	CAACTTGCGCTCATCTTAGGCTTT
Mouse *IFNγ*	Forward	ACGGCACAGTCATTGAAAGC
	Reverse	TGTCACCATCCTTTTGCCAGT
Mouse *IL-1β*	Forward	GAAGTTGACGGACCCCAAAA
	Reverse	GCCTGCCTGAAGCTCTTGTT
Mouse *IL-4rα*	Forward	GGGCTGTCTGATTTTGCTGT
	Reverse	TGGAAGTGCGGATGTAGTCA
Mouse *IL-6*	Forward	AGAAAACAATCTGAAACTTCCAGAGAT
	Reverse	GAAGACCAGAGGAAATTTTCAATAGG
Mouse *IL-10*	Forward	AGGCGCTGTCATCGATTTCT

	Reverse	ATGGCCTTGTAGACACCTTGG
Mouse *IL-12p40*	Forward	TTGTTCGAATCCAGCGCAAG
	Reverse	AGACATTCCCGCCTTTGCAT
Mouse *IL-13*	Forward	TGCTTGCCTTGGTGGTCT
	Reverse	GGGGAGTCTGGTCTTGTGTG
Mouse *IL-13rα*	Forward	CACAGTCAGAGTAAGAGTCAAAACA
	Reverse	ATGGTGGTGTAGAAGGTGGA
Mouse *IL-17*	Forward	GTGTCAATGCGGAGGGAAAG
	Reverse	CCCACCAGCATCTTCTCGAC
Mouse *Mcpt4* (mMCP4)	Forward	GTGACCGACACTGGCAAGAT
	Reverse	GGCCATGTAAGGGCGAGAAT
Mouse *Cma1* (mMCP5)	Forward	CTTACTCTTCATCTGCTGCTCC
	Reverse	ACTCCGTGCCTCCAATGAT
Mouse *Mcpt6* (mMCP6)	Forward	CACTGTCCCTCCTGGCTAGT
	Reverse	GAAGCCTCATGTCCTCCCAC
Mouse *MMP9*	Forward	CGAACTTCGACACTGACAAGAAGT
	Reverse	GCACGCTGGAATGATCTAAGC
Mouse *Ndst2*	Forward	CCTTTCTGAACTACACGAGGAC
	Reverse	ATCCGTAAGGAAAAGGCGAGA
Mouse *NRC1*	Forward	GCACCTACCGACCCTACTTC
	Reverse	GGCGGAGTCCTTTTGTAATCCT
Mouse *PAR2*	Forward	TGCTGGGAGGTATCACCCTTC
	Reverse	GCTGGGTTTCTAATCTGCCAAT
Mouse *Prss31*	Forward	GATGACTCTGGAGGGCCACTA
	Reverse	ATAGGCAGTAACCCGGGCAT
Mouse *RORγt*	Forward	CCGCTGAGAGGGCTTCAC
	Reverse	TGCAGGAGTAGGCCACATTACA
Mouse *STAT1*	Forward	CCCGAATTTGACAGTATGATGA
	Reverse	GAAGGAACAGTAGCAGGAAGGA
Mouse *STAT6*	Forward	GCACCTTGGAGAGCATCTATCA
	Reverse	TTGAGTTCTTCCTTGCTTCCGATG
Mouse *Tbet*	Forward	TCAACCAGCACCAGACAGAGATG
	Reverse	CACCAAGACCACATCCACAAACA
Mouse *TLR2*	Forward	TGTAGGGGCTTCACTTCTCTGCTT
	Reverse	AGACTCCTGAGCAGAACAGCGTTT
Mouse *TLR3*	Forward	TCCAACTGGAGAACCTCCAAGAAC
	Reverse	TTCAAGAGGAGGGCGAATAACTTG
Mouse *TNFα*	Forward	TCTGTCTACTGAACTTCGGGGTGA

	Reverse	TTGTCTTTGAGATCCATGCCGTT

Table 6.1 - Oligonucleotide sequences of the primer pairs used for quantitative polymerase chain reaction analyses of gene expression.

6.2. Antibody cocktail and gating strategy used for determining the percentages and numbers of immune cells in the uterus of WT, MC-deficient, Ndst2-deficient and Prss31-deficient mice

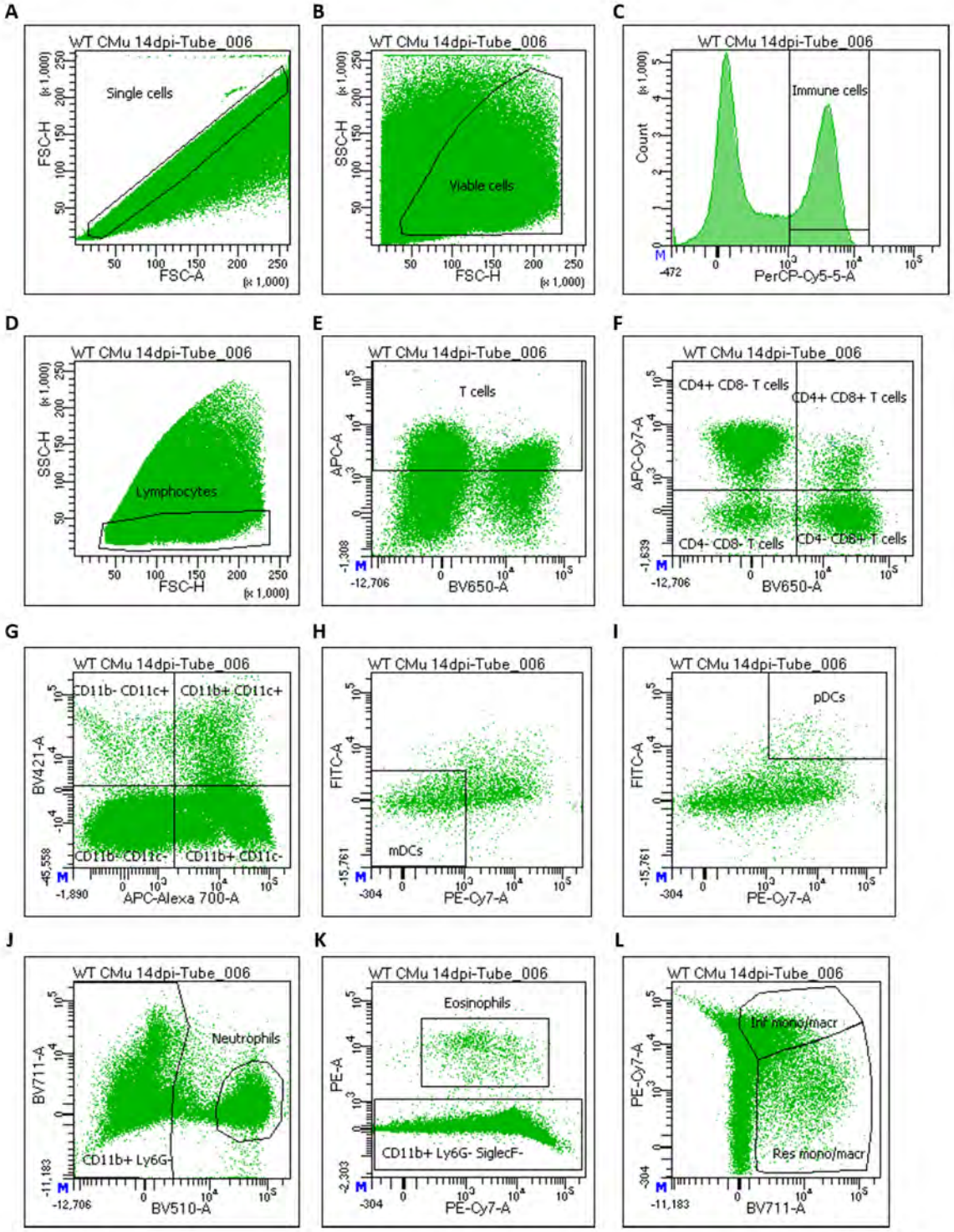


Figure 6.1 - Gating strategy for characterising the immune cells in the uterus of wild type (WT), Mast cell (MC)-deficient mice, *N*-deacetylase/*N*-sulphotransferase-2- (Ndst2-)deficient mice and protease serine member S31- (Prss31-) deficient mice. Female WT (WT and *Cpa3-Cre;Mcl-1*$^{WT/WT}$), MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$), Ndst2-deficient and Prss31-deficient mice were pre-treated with progesterone

subcutaneously. Seven days later, mice were infected intra-vaginally with $5x10^4$ infection forming units of *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Single cell suspensions from the uterus were obtained and counted. Samples were blocked with Fcblock to avoid unspecific binding and then stained with a cocktail of antibodies specific for surface markers conjugated with flurochromes. Cells were fixed with 4% paraformaldehyse and fluorescence was measured using a BD LSRFortessaTM X-20 cell analyser (BD Biosciences) and analysed using BD FACSDivaTM Software. **(A)** Single cells were first selected to remove doublets using the forward scatter (FSC) height (H) and area (A). Then **(B)** viable cells were selected from the single cells, using FSC and side scatter (SSC) profile. **(C)** $CD45^+$ cells were selected ($PerCPCy5\text{-}5^+$) from viable cells to determine the immune cells in the uterus. **(D)** Immune cells of low SSC were selected as a rough gating for lymphocytes. **(E)** Gating of $CD3^+$ lymphocytes (APC^+) allowed to determine T cells, **(F)** that were further divided according to their expression of CD4 (APC-Cy7) and CD8 (BV650) to evaluate $CD4^+$ T cells ($CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$) and $CD8^+$ T cells ($CD45^+$ $CD3^+$ $CD4^-$ $CD8^+$). **(G)** Immune cells were then used to separate cells expressing CD11b (APC-Alex 700) or CD11c (BV421). **(H)** Myeloid dendritic cells (mDCs) were determined as $CD11b^+$ $CD11c^+$ cells that are not expressing Ly6C (PE-Cy7) and PDCA (FITC) ($CD45^+$ $CD11b^+$ $CD11c^+$ $Ly6C^-$ $PDCA^-$). **(I)** Plamacytoid dendritic cells (pDCs) were determined as $CD11b^-$ $CD11c^+$ cells that are expressing Ly6C (PE-Cy7) and PDCA (FITC) ($CD45^+$ $CD11b^-$ $CD11c^+$ $Ly6C^+$ $PDCA^+$). **(J)** Neutrophils were determined as $CD11b^+$ cells expressing Ly6G (BV510) ($CD45^+$ $CD11b^+$ $Ly6G^+$) and $CD11b^+$ cells not expressing Ly6G were gated. **(K)** The $CD11b^+$ $Ly6G^-$ cells were then separated according to their expression of SiglecF (PE) in eosinophils ($CD45^+$ $CD11b^+$ $Ly6G^-$ $SiglecF^+$) and $CD11b^+$ $Ly6G^-$ $SiglecF^-$ cells, **(L)** that were then gated according to their expression of F4/80 (BV711) and Ly6C (PE-Cy7) to determine infiltrating monocytes and macrophages (Inf mono/macr; $CD45^+$ $CD11b^+$ $Ly6G^-$ $SiglecF^-$ $F4/80^+$ $Ly6C^+$) and resident monocytes and macrophages (Res mono/macr; $CD45^+$ $CD11b^+$ $Ly6G^-$ $SiglecF^-$ $F4/80^+$ $Ly6C^-$).

Antibody targets	Fluorochromes
CD45	PerCP
CD3	APC
CD4	APC-Cy7
CD8	BV650
CD11b	AF700
CD11c	BV421
F4/80	BV711
Ly6C	PE-Cy7
Ly6G	BV510
PDCA	FITC
SiglecF	PE
c-kit	BV786
FcεRIα	Biotin
Streptavidin	BV605

Table 6.2 – Panel of the antibodies used for extracellular staining of uterine cells of wild type, mast cell-deficient, *N*-deacetylase/*N*-sulphotransferase-2-deficient and protease serine member S31-deficient mice.

6.3. Antibody cocktail and gating strategy used for determining the percentages and numbers of myeloid cells in the bone marrow of WT and MC-deficient mice

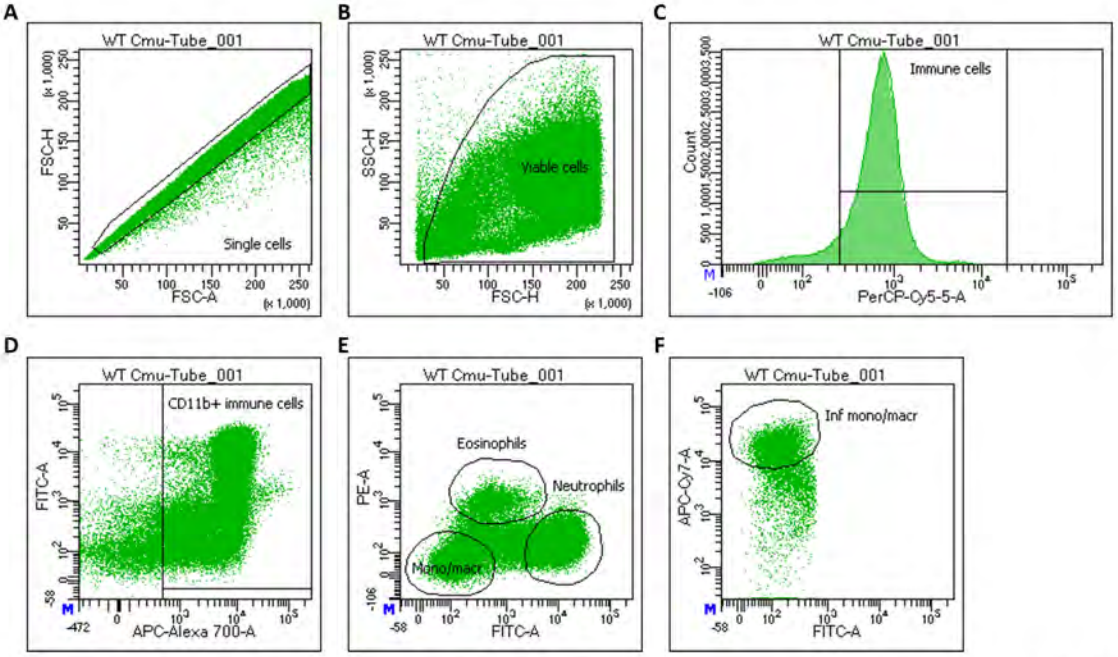


Figure 6.2 - Gating strategy for characterising the myeloid immune cells in the bone marrow of wild type (WT) and Mast cell (MC)-deficient mice. Female WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) and MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) mice were pre-treated with progesterone subcutaneously. Seven days later, mice were infected intra-vaginally with $5x10^4$ infection forming units of *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Bone marrow cells were obtained and counted. Samples were blocked with Fcblock to avoid unspecific binding and then stained with a cocktail of antibodies specific for surface markers conjugated with flurochromes. Cells were fixed with 4% paraformaldehyse and fluorescence was measured using a BD LSRFortessaTM X-20 cell analyser (BD Biosciences) and analysed using BD FACSDivaTM Software. **(A)** Single cells were first selected to remove doublets using the forward scatter (FSC) height (H) and area (A). Then **(B)** viable cells were selected from the single cells, using FSC and side scatter (SSC) profile. **(C)** $CD45^+$ cells were selected (PerCPCy5-5^+) from viable cells to determine the immune cells in the bone marrow. **(D)** Gating of $CD11b^+$ immune cells (APC-Alexa 700^+) allowed to gate myeloid cells, **(E)** that were further divided according to their expression of SiglecF (PE) and Ly6G (FITC) to determine eosinophils ($CD45^+$ $CD11b^+$ $SiglecF^+$), neutrophils ($CD45^+$ $CD11b^+$ $SiglecF^-$ $Ly6G^+$) and monocytes and macrophages (Mono/macr; $CD45^+$ $CD11b^+$ $SiglecF^-$ $Ly6G^-$). **(F)** Monocytes and macrophages were then separated between resident populations ($CD45^+$ $CD11b^+$ $SiglecF^-$ $Ly6G^-$ $Ly6C^-$) and inflammatory populations ($CD45^+$ $CD11b^+$ $SiglecF^-$ $Ly6G^-$ $Ly6C^+$) according to their expression of Ly6C (APC-Cy7).

Antibody targets	Fluorochromes
CD45	PerCP
CD11b	AF700
Ly6C	APC-Cy7
Ly6G	FITC
SiglecF	PE

Table 6.3 – Myeloid panel, with the antibodies and their associated fluorochromes used for extracellular staining of bone marrow cells of wild type and mast cell deficient mice.

6.4. Antibody cocktail and gating strategy used for determining the percentages and numbers of lymphoid cells in the bone marrow of WT and MC-deficient mice

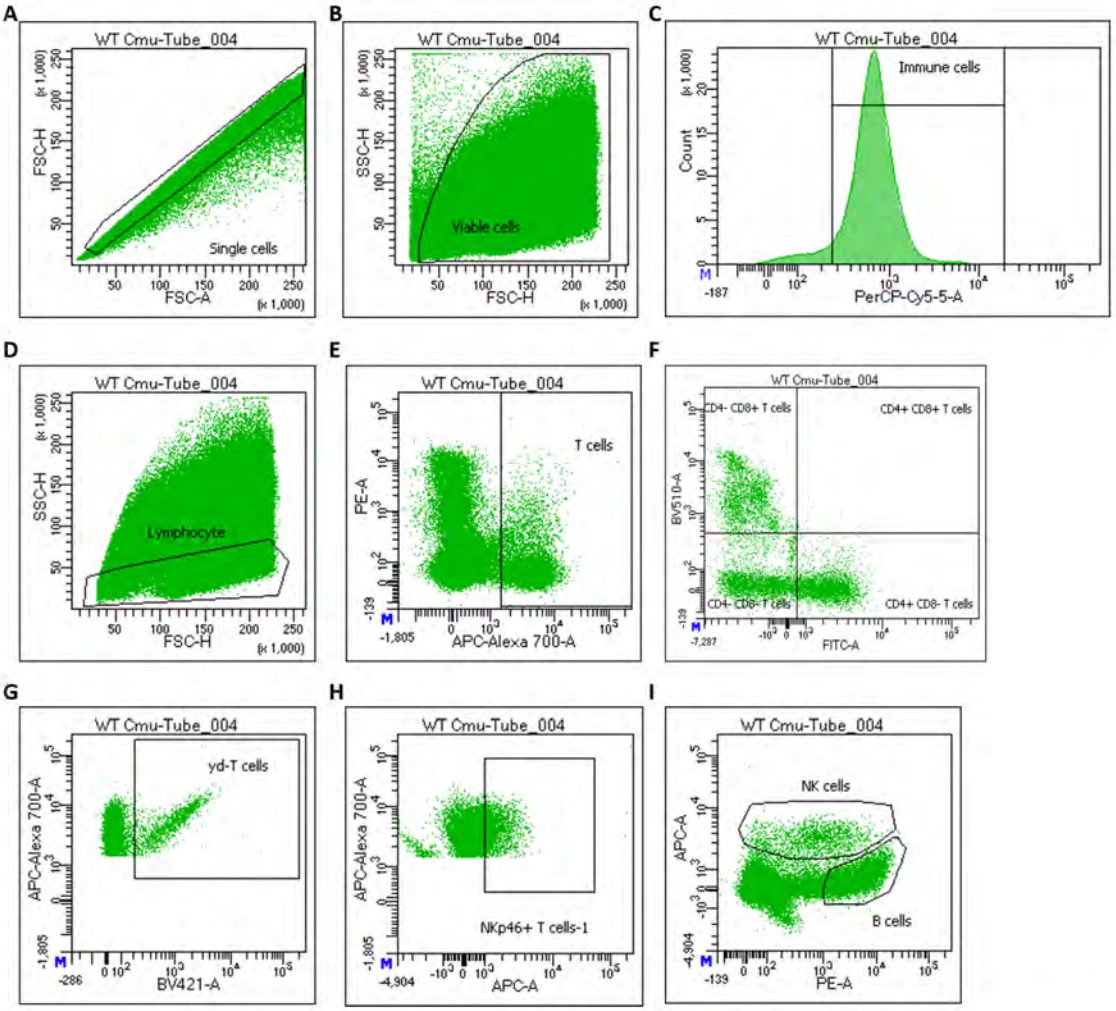


Figure 6.3 – Gating strategy for characterising the lymphoid immune cells in the bone marrow of wild type (WT) and mast cell- (MC-) deficient mice. Female WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) and MC-deficient (*Cpa3-Cre;Mcl-1*$^{fl/fl}$) mice were pre-treated with progesterone subcutaneously. Seven days later, mice were infected intra-vaginally with $5x10^4$ infection forming units of *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Bone marrow cells were obtained and counted. Samples were blocked with Fcblock to avoid unspecific binding and then stained with a cocktail of antibodies specific for surface markers conjugated with flurochromes. Cells were fixed with 4% paraformaldehyse and fluorescence was measured using a BD LSRFortessaTM X-20 cell analyser (BD Biosciences) and analysed using BD FACSDivaTM Software. **(A)** Single cells were first selected to remove doublets using the forward scatter (FSC) height (H) and area (A). Then **(B)** viable cells were selected from the single cells, using FSC and side scatter (SSC) profile. **(C)** $CD45^+$ cells were selected ($PerCPCy5\text{-}5^+$) from viable cells to determine the immune cells in the bone marrow. **(D)** Immune cells of low SSC were selected as a rough gating for lymphocytes. **(E)** Gating of $CD3^+$ lymphocytes ($APC\text{-}Alexa\ 700^+$) allowed to determine T cells, **(F)** that were further divided according to their expression of CD4 (FITC) and CD8 (BV510) to evaluate $CD4^+$ T cells ($CD45^+\ CD3^+\ CD4^+\ CD8^-$) and $CD8^+$ T cells ($CD45^+\ CD3^+\ CD4^-\ CD8^+$); **(G)** to their expression of γδTCR ($BV421^+$) to determine the γδ T cells ($CD45^+\ CD3^+\ \gamma\delta TCR^+$); and **(H)** to their expression of NKp46 (APC^+) to determine NKT cells ($CD45^+\ CD3^+\ NKp46^+$). **(I)** $CD3^-$ lymphocytes were then used to determine

natural killer (NK) cells that are $CD45^+$ $CD3^-$ $NKp46^+$ (APC^+) and B cells that are $CD45^+$ $CD3^-$ $NKp46^-$ $B220^+$ (PE^+).

Antibody targets	Fluorochromes
CD45	PerCP
CD3	AF700
CD4	FITC
CD8	BV510
γδTCR	BV421
NKp46	APC
B220	PE

Table 6.4 – Mature lymphoid panel, with the antibodies and their associated fluorochromes, used for extracellular staining of bone marrow cells of wild type and mast cell deficient mice.

6.5. Problem encountered in evaluating the number of MCs in the uterus by flow cytometry

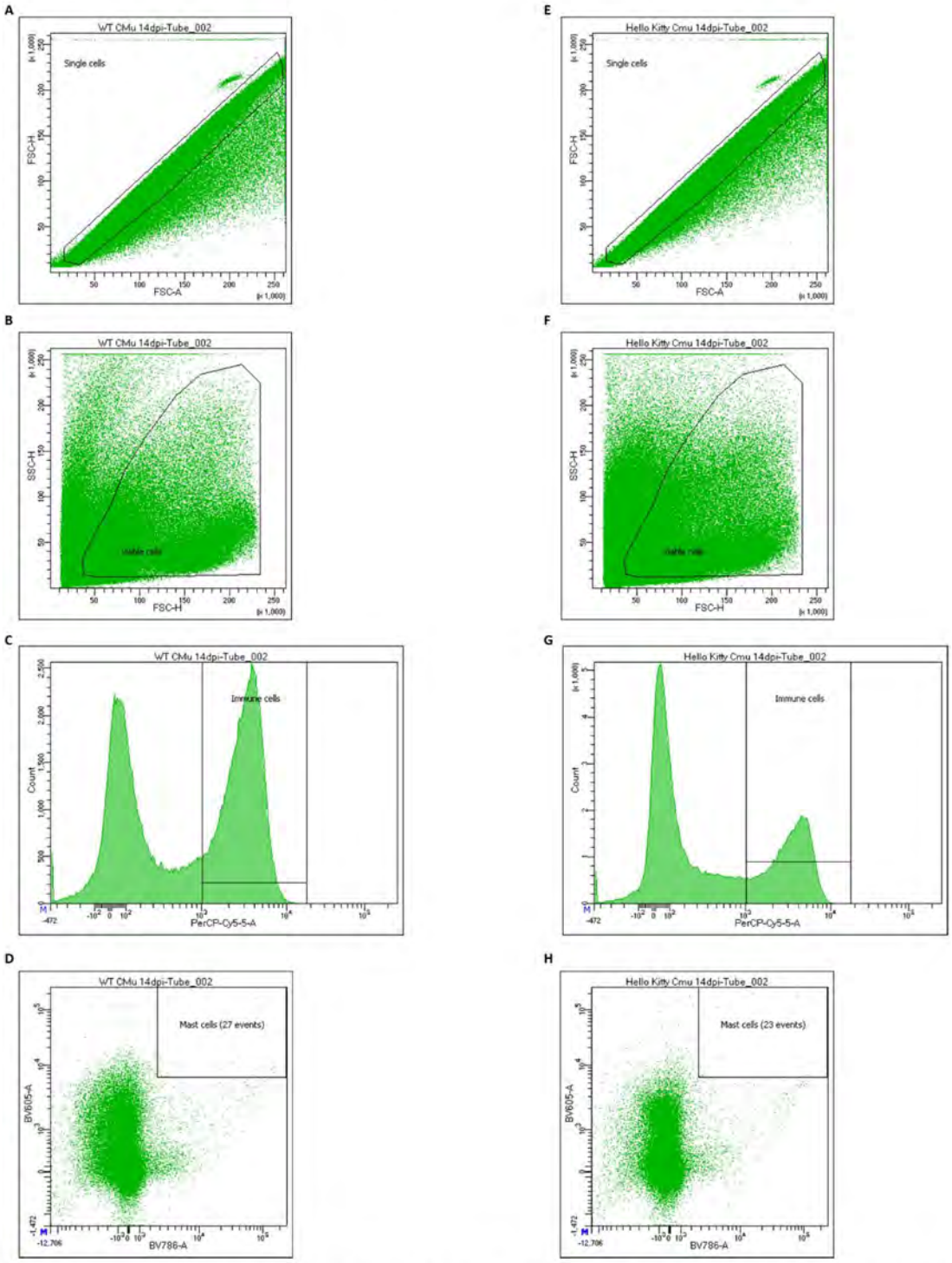


Figure 6.4 – Representative plots showing the absence of staining for mast cell (MC) in the uterus of wild type (WT) and MC-deficient mice. (A, B, C, D) WT (*Cpa3-Cre;Mcl-1*$^{WT/WT}$) and **(E, F, G, H)** MC-deficient (Hello Kitty; *Cpa3-Cre;Mcl-1*$^{fl/fl}$) female mice were pre-treated with progesterone subcutaneously. Seven days later, mice were infected intra-vaginally with $5x10^4$ infection forming units of *Chlamydia muridarum* (Cmu) and sacrificed at 14 days post infection. Single cell suspensions from the uterus were obtained and counted. Samples were blocked with Fcblock to avoid unspecific binding and then stained with a cocktail of antibodies specific for surface markers conjugated with flurochromes. Cells were fixed

with 4% paraformaldehyse and fluorescence was measured using a BD LSRFortessaTM X-20 cell analyser (BD Biosciences) and analysed using BD FACSDivaTM Software. **(A, E)** Single cells were first selected to remove doublets using the forward scatter (FSC) height (H) and area (A). Then **(B, F)** viable cells were selected from the single cells, using FSC and side scatter (SSC) profile. **(C, G)** $CD45^+$ cells were selected ($PerCPCy5\text{-}5^+$) from viable cells to determine the immune cells in the uterus. **(D, H)** Gating of immune cells according to their expression of FcεRI (BV605) and c-kit (BV786) should identify MC ($CD45^+$ $Fc\varepsilon RI^+$ $c\text{-}kit^+$) but **(D)** WT mice appear to have no real positive staining, similarly to **(H)** MC-deficient mice.

References

1. World Health Organization. Report on global sexually transmitted infection surveillance. 2018.

2. World Health Organization. Sexually transmitted infections (STIs) 2015 [Available from: http://www.who.int/mediacentre/factsheets/fs110/en/.

3. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. Bull World Health Organ. 2019;97(8):548-62P.

4. World Health Organization. Hepatitis B [18/07/2018]. World Health Organization Website: World Health Organization; 2018 [Fact sheet]. Available from: https://www.who.int/en/news-room/fact-sheets/detail/hepatitis-b.

5. World Health Organization. Herpes simplex virus World Health Organization Website: World Health Organization 2017 [updated 31/01/2017. Fact sheet]. Available from: https://www.who.int/en/news-room/fact-sheets/detail/herpes-simplex-virus.

6. World Health Organization. HIV/AIDS World Health Organization Website: World Health Organization; 2018 [updated 19/07/2018. Fact sheet]. Available from: https://www.who.int/en/news-room/fact-sheets/detail/hiv-aids.

7. Kirby Institute. HIV, viral hepatitis and sexually transmissible infections in Australia: Annual surveillance report 2018. Sydney: Kirby Institute, UNSW Sydney: Sydney: Kirby Institute, UNSW Sydney, Sydney: Kirby Institute US; 2018 2018.

8. Ward J, Goller J, Ali H, Bowring A, Couzos S, Saunders M, et al. Chlamydia among Australian Aboriginal and/or Torres Strait Islander people attending sexual health services, general practices and Aboriginal community controlled health services. BMC Health Serv Res. 2014;14:285.

9. O'Connor CC, Ali H, Guy RJ, Templeton DJ, Fairley CK, Chen MY, et al. High chlamydia positivity rates in Indigenous people attending Australian sexual health services. Med J Aust. 2014;200(10):595-8.

10. Louv WC, Austin H, Perlman J, Alexander WJ. Oral contraceptive use and the risk of chlamydial and gonococcal infections. Am J Obstet Gynecol. 1989;160(2):396-402.

11. Van Der Pol B, Unemo M. Chlamydial infections. In: Unemo M, Ballard R, Ison C, Lewis D, Ndowa F, Peeling R, editors. Laboratory diagnosis of sexually transmitted infections, including human immunodeficiency virus. Geneva: World Health Organization; 2013. p. 55-72.

12. Meyer T. Diagnostic Procedures to Detect Chlamydia trachomatis Infections. Microorganisms. 2016;4(3).

13. Kelly H, Coltart CEM, Pant Pai N, Klausner JD, Unemo M, Toskin I, et al. Systematic reviews of point-of-care tests for the diagnosis of urogenital Chlamydia trachomatis infections. Sex Transm Infect. 2017;93(S4):S22-S30.

14. World Health Organization. WHO guidelines for the treatment of Chlamydia trachomatis. 2016.

15. Australasian Society for HIV, Viral Hepatitis and Sexual Health Medicine,. Australian STI management guidelines for use in primary care: Chlamydia 2018 [updated 29/03/2018. Available from: http://www.sti.guidelines.org.au/sexually-transmissible-infections/chlamydia#follow-up.

16. Fifer H, Cole M, Hughes G, Padfield S, Smolarchuk C, Woodford N, et al. Sustained transmission of high-level azithromycin-resistant Neisseria gonorrhoeae in England: an observational study. Lancet Infect Dis. 2018;18(5):573-81.

17. World Health Organization. WHO guidelines for the treatment of Neisseria gonorrhoeae. 2016.

18. Sandoz KM, Rockey DD. Antibiotic resistance in Chlamydiae. Future Microbiol. 2010;5(9):1427-42.

19. Joseph SJ, Marti H, Didelot X, Read TD, Dean D. Tetracycline Selective Pressure and Homologous Recombination Shape the Evolution of Chlamydia suis: A Recently Identified Zoonotic Pathogen. Genome Biol Evol. 2016;8(8):2613-23.

20. Mestrovic T, Ljubin-Sternak S. Molecular mechanisms of Chlamydia trachomatis resistance to antimicrobial drugs. Front Biosci (Landmark Ed). 2018;23:656-70.

21. Elwell C, Mirrashidi K, Engel J. Chlamydia cell biology and pathogenesis. Nat Rev Microbiol. 2016;14(6):385-400.

22. Beagley KW, Timms P. Chlamydia trachomatis infection: incidence, health costs and prospects for vaccine development. J Reprod Immunol. 2000;48(1):47-68.

23. Haggerty CL, Gottlieb SL, Taylor BD, Low N, Xu F, Ness RB. Risk of sequelae after Chlamydia trachomatis genital infection in women. J Infect Dis. 2010;201 Suppl 2:S134-55.

24. Redgrove KA, McLaughlin EA. The Role of the Immune Response in Chlamydia trachomatis Infection of the Male Genital Tract: A Double-Edged Sword. Front Immunol. 2014;5:534.

25. Hillis SD, Owens LM, Marchbanks PA, Amsterdam LF, Mac Kenzie WR. Recurrent chlamydial infections increase the risks of hospitalization for ectopic pregnancy and pelvic inflammatory disease. Am J Obstet Gynecol. 1997;176(1 Pt 1):103-7.

26. Peipert JF. Clinical practice. Genital chlamydial infections. N Engl J Med. 2003;349(25):2424-30.

27. Shao R, Wang X, Wang W, Stener-Victorin E, Mallard C, Brannstrom M, et al. From mice to women and back again: causalities and clues for Chlamydia-induced tubal ectopic pregnancy. Fertil Steril. 2012;98(5):1175-85.

28. Zar HJ. Neonatal chlamydial infections: prevention and treatment. Paediatr Drugs. 2005;7(2):103-10.

29. Gencay M, Koskiniemi M, Fellman V, Ammala P, Vaheri A, Puolakkainen M. Chlamydia trachomatis infection in mothers with preterm delivery and in their newborn infants. APMIS. 2001;109(9):636-40.

30. Rours IG, Hammerschlag MR, Ott A, De Faber TJ, Verbrugh HA, de Groot R, et al. Chlamydia trachomatis as a cause of neonatal conjunctivitis in Dutch infants. Pediatrics. 2008;121(2):e321-6.

31. Bekler C, Kultursay N, Ozacar T, Sayiner A, Yalaz M, Akisu M. Chlamydial infections in term and preterm neonates. Jpn J Infect Dis. 2012;65(1):1-6.

32. Pendle S, Gowers A. Reactive arthritis associated with proctitis due to Chlamydia trachomatis serovar L2b. Sex Transm Dis. 2012;39(1):79-80.

33. Gaston JS. Immunological basis of Chlamydia induced reactive arthritis. Sex Transm Infect. 2000;76(3):156-61.

34. Buckner LR, Amedee AM, Albritton HL, Kozlowski PA, Lacour N, McGowin CL, et al. Chlamydia trachomatis Infection of Endocervical Epithelial Cells Enhances Early HIV Transmission Events. PLoS One. 2016;11(1):e0146663.

35. Madeleine MM, Anttila T, Schwartz SM, Saikku P, Leinonen M, Carter JJ, et al. Risk of cervical cancer associated with Chlamydia trachomatis antibodies by histology, HPV type and HPV cofactors. Int J Cancer. 2007;120(3):650-5.

36. Smith JS, Bosetti C, Munoz N, Herrero R, Bosch FX, Eluf-Neto J, et al. Chlamydia trachomatis and invasive cervical cancer: a pooled analysis of the IARC multicentric case-control study. Int J Cancer. 2004;111(3):431-9.

37. Smith JS, Munoz N, Herrero R, Eluf-Neto J, Ngelangel C, Franceschi S, et al. Evidence for Chlamydia trachomatis as a human papillomavirus cofactor in the etiology of invasive cervical cancer in Brazil and the Philippines. J Infect Dis. 2002;185(3):324-31.

38. Trabert B, Waterboer T, Idahl A, Brenner N, Brinton LA, Butt J, et al. Antibodies Against Chlamydia trachomatis and Ovarian Cancer Risk in Two Independent Populations. J Natl Cancer Inst. 2018.

39. Tuite AR, Jayaraman GC, Allen VG, Fisman DN. Estimation of the burden of disease and costs of genital Chlamydia trachomatis infection in Canada. Sex Transm Dis. 2012;39(4):260-7.

40. Shaw K, Coleman D, O'Sullivan M, Stephens N. Public health policies and management strategies for genital Chlamydia trachomatis infection. Risk Manag Healthc Policy. 2011;4:57-65.

41. Starnbach MN. Action Needed on Chlamydia Vaccines. Trends Microbiol. 2018;26(8):639-40.

42. Hafner LM, Wilson DP, Timms P. Development status and future prospects for a vaccine against Chlamydia trachomatis infection. Vaccine. 2014;32(14):1563-71.

43. Vasilevsky S, Greub G, Nardelli-Haefliger D, Baud D. Genital Chlamydia trachomatis: understanding the roles of innate and adaptive immunity in vaccine research. Clin Microbiol Rev. 2014;27(2):346-70.

44. Nunes A, Gomes JP. Evolution, phylogeny, and molecular epidemiology of Chlamydia. Infect Genet Evol. 2014;23:49-64.

45. Moulder JW. The relation of the psittacosis group (Chlamydiae) to bacteria and viruses. Annu Rev Microbiol. 1966;20:107-30.

46. Sachse K, Bavoil PM, Kaltenboeck B, Stephens RS, Kuo CC, Rossello-Mora R, et al. Emendation of the family Chlamydiaceae: proposal of a single genus, Chlamydia, to include all currently recognized species. Syst Appl Microbiol. 2015;38(2):99-103.

47. Bayramova F, Jacquier N, Greub G. Insight in the biology of Chlamydia-related bacteria. Microbes Infect. 2018;20(7-8):432-40.

48. Borel N, Polkinghorne A, Pospischil A. A Review on Chlamydial Diseases in Animals: Still a Challenge for Pathologists? Vet Pathol. 2018;55(3):374-90.

49. Miyairi I, Mahdi OS, Ouellette SP, Belland RJ, Byrne GI. Different growth rates of Chlamydia trachomatis biovars reflect pathotype. J Infect Dis. 2006;194(3):350-7.

50. Harris SR, Clarke IN, Seth-Smith HM, Solomon AW, Cutcliffe LT, Marsh P, et al. Whole-genome analysis of diverse Chlamydia trachomatis strains identifies phylogenetic relationships masked by current clinical typing. Nat Genet. 2012;44(4):413-9, S1.

51. Read TD, Brunham RC, Shen C, Gill SR, Heidelberg JF, White O, et al. Genome sequences of Chlamydia trachomatis MoPn and Chlamydia pneumoniae AR39. Nucleic Acids Res. 2000;28(6):1397-406.

52. Batteiger BE, Newhall WJt, Jones RB. Differences in outer membrane proteins of the lymphogranuloma venereum and trachoma biovars of Chlamydia trachomatis. Infect Immun. 1985;50(2):488-94.

53. Caldwell HD, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect Immun. 1981;31(3):1161-76.

54. Abdelsamed H, Peters J, Byrne GI. Genetic variation in Chlamydia trachomatis and their hosts: impact on disease severity and tissue tropism. Future Microbiol. 2013;8(9):1129-46.

55. Kari L, Whitmire WM, Carlson JH, Crane DD, Reveneau N, Nelson DE, et al. Pathogenic diversity among Chlamydia trachomatis ocular strains in nonhuman primates is affected by subtle genomic variations. J Infect Dis. 2008;197(3):449-56.

56. World Health Organization. Trachoma 2015 [Available from: http://www.who.int/mediacentre/factsheets/fs382/en/.

57. Hafner L, Beagley K, Timms P. Chlamydia trachomatis infection: host immune responses and potential vaccines. Mucosal Immunol. 2008;1(2):116-30.

58. Mabey D, Peeling RW. Lymphogranuloma venereum. Sex Transm Infect. 2002;78(2):90-2.

59. Ceovic R, Gulin SJ. Lymphogranuloma venereum: diagnostic and treatment challenges. Infect Drug Resist. 2015;8:39-47.

60. Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, et al. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science. 1998;282(5389):754-9.

61. Gong S, Yang Z, Lei L, Shen L, Zhong G. Characterization of Chlamydia trachomatis plasmid-encoded open reading frames. J Bacteriol. 2013;195(17):3819-26.

62. Song L, Carlson JH, Whitmire WM, Kari L, Virtaneva K, Sturdevant DE, et al. Chlamydia trachomatis plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. Infect Immun. 2013;81(3):636-44.

63. Yeow TC, Wong WF, Sabet NS, Sulaiman S, Shahhosseini F, Tan GM, et al. Prevalence of plasmid-bearing and plasmid-free Chlamydia trachomatis infection among women who visited obstetrics and gynecology clinics in Malaysia. BMC Microbiol. 2016;16:45.

64. Farencena A, Comanducci M, Donati M, Ratti G, Cevenini R. Characterization of a new isolate of Chlamydia trachomatis which lacks the common plasmid and has properties of biovar trachoma. Infect Immun. 1997;65(7):2965-9.

65. O'Connell CM, Ingalls RR, Andrews CW, Jr., Scurlock AM, Darville T. Plasmid-deficient Chlamydia muridarum fail to induce immune pathology and protect against oviduct disease. J Immunol. 2007;179(6):4027-34.

66. Olivares-Zavaleta N, Whitmire W, Gardner D, Caldwell HD. Immunization with the attenuated plasmidless Chlamydia trachomatis L2(25667R) strain provides partial protection in a murine model of female genitourinary tract infection. Vaccine. 2010;28(6):1454-62.

67. Ramsey KH, Schripsema JH, Smith BJ, Wang Y, Jham BC, O'Hagan KP, et al. Plasmid CDS5 influences infectivity and virulence in a mouse model of Chlamydia trachomatis urogenital infection. Infect Immun. 2014;82(8):3341-9.

68. Sigar IM, Schripsema JH, Wang Y, Clarke IN, Cutcliffe LT, Seth-Smith HM, et al. Plasmid deficiency in urogenital isolates of Chlamydia trachomatis reduces infectivity and virulence in a mouse model. Pathog Dis. 2014;70(1):61-9.

69. Sturdevant GL, Zhou B, Carlson JH, Whitmire WM, Song L, Caldwell HD. Infectivity of urogenital Chlamydia trachomatis plasmid-deficient, CT135-null, and double-deficient strains in female mice. Pathog Dis. 2014;71(1):90-2.

70. Lei L, Chen J, Hou S, Ding Y, Yang Z, Zeng H, et al. Reduced live organism recovery and lack of hydrosalpinx in mice infected with plasmid-free Chlamydia muridarum. Infect Immun. 2014;82(3):983-92.

71. Hou S, Yue L, Xu R, Zhu C, Shan S, Wang H, et al. Chlamydia muridarum plasmid induces mouse oviduct pathology by promoting chlamydial survival and ascending infection and triggering host inflammation. Eur J Dermatol. 2018;28(5):628-36.

72. Liu Y, Huang Y, Yang Z, Sun Y, Gong S, Hou S, et al. Plasmid-encoded Pgp3 is a major virulence factor for Chlamydia muridarum to induce hydrosalpinx in mice. Infect Immun. 2014;82(12):5327-35.

73. Huang Y, Sun Y, Qin T, Liu Y. The Structural Integrity of Plasmid-Encoded Pgp3 Is Essential for Induction of Hydrosalpinx by Chlamydia muridarum. Front Cell Infect Microbiol. 2019;9:13.

74. Li Z, Chen D, Zhong Y, Wang S, Zhong G. The chlamydial plasmid-encoded protein pgp3 is secreted into the cytosol of Chlamydia-infected cells. Infect Immun. 2008;76(8):3415-28.

75. Hou S, Dong X, Yang Z, Li Z, Liu Q, Zhong G. Chlamydial plasmid-encoded virulence factor Pgp3 neutralizes the antichlamydial activity of human cathelicidin LL-37. Infect Immun. 2015;83(12):4701-9.

76. Luan X, Peng B, Li Z, Tang L, Chen C, Chen L, et al. Vaccination with MIP or Pgp3 induces cross-serovar protection against chlamydial genital tract infection in mice. Immunobiology. 2019;224(2):223-30.

77. Donati M, Sambri V, Comanducci M, Di Leo K, Storni E, Giacani L, et al. DNA immunization with pgp3 gene of Chlamydia trachomatis inhibits the spread of chlamydial infection from the lower to the upper genital tract in C3H/HeN mice. Vaccine. 2003;21(11-12):1089-93.

78. Betts-Hampikian HJ, Fields KA. Disulfide bonding within components of the Chlamydia type III secretion apparatus correlates with development. J Bacteriol. 2011;193(24):6950-9.

79. Ingalls RR, Rice PA, Qureshi N, Takayama K, Lin JS, Golenbock DT. The inflammatory cytokine response to Chlamydia trachomatis infection is endotoxin mediated. Infect Immun. 1995;63(8):3125-30.

80. Abdelrahman YM, Belland RJ. The chlamydial developmental cycle. FEMS Microbiol Rev. 2005;29(5):949-59.

81. Wuppermann FN, Hegemann JH, Jantos CA. Heparan sulfate-like glycosaminoglycan is a cellular receptor for Chlamydia pneumoniae. J Infect Dis. 2001;184(2):181-7.

82. Fechtner T, Stallmann S, Moelleken K, Meyer KL, Hegemann JH. Characterization of the interaction between the chlamydial adhesin OmcB and the human host cell. J Bacteriol. 2013;195(23):5323-33.

83. Kim JH, Jiang S, Elwell CA, Engel JN. Chlamydia trachomatis co-opts the FGF2 signaling pathway to enhance infection. PLoS Pathog. 2011;7(10):e1002285.

84. Taraktchoglou M, Pacey AA, Turnbull JE, Eley A. Infectivity of Chlamydia trachomatis serovar LGV but not E is dependent on host cell heparan sulfate. Infect Immun. 2001;69(2):968-76.

85. Fadel S, Eley A. Differential glycosaminoglycan binding of Chlamydia trachomatis OmcB protein from serovars E and LGV. J Med Microbiol. 2008;57(Pt 9):1058-61.

86. Stephens RS, Poteralski JM, Olinger L. Interaction of Chlamydia trachomatis with mammalian cells is independent of host cell surface heparan sulfate glycosaminoglycans. Infect Immun. 2006;74(3):1795-9.

87. Conant CG, Stephens RS. Chlamydia attachment to mammalian cells requires protein disulfide isomerase. Cell Microbiol. 2007;9(1):222-32.

88. Panzetta ME, Valdivia RH, Saka HA. Chlamydia Persistence: A Survival Strategy to Evade Antimicrobial Effects in-vitro and in-vivo. Front Microbiol. 2018;9:3101.

89. Hogan RJ, Mathews SA, Mukhopadhyay S, Summersgill JT, Timms P. Chlamydial persistence: beyond the biphasic paradigm. Infect Immun. 2004;72(4):1843-55.

90. De Clercq E, Kalmar I, Vanrompay D. Animal models for studying female genital tract infection with Chlamydia trachomatis. Infect Immun. 2013;81(9):3060-7.

91. Batzoglou S, Pachter L, Mesirov JP, Berger B, Lander ES. Human and mouse gene structure: comparative analysis and application to exon prediction. Genome Res. 2000;10(7):950-8.

92. Kobayashi A, Behringer RR. Developmental genetics of the female reproductive tract in mammals. Nat Rev Genet. 2003;4(12):969-80.

93. Chen J, Zhang H, Zhou Z, Yang Z, Ding Y, Zhou Z, et al. Chlamydial induction of hydrosalpinx in 11 strains of mice reveals multiple host mechanisms for preventing upper genital tract pathology. PLoS One. 2014;9(4):e95076.

94. Morrison RP, Caldwell HD. Immunity to murine chlamydial genital infection. Infect Immun. 2002;70(6):2741-51.

95. Vicetti Miguel RD, Quispe Calla NE, Pavelko SD, Cherpes TL. Intravaginal Chlamydia trachomatis Challenge Infection Elicits TH1 and TH17 Immune Responses in Mice That Promote Pathogen Clearance and Genital Tract Damage. PLoS One. 2016;11(9):e0162445.

96. Gondek DC, Olive AJ, Stary G, Starnbach MN. CD4+ T cells are necessary and sufficient to confer protection against Chlamydia trachomatis infection in the murine upper genital tract. J Immunol. 2012;189(5):2441-9.

97. Shah AA, Schripsema JH, Imtiaz MT, Sigar IM, Kasimos J, Matos PG, et al. Histopathologic changes related to fibrotic oviduct occlusion after genital tract infection of mice with Chlamydia muridarum. Sex Transm Dis. 2005;32(1):49-56.

98. Barron AL, White HJ, Rank RG, Soloff BL, Moses EB. A new animal model for the study of Chlamydia trachomatis genital infections: infection of mice with the agent of mouse pneumonitis. J Infect Dis. 1981;143(1):63-6.

99. Lyons JM, Ito JI, Jr., Pena AS, Morre SA. Differences in growth characteristics and elementary body associated cytotoxicity between Chlamydia trachomatis oculogenital serovars D and H and Chlamydia muridarum. J Clin Pathol. 2005;58(4):397-401.

100. Morre SA, Lyons JM, Ito JI, Jr. Murine models of Chlamydia trachomatis genital tract infection: use of mouse pneumonitis strain versus human strains. Infect Immun. 2000;68(12):7209-11.

101. Ramsey KH, DeWolfe JL, Salyer RD. Disease outcome subsequent to primary and secondary urogenital infection with murine or human biovars of Chlamydia trachomatis. Infect Immun. 2000;68(12):7186-9.

102. Wira CR, Fahey JV, Rodriguez-Garcia M, Shen Z, Patel MV. Regulation of mucosal immunity in the female reproductive tract: the role of sex hormones in immune protection against sexually transmitted pathogens. Am J Reprod Immunol. 2014;72(2):236-58.

103. Wira CR, Fahey JV, Ghosh M, Patel MV, Hickey DK, Ochiel DO. Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. Am J Reprod Immunol. 2010;63(6):544-65.

104. Clark GF, Schust DJ. Manifestations of immune tolerance in the human female reproductive tract. Front Immunol. 2013;4:26.

105. Mirmonsef P, Gilbert D, Zariffard MR, Hamaker BR, Kaur A, Landay AL, et al. The effects of commensal bacteria on innate immune responses in the female genital tract. Am J Reprod Immunol. 2011;65(3):190-5.

106. Wira CR, Grant-Tschudy KS, Crane-Godreau MA. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. Am J Reprod Immunol. 2005;53(2):65-76.

107. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunol Rev. 2005;206:306-35.

108. Yasin B, Lehrer RI, Harwig SS, Wagar EA. Protegrins: structural requirements for inactivating elementary bodies of Chlamydia trachomatis. Infect Immun. 1996;64(11):4863-6.

109. Yasin B, Harwig SS, Lehrer RI, Wagar EA. Susceptibility of Chlamydia trachomatis to protegrins and defensins. Infect Immun. 1996;64(3):709-13.

110. Quayle AJ. The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. J Reprod Immunol. 2002;57(1-2):61-79.

111. Hou S, Sun X, Dong X, Lin H, Tang L, Xue M, et al. Chlamydial plasmid-encoded virulence factor Pgp3 interacts with human cathelicidin peptide LL-37 to modulate immune response. Microbes Infect. 2019;21(1):50-5.

112. Rasmussen SJ, Eckmann L, Quayle AJ, Shen L, Zhang YX, Anderson DJ, et al. Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis. J Clin Invest. 1997;99(1):77-87.

113. Lu H, Shen C, Brunham RC. Chlamydia trachomatis infection of epithelial cells induces the activation of caspase-1 and release of mature IL-18. J Immunol. 2000;165(3):1463-9.

114. Darville T, O'Neill JM, Andrews CW, Jr., Nagarajan UM, Stahl L, Ojcius DM. Toll-like receptor-2, but not Toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection. J Immunol. 2003;171(11):6187-97.

115. Derbigny WA, Kerr MS, Johnson RM. Pattern recognition molecules activated by Chlamydia muridarum infection of cloned murine oviduct epithelial cell lines. J Immunol. 2005;175(9):6065-75.

116. Derbigny WA, Johnson RM, Toomey KS, Ofner S, Jayarapu K. The Chlamydia muridarum-induced IFN-beta response is TLR3-dependent in murine oviduct epithelial cells. J Immunol. 2010;185(11):6689-97.

117. Kumar R, Gong H, Liu L, Ramos-Solis N, Seye CI, Derbigny WA. TLR3 deficiency exacerbates the loss of epithelial barrier function during genital tract Chlamydia muridarum infection. PLoS One. 2019;14(1):e0207422.

118. Carrasco SE, Hu S, Imai DM, Kumar R, Sandusky GE, Yang XF, et al. Toll-like receptor 3 (TLR3) promotes the resolution of Chlamydia muridarum genital tract infection in congenic C57BL/6N mice. PLoS One. 2018;13(4):e0195165.

119. Buchholz KR, Stephens RS. The cytosolic pattern recognition receptor NOD1 induces inflammatory interleukin-8 during Chlamydia trachomatis infection. Infect Immun. 2008;76(7):3150-5.

120. Hvid M, Baczynska A, Deleuran B, Fedder J, Knudsen HJ, Christiansen G, et al. Interleukin-1 is the initiator of Fallopian tube destruction during Chlamydia trachomatis infection. Cell Microbiol. 2007;9(12):2795-803.

121. Agrawal T, Gupta R, Dutta R, Srivastava P, Bhengraj AR, Salhan S, et al. Protective or pathogenic immune response to genital chlamydial infection in women--a possible role of cytokine secretion profile of cervical mucosal cells. Clin Immunol. 2009;130(3):347-54.

122. Tseng CT, Rank RG. Role of NK cells in early host response to chlamydial genital infection. Infect Immun. 1998;66(12):5867-75.

123. Hook CE, Telyatnikova N, Goodall JC, Braud VM, Carmichael AJ, Wills MR, et al. Effects of Chlamydia trachomatis infection on the expression of natural killer (NK) cell ligands and susceptibility to NK cell lysis. Clin Exp Immunol. 2004;138(1):54-60.

124. Mavoungou E, Poaty-Mavoungou V, Toure FS, Sall A, Delicat A, Yaba P, et al. Impairment of natural killer cell activity in Chlamydia trachomatis infected individuals. Trop Med Int Health. 1999;4(11):719-27.

125. Hook CE, Matyszak MK, Gaston JS. Infection of epithelial and dendritic cells by Chlamydia trachomatis results in IL-18 and IL-12 production, leading to interferon-gamma production by human natural killer cells. FEMS Immunol Med Microbiol. 2005;45(2):113-20.

126. Akira S. The role of IL-18 in innate immunity. Curr Opin Immunol. 2000;12(1):59-63.

127. Lijek RS, Helble JD, Olive AJ, Seiger KW, Starnbach MN. Pathology after Chlamydia trachomatis infection is driven by nonprotective immune cells that are distinct from protective populations. Proc Natl Acad Sci U S A. 2018;115(9):2216-21.

128. Darville T, Hiltke TJ. Pathogenesis of genital tract disease due to Chlamydia trachomatis. J Infect Dis. 2010;201 Suppl 2:S114-25.

129. Barteneva N, Theodor I, Peterson EM, de la Maza LM. Role of neutrophils in controlling early stages of a Chlamydia trachomatis infection. Infect Immun. 1996;64(11):4830-3.

130. Lehr S, Vier J, Hacker G, Kirschnek S. Activation of neutrophils by Chlamydia trachomatis-infected epithelial cells is modulated by the chlamydial plasmid. Microbes Infect. 2018;20(5):284-92.

131. Rajeeve K, Das S, Prusty BK, Rudel T. Chlamydia trachomatis paralyses neutrophils to evade the host innate immune response. Nat Microbiol. 2018;3(7):824-35.

132. Naglak EK, Morrison SG, Morrison RP. Neutrophils Are Central to Antibody-Mediated Protection against Genital Chlamydia. Infect Immun. 2017;85(10).

133. Frazer LC, O'Connell CM, Andrews CW, Jr., Zurenski MA, Darville T. Enhanced neutrophil longevity and recruitment contribute to the severity of oviduct pathology during Chlamydia muridarum infection. Infect Immun. 2011;79(10):4029-41.

134. Lee HY, Schripsema JH, Sigar IM, Murray CM, Lacy SR, Ramsey KH. A link between neutrophils and chronic disease manifestations of Chlamydia muridarum urogenital infection of mice. FEMS Immunol Med Microbiol. 2010;59(1):108-16.

135. Ramsey KH, Sigar IM, Schripsema JH, Shaba N, Cohoon KP. Expression of matrix metalloproteinases subsequent to urogenital Chlamydia muridarum infection of mice. Infect Immun. 2005;73(10):6962-73.

136. Imtiaz MT, Distelhorst JT, Schripsema JH, Sigar IM, Kasimos JN, Lacy SR, et al. A role for matrix metalloproteinase-9 in pathogenesis of urogenital Chlamydia muridarum infection in mice. Microbes Infect. 2007;9(14-15):1561-6.

137. Ault KA, Kelly KA, Ruther PE, Izzo AA, Izzo LS, Sigar IM, et al. Chlamydia trachomatis enhances the expression of matrix metalloproteinases in an in vitro model of the human fallopian tube infection. Am J Obstet Gynecol. 2002;187(5):1377-83.

138. Wiesenfeld HC, Heine RP, Krohn MA, Hillier SL, Amortegui AA, Nicolazzo M, et al. Association between elevated neutrophil defensin levels and endometritis. J Infect Dis. 2002;186(6):792-7.

139. Spencer LA, Weller PF. Eosinophils and Th2 immunity: contemporary insights. Immunol Cell Biol. 2010;88(3):250-6.

140. Vicetti Miguel RD, Quispe Calla NE, Dixon D, Foster RA, Gambotto A, Pavelko SD, et al. IL-4-secreting eosinophils promote endometrial stromal cell proliferation and prevent Chlamydia-induced upper genital tract damage. Proc Natl Acad Sci U S A. 2017.

141. Rajaram K, Nelson DE. Chlamydia muridarum infection of macrophages elicits bactericidal nitric oxide production via reactive oxygen species and cathepsin B. Infect Immun. 2015;83(8):3164-75.

142. Lausen M, Christiansen G, Bouet Guldbaek Poulsen T, Birkelund S. Immunobiology of monocytes and macrophages during Chlamydia trachomatis infection. Microbes Infect. 2019;21(2):73-84.

143. Al-Zeer MA, Al-Younes HM, Lauster D, Abu Lubad M, Meyer TF. Autophagy restricts Chlamydia trachomatis growth in human macrophages via IFNG-inducible guanylate binding proteins. Autophagy. 2013;9(1):50-62.

144. Sun HS, Eng EW, Jeganathan S, Sin AT, Patel PC, Gracey E, et al. Chlamydia trachomatis vacuole maturation in infected macrophages. J Leukoc Biol. 2012;92(4):815-27.

145. Tietzel I, Quayle AJ, Carabeo RA. Alternatively Activated Macrophages Are Host Cells for Chlamydia trachomatis and Reverse Anti-chlamydial Classically Activated Macrophages. Front Microbiol. 2019;10:919.

146. Gracey E, Lin A, Akram A, Chiu B, Inman RD. Intracellular survival and persistence of Chlamydia muridarum is determined by macrophage polarization. PLoS One. 2013;8(8):e69421.

147. Zuck M, Ellis T, Venida A, Hybiske K. Extrusions are phagocytosed and promote Chlamydia survival within macrophages. Cell Microbiol. 2017;19(4).

148. Zuck M, Sherrid A, Suchland R, Ellis T, Hybiske K. Conservation of extrusion as an exit mechanism for Chlamydia. Pathog Dis. 2016;74(7).

149. Prantner D, Darville T, Sikes JD, Andrews CW, Jr., Brade H, Rank RG, et al. Critical role for interleukin-1beta (IL-1beta) during Chlamydia muridarum genital infection and bacterial replication-independent secretion of IL-1beta in mouse macrophages. Infect Immun. 2009;77(12):5334-46.

150. Chen L, Liu X, Yu X, Ren R, Wang C, Zhao R, et al. Chlamydia muridarum Infection of Macrophages Stimulates IL-1beta Secretion and Cell Death via Activation of Caspase-1 in an RIP3-Independent Manner. Biomed Res Int. 2017;2017:1592365.

151. Lu H, Zhong G. Interleukin-12 production is required for chlamydial antigen-pulsed dendritic cells to induce protection against live Chlamydia trachomatis infection. Infect Immun. 1999;67(4):1763-9.

152. Agrawal T, Vats V, Wallace PK, Singh A, Salhan S, Mittal A. Recruitment of myeloid and plasmacytoid dendritic cells in cervical mucosa during Chlamydia trachomatis infection. Clin Microbiol Infect. 2009;15(1):50-9.

153. Moniz RJ, Chan AM, Gordon LK, Braun J, Arditi M, Kelly KA. Plasmacytoid dendritic cells modulate nonprotective T-cell responses to genital infection by Chlamydia muridarum. FEMS Immunol Med Microbiol. 2010;58(3):397-404.

154. Moniz RJ, Chan AM, Kelly KA. Identification of dendritic cell subsets responding to genital infection by Chlamydia muridarum. FEMS Immunol Med Microbiol. 2009;55(2):226-36.

155. Ojcius DM, Bravo de Alba Y, Kanellopoulos JM, Hawkins RA, Kelly KA, Rank RG, et al. Internalization of Chlamydia by dendritic cells and stimulation of Chlamydia-specific T cells. J Immunol. 1998;160(3):1297-303.

156. Kaiko GE, Phipps S, Hickey DK, Lam CE, Hansbro PM, Foster PS, et al. Chlamydia muridarum infection subverts dendritic cell function to promote Th2 immunity and airways hyperreactivity. J Immunol. 2008;180(4):2225-32.

157. Matyszak MK, Young JL, Gaston JS. Uptake and processing of Chlamydia trachomatis by human dendritic cells. Eur J Immunol. 2002;32(3):742-51.

158. Loomis WP, Starnbach MN. T cell responses to Chlamydia trachomatis. Curr Opin Microbiol. 2002;5(1):87-91.

159. Farris CM, Morrison SG, Morrison RP. CD4+ T cells and antibody are required for optimal major outer membrane protein vaccine-induced immunity to Chlamydia muridarum genital infection. Infect Immun. 2010;78(10):4374-83.

160. Li LX, McSorley SJ. B cells enhance antigen-specific CD4 T cell priming and prevent bacteria dissemination following Chlamydia muridarum genital tract infection. PLoS Pathog. 2013;9(10):e1003707.

161. Li W, Murthy AK, Guentzel MN, Seshu J, Forsthuber TG, Zhong G, et al. Antigen-specific CD4+ T cells produce sufficient IFN-gamma to mediate robust protective immunity against genital Chlamydia muridarum infection. J Immunol. 2008;180(5):3375-82.

162. Morrison SG, Su H, Caldwell HD, Morrison RP. Immunity to murine Chlamydia trachomatis genital tract reinfection involves B cells and CD4(+) T cells but not CD8(+) T cells. Infect Immun. 2000;68(12):6979-87.

163. Perry LL, Feilzer K, Caldwell HD. Immunity to Chlamydia trachomatis is mediated by T helper 1 cells through IFN-gamma-dependent and -independent pathways. J Immunol. 1997;158(7):3344-52.

164. Morrison RP, Feilzer K, Tumas DB. Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in Chlamydia trachomatis genital tract infection. Infect Immun. 1995;63(12):4661-8.

165. Bakshi RK, Gupta K, Jordan SJ, Chi X, Lensing SY, Press CG, et al. An Adaptive Chlamydia trachomatis-Specific IFN-gamma-Producing CD4(+) T Cell Response Is Associated With Protection Against Chlamydia Reinfection in Women. Front Immunol. 2018;9:1981.

166. Su H, Caldwell HD. CD4+ T cells play a significant role in adoptive immunity to Chlamydia trachomatis infection of the mouse genital tract. Infect Immun. 1995;63(9):3302-8.

167. Johansson M, Schon K, Ward M, Lycke N. Genital tract infection with Chlamydia trachomatis fails to induce protective immunity in gamma interferon receptor-deficient mice despite a strong local immunoglobulin A response. Infection and Immunity. 1997;65(3):1032-44.

168. Jordan SJ, Gupta K, Ogendi BMO, Bakshi RK, Kapil R, Press CG, et al. The Predominant CD4(+) Th1 Cytokine Elicited to Chlamydia trachomatis Infection in Women Is Tumor Necrosis Factor Alpha and Not Interferon Gamma. Clin Vaccine Immunol. 2017;24(4).

169. Ito JI, Lyons JM. Role of gamma interferon in controlling murine chlamydial genital tract infection. Infect Immun. 1999;67(10):5518-21.

170. Cotter TW, Ramsey KH, Miranpuri GS, Poulsen CE, Byrne GI. Dissemination of Chlamydia trachomatis chronic genital tract infection in gamma interferon gene knockout mice. Infect Immun. 1997;65(6):2145-52.

171. Rank RG, Ramsey KH, Pack EA, Williams DM. Effect of gamma interferon on resolution of murine chlamydial genital infection. Infect Immun. 1992;60(10):4427-9.

172. Ramsey KH, Sigar IM, Rana SV, Gupta J, Holland SM, Byrne GI. Role for inducible nitric oxide synthase in protection from chronic Chlamydia trachomatis urogenital disease in mice and its regulation by oxygen free radicals. Infect Immun. 2001;69(12):7374-9.

173. Naglak EK, Morrison SG, Morrison RP. IFNgamma is Required for Optimal Antibody-Mediated Immunity against Genital Chlamydia Infection. Infect Immun. 2016;84(11):3232-42.

174. Beatty WL, Belanger TA, Desai AA, Morrison RP, Byrne GI. Role of tryptophan in gamma interferon-mediated chlamydial persistence. Ann N Y Acad Sci. 1994;730:304-6.

175. Scurlock AM, Frazer LC, Andrews CW, Jr., O'Connell CM, Foote IP, Bailey SL, et al. Interleukin-17 contributes to generation of Th1 immunity and neutrophil recruitment during Chlamydia muridarum genital tract infection but is not required for macrophage influx or normal resolution of infection. Infect Immun. 2011;79(3):1349-62.

176. Vicetti Miguel RD, Harvey SA, LaFramboise WA, Reighard SD, Matthews DB, Cherpes TL. Human female genital tract infection by the obligate intracellular bacterium Chlamydia trachomatis elicits robust Type 2 immunity. PLoS One. 2013;8(3):e58565.

177. Asquith KL, Horvat JC, Kaiko GE, Carey AJ, Beagley KW, Hansbro PM, et al. Interleukin-13 promotes susceptibility to chlamydial infection of the respiratory and genital tracts. PLoS Pathog. 2011;7(5):e1001339.

178. Chen L, Lei L, Chang X, Li Z, Lu C, Zhang X, et al. Mice deficient in MyD88 Develop a Th2-dominant response and severe pathology in the upper genital tract following Chlamydia muridarum infection. J Immunol. 2010;184(5):2602-10.

179. Johnson RM, Yu H, Strank NO, Karunakaran K, Zhu Y, Brunham RC. B Cell Presentation of Chlamydia Antigen Selects Out Protective CD4gamma13 T Cells: Implications for Genital Tract Tissue-Resident Memory Lymphocyte Clusters. Infect Immun. 2018;86(2).

180. Moore-Connors JM, Fraser R, Halperin SA, Wang J. CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 responses and genital tract inflammation upon intracellular Chlamydia muridarum infection. J Immunol. 2013;191(6):3430-9.

181. Omosun Y, McKeithen D, Ryans K, Kibakaya C, Blas-Machado U, Li D, et al. Interleukin-10 modulates antigen presentation by dendritic cells through regulation of NLRP3 inflammasome assembly during Chlamydia infection. Infect Immun. 2015;83(12):4662-72.

182. Murthy AK, Li W, Chaganty BK, Kamalakaran S, Guentzel MN, Seshu J, et al. Tumor necrosis factor alpha production from CD8+ T cells mediates oviduct pathological sequelae following primary genital Chlamydia muridarum infection. Infect Immun. 2011;79(7):2928-35.

183. Vlcek KR, Li W, Manam S, Zanotti B, Nicholson BJ, Ramsey KH, et al. The contribution of Chlamydia-specific CD8(+) T cells to upper genital tract pathology. Immunol Cell Biol. 2016;94(2):208-12.

184. Magee DM, Williams DM, Smith JG, Bleicker CA, Grubbs BG, Schachter J, et al. Role of CD8 T cells in primary Chlamydia infection. Infect Immun. 1995;63(2):516-21.

185. Li LX, McSorley SJ. A re-evaluation of the role of B cells in protective immunity to Chlamydia infection. Immunol Lett. 2015;164(2):88-93.

186. Cotter TW, Meng Q, Shen ZL, Zhang YX, Su H, Caldwell HD. Protective efficacy of major outer membrane protein-specific immunoglobulin A (IgA) and IgG monoclonal antibodies in a murine model of Chlamydia trachomatis genital tract infection. Infect Immun. 1995;63(12):4704-14.

187. van den Broek IV, Land JA, van Bergen JE, Morre SA, van der Sande MA. Chlamydia trachomatis Antibody Testing in Vaginal Mucosal Material versus Blood Samples of Women Attending a Fertility Clinic and an STI Clinic. Obstet Gynecol Int. 2014;2014:601932.

188. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol. 2010;10(6):440-52.

189. da Silva EZ, Jamur MC, Oliver C. Mast cell function: a new vision of an old cell. J Histochem Cytochem. 2014;62(10):698-738.

190. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. 2014;14(7):478-94.

191. Krystel-Whittemore M, Dileepan KN, Wood JG. Mast Cell: A Multi-Functional Master Cell. Front Immunol. 2015;6:620.

192. Hallgren J, Gurish MF. Mast cell progenitor trafficking and maturation. Adv Exp Med Biol. 2011;716:14-28.

193. Dahlin JS, Hallgren J. Mast cell progenitors: origin, development and migration to tissues. Mol Immunol. 2015;63(1):9-17.

194. Kambe N, Miyachi Y. A possible mechanism of mast cell proliferation in mastocytosis. J Dermatol. 2002;29(1):1-9.

195. Ryan JJ, Kashyap M, Bailey D, Kennedy S, Speiran K, Brenzovich J, et al. Mast cell homeostasis: a fundamental aspect of allergic disease. Crit Rev Immunol. 2007;27(1):15-32.

196. Crivellato E, Ribatti D. The mast cell: an evolutionary perspective. Biol Rev Camb Philos Soc. 2010;85(2):347-60.

197. Marshall JS. Mast-cell responses to pathogens. Nat Rev Immunol. 2004;4(10):787-99.

198. Marshall JS, King CA, McCurdy JD. Mast cell cytokine and chemokine responses to bacterial and viral infection. Curr Pharm Des. 2003;9(1):11-24.

199. Douaiher J, Succar J, Lancerotto L, Gurish MF, Orgill DP, Hamilton MJ, et al. Development of mast cells and importance of their tryptase and chymase serine proteases in inflammation and wound healing. Adv Immunol. 2014;122:211-52.

200. Pejler G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. Blood. 2010;115(24):4981-90.

201. Dai H, Korthuis RJ. Mast Cell Proteases and Inflammation. Drug Discov Today Dis Models. 2011;8(1):47-55.

202. Stevens RL, Adachi R. Protease-proteoglycan complexes of mouse and human mast cells and importance of their beta-tryptase-heparin complexes in inflammation and innate immunity. Immunol Rev. 2007;217:155-67.

203. Wolters PJ, Pham CT, Muilenburg DJ, Ley TJ, Caughey GH. Dipeptidyl peptidase I is essential for activation of mast cell chymases, but not tryptases, in mice. J Biol Chem. 2001;276(21):18551-6.

204. Le QT, Gomez G, Zhao W, Hu J, Xia HZ, Fukuoka Y, et al. Processing of human protryptase in mast cells involves cathepsins L, B, and C. J Immunol. 2011;187(4):1912-8.

205. Heutinck KM, ten Berge IJ, Hack CE, Hamann J, Rowshani AT. Serine proteases of the human immune system in health and disease. Mol Immunol. 2010;47(11-12):1943-55.

206. Hedstrom L. Serine protease mechanism and specificity. Chem Rev. 2002;102(12):4501-24.

207. Song J, Tan H, Boyd SE, Shen H, Mahmood K, Webb GI, et al. Bioinformatic approaches for predicting substrates of proteases. J Bioinform Comput Biol. 2011;9(1):149-78.

208. Reimer J. Hematopoietic Serine Proteases from the Mast Cell Chymase and Tryptase Loci - a Functional and Evolutionary Analysis. Uppsala (Sweden): Uppsala University; 2008.

209. Karlson U, Pejler G, Tomasini-Johansson B, Hellman L. Extended substrate specificity of rat mast cell protease 5, a rodent alpha-chymase with elastase-like primary specificity. J Biol Chem. 2003;278(41):39625-31.

210. Kunori Y, Koizumi M, Masegi T, Kasai H, Kawabata H, Yamazaki Y, et al. Rodent alpha-chymases are elastase-like proteases. Eur J Biochem. 2002;269(23):5921-30.

211. Lyons JJ, Yu X, Hughes JD, Le QT, Jamil A, Bai Y, et al. Elevated basal serum tryptase identifies a multisystem disorder associated with increased TPSAB1 copy number. Nat Genet. 2016;48(12):1564-9.

212. Wong GW, Tang Y, Feyfant E, Sali A, Li L, Li Y, et al. Identification of a new member of the tryptase family of mouse and human mast cell proteases which possesses a novel COOH-terminal hydrophobic extension. J Biol Chem. 1999;274(43):30784-93.

213. Payne V, Kam PC. Mast cell tryptase: a review of its physiology and clinical significance. Anaesthesia. 2004;59(7):695-703.

214. Harris JL, Niles A, Burdick K, Maffitt M, Backes BJ, Ellman JA, et al. Definition of the extended substrate specificity determinants for beta-tryptases I and II. J Biol Chem. 2001;276(37):34941-7.

215. Fukuoka Y, Schwartz LB. Human beta-tryptase: detection and characterization of the active monomer and prevention of tetramer reconstitution by protease inhibitors. Biochemistry. 2004;43(33):10757-64.

216. Pereira PJ, Bergner A, Macedo-Ribeiro S, Huber R, Matschiner G, Fritz H, et al. Human beta-tryptase is a ring-like tetramer with active sites facing a central pore. Nature. 1998;392(6673):306-11.

217. Huang C, Li L, Krilis SA, Chanasyk K, Tang Y, Li Z, et al. Human tryptases alpha and beta/II are functionally distinct due, in part, to a single amino acid difference in one of the surface loops that forms the substrate-binding cleft. J Biol Chem. 1999;274(28):19670-6.

218. Soto D, Malmsten C, Blount JL, Muilenburg DJ, Caughey GH. Genetic deficiency of human mast cell alpha-tryptase. Clin Exp Allergy. 2002;32(7):1000-6.

219. Pallaoro M, Fejzo MS, Shayesteh L, Blount JL, Caughey GH. Characterization of genes encoding known and novel human mast cell tryptases on chromosome 16p13.3. J Biol Chem. 1999;274(6):3355-62.

220. Wang HW, McNeil HP, Husain A, Liu K, Tedla N, Thomas PS, et al. Delta tryptase is expressed in multiple human tissues, and a recombinant form has proteolytic activity. J Immunol. 2002;169(9):5145-52.

221. Ghildyal N, Friend DS, Stevens RL, Austen KF, Huang C, Penrose JF, et al. Fate of two mast cell tryptases in V3 mastocytosis and normal BALB/c mice undergoing passive systemic anaphylaxis: prolonged retention of exocytosed mMCP-6 in connective tissues, and rapid accumulation of enzymatically active mMCP-7 in the blood. J Exp Med. 1996;184(3):1061-73.

222. Huang C, Wong GW, Ghildyal N, Gurish MF, Sali A, Matsumoto R, et al. The tryptase, mouse mast cell protease 7, exhibits anticoagulant activity in vivo and in vitro due to its ability to degrade fibrinogen in the presence of the diverse array of protease inhibitors in plasma. J Biol Chem. 1997;272(50):31885-93.

223. Matsumoto R, Sali A, Ghildyal N, Karplus M, Stevens RL. Packaging of proteases and proteoglycans in the granules of mast cells and other hematopoietic cells. A cluster of histidines on mouse mast cell protease 7 regulates its binding to heparin serglycin proteoglycans. J Biol Chem. 1995;270(33):19524-31.

224. Caughey GH, Raymond WW, Blount JL, Hau LW, Pallaoro M, Wolters PJ, et al. Characterization of human gamma-tryptases, novel members of the chromosome 16p mast cell tryptase and prostasin gene families. J Immunol. 2000;164(12):6566-75.

225. Yuan J, Beltman J, Gjerstad E, Nguyen MT, Sampang J, Chan H, et al. Expression and characterization of recombinant gamma-tryptase. Protein Expr Purif. 2006;49(1):47-54.

226. Wong GW, Foster PS, Yasuda S, Qi JC, Mahalingam S, Mellor EA, et al. Biochemical and functional characterization of human transmembrane tryptase (TMT)/tryptase gamma. TMT is an exocytosed mast cell protease that induces airway hyperresponsiveness in vivo via an interleukin-13/interleukin-4 receptor alpha/signal transducer and activator of transcription (STAT) 6-dependent pathway. J Biol Chem. 2002;277(44):41906-15.

227. Hunt JE, Stevens RL, Austen KF, Zhang J, Xia Z, Ghildyal N. Natural disruption of the mouse mast cell protease 7 gene in the C57BL/6 mouse. J Biol Chem. 1996;271(5):2851-5.

228. Caughey GH. Mast cell proteases as pharmacological targets. Eur J Pharmacol. 2016;778:44-55.

229. Caughey GH, Schaumberg TH, Zerweck EH, Butterfield JH, Hanson RD, Silverman GA, et al. The human mast cell chymase gene (CMA1): mapping to the cathepsin G/granzyme gene cluster and lineage-restricted expression. Genomics. 1993;15(3):614-20.

230. Gallwitz M, Hellman L. Rapid lineage-specific diversification of the mast cell chymase locus during mammalian evolution. Immunogenetics. 2006;58(8):641-54.

231. Gallwitz M, Reimer JM, Hellman L. Expansion of the mast cell chymase locus over the past 200 million years of mammalian evolution. Immunogenetics. 2006;58(8):655-69.

232. Karlson U, Pejler G, Froman G, Hellman L. Rat mast cell protease 4 is a beta-chymase with unusually stringent substrate recognition profile. J Biol Chem. 2002;277(21):18579-85.

233. Tchougounova E, Pejler G, Abrink M. The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover. J Exp Med. 2003;198(3):423-31.

234. Andersson MK, Enoksson M, Gallwitz M, Hellman L. The extended substrate specificity of the human mast cell chymase reveals a serine protease with well-defined substrate recognition profile. Int Immunol. 2009;21(1):95-104.

235. Pejler G, Knight SD, Henningsson F, Wernersson S. Novel insights into the biological function of mast cell carboxypeptidase A. Trends Immunol. 2009;30(8):401-8.

236. Gomez-Ortiz M, Gomis-Ruth FX, Huber R, Aviles FX. Inhibition of carboxypeptidase A by excess zinc: analysis of the structural determinants by X-ray crystallography. FEBS Lett. 1997;400(3):336-40.

237. Lilla JN, Chen CC, Mukai K, BenBarak MJ, Franco CB, Kalesnikoff J, et al. Reduced mast cell and basophil numbers and function in Cpa3-Cre; Mcl-1fl/fl mice. Blood. 2011;118(26):6930-8.

238. Springman EB, Dikov MM, Serafin WE. Mast cell procarboxypeptidase A. Molecular modeling and biochemical characterization of its processing within secretory granules. J Biol Chem. 1995;270(3):1300-7.

239. Stevens RL, McNeil HP, Wensing LA, Shin K, Wong GW, Hansbro PM, et al. Experimental Arthritis Is Dependent on Mouse Mast Cell Protease-5. J Biol Chem. 2017;292(13):5392-404.

240. Feyerabend TB, Hausser H, Tietz A, Blum C, Hellman L, Straus AH, et al. Loss of histochemical identity in mast cells lacking carboxypeptidase A. Mol Cell Biol. 2005;25(14):6199-210.

241. Ronnberg E, Melo FR, Pejler G. Mast cell proteoglycans. J Histochem Cytochem. 2012;60(12):950-62.

242. Abrink M, Grujic M, Pejler G. Serglycin is essential for maturation of mast cell secretory granule. J Biol Chem. 2004;279(39):40897-905.

243. Duelli A, Ronnberg E, Waern I, Ringvall M, Kolset SO, Pejler G. Mast cell differentiation and activation is closely linked to expression of genes coding for the serglycin proteoglycan core protein and a distinct set of chondroitin sulfate and heparin sulfotransferases. J Immunol. 2009;183(11):7073-83.

244. Humphries DE, Wong GW, Friend DS, Gurish MF, Qiu WT, Huang C, et al. Heparin is essential for the storage of specific granule proteases in mast cells. Nature. 1999;400(6746):769-72.

245. Forsberg E, Pejler G, Ringvall M, Lunderius C, Tomasini-Johansson B, Kusche-Gullberg M, et al. Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. Nature. 1999;400(6746):773-6.

246. Deligny A, Dierker T, Dagalv A, Lundequist A, Eriksson I, Nairn AV, et al. NDST2 (N-Deacetylase/N-Sulfotransferase-2) Enzyme Regulates Heparan Sulfate Chain Length. J Biol Chem. 2016;291(36):18600-7.

247. Ledin J, Ringvall M, Thuveson M, Eriksson I, Wilen M, Kusche-Gullberg M, et al. Enzymatically active N-deacetylase/N-sulfotransferase-2 is present in liver but does not contribute to heparan sulfate N-sulfation. J Biol Chem. 2006;281(47):35727-34.

248. Pallerla SR, Lawrence R, Lewejohann L, Pan Y, Fischer T, Schlomann U, et al. Altered heparan sulfate structure in mice with deleted NDST3 gene function. J Biol Chem. 2008;283(24):16885-94.

249. Aikawa J, Grobe K, Tsujimoto M, Esko JD. Multiple isozymes of heparan sulfate/heparin GlcNAc N-deacetylase/GlcN N-sulfotransferase. Structure and activity of the fourth member, NDST4. J Biol Chem. 2001;276(8):5876-82.

250. Lencz T, Guha S, Liu C, Rosenfeld J, Mukherjee S, DeRosse P, et al. Genome-wide association study implicates NDST3 in schizophrenia and bipolar disorder. Nat Commun. 2013;4:2739.

251. Knight PA, Brown JK, Pemberton AD. Innate immune response mechanisms in the intestinal epithelium: potential roles for mast cells and goblet cells in the expulsion of adult Trichinella spiralis. Parasitology. 2008;135(6):655-70.

252. Xing W, Austen KF, Gurish MF, Jones TG. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. Proc Natl Acad Sci U S A. 2011;108(34):14210-5.

253. Sutherland RE, Olsen JS, McKinstry A, Villalta SA, Wolters PJ. Mast cell IL-6 improves survival from Klebsiella pneumonia and sepsis by enhancing neutrophil killing. J Immunol. 2008;181(8):5598-605.

254. Shelburne CP, Nakano H, St John AL, Chan C, McLachlan JB, Gunn MD, et al. Mast cells augment adaptive immunity by orchestrating dendritic cell trafficking through infected tissues. Cell Host Microbe. 2009;6(4):331-42.

255. Siebenhaar F, Syska W, Weller K, Magerl M, Zuberbier T, Metz M, et al. Control of Pseudomonas aeruginosa skin infections in mice is mast cell-dependent. Am J Pathol. 2007;170(6):1910-6.

256. Xu X, Zhang D, Lyubynska N, Wolters PJ, Killeen NP, Baluk P, et al. Mast cells protect mice from Mycoplasma pneumonia. Am J Respir Crit Care Med. 2006;173(2):219-25.

257. Jiang AP, Jiang JF, Wei JF, Guo MG, Qin Y, Guo QQ, et al. Human Mucosal Mast Cells Capture HIV-1 and Mediate Viral trans-Infection of CD4+ T Cells. J Virol. 2015;90(6):2928-37.

258. Graham AC, Hilmer KM, Zickovich JM, Obar JJ. Inflammatory response of mast cells during influenza A virus infection is mediated by active infection and RIG-I signaling. J Immunol. 2013;190(9):4676-84.

259. Cruz A, Mendes EA, de Andrade MV, do Nascimento VC, Cartelle CT, Arantes RM, et al. Mast cells are crucial in the resistance against Toxoplasma gondii oral infection. Eur J Immunol. 2014;44(10):2949-54.

260. Dudeck A, Suender CA, Kostka SL, von Stebut E, Maurer M. Mast cells promote Th1 and Th17 responses by modulating dendritic cell maturation and function. Eur J Immunol. 2011;41(7):1883-93.

261. Furuta T, Kikuchi T, Iwakura Y, Watanabe N. Protective roles of mast cells and mast cell-derived TNF in murine malaria. J Immunol. 2006;177(5):3294-302.

262. Chiba N, Shimada K, Chen S, Jones HD, Alsabeh R, Slepenkin AV, et al. Mast cells play an important role in chlamydia pneumoniae lung infection by facilitating immune cell recruitment into the airway. J Immunol. 2015;194(8):3840-51.

263. Urb M, Sheppard DC. The role of mast cells in the defence against pathogens. PLoS Pathog. 2012;8(4):e1002619.

264. Reher TM, Brunskole I, Neumann D, Seifert R. Evidence for ligand-specific conformations of the histamine H(2)-receptor in human eosinophils and neutrophils. Biochem Pharmacol. 2012;84(9):1174-85.

265. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med. 2001;193(2):255-61.

266. Kondeti V, Al-Azzam N, Duah E, Thodeti CK, Boyce JA, Paruchuri S. Leukotriene D4 and prostaglandin E2 signals synergize and potentiate vascular inflammation in a mast cell-dependent manner through cysteinyl leukotriene receptor 1 and E-prostanoid receptor 3. J Allergy Clin Immunol. 2016;137(1):289-98.

267. Peters-Golden M, Canetti C, Mancuso P, Coffey MJ. Leukotrienes: underappreciated mediators of innate immune responses. J Immunol. 2005;174(2):589-94.

268. Ashina K, Tsubosaka Y, Nakamura T, Omori K, Kobayashi K, Hori M, et al. Histamine Induces Vascular Hyperpermeability by Increasing Blood Flow and Endothelial Barrier Disruption In Vivo. PLoS One. 2015;10(7):e0132367.

269. Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, et al. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. Blood. 2013;121(6):1008-15.

270. Kubes P, Gaboury JP. Rapid mast cell activation causes leukocyte-dependent and -independent permeability alterations. Am J Physiol. 1996;271(6 Pt 2):H2438-46.

271. Haribabu B, Verghese MW, Steeber DA, Sellars DD, Bock CB, Snyderman R. Targeted disruption of the leukotriene B(4) receptor in mice reveals its role in inflammation and platelet-activating factor-induced anaphylaxis. J Exp Med. 2000;192(3):433-8.

272. Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. Nature. 1996;381(6577):77-80.

273. Malaviya R, Abraham SN. Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis. J Leukoc Biol. 2000;67(6):841-6.

274. Thakurdas SM, Melicoff E, Sansores-Garcia L, Moreira DC, Petrova Y, Stevens RL, et al. The mast cell-restricted tryptase mMCP-6 has a critical immunoprotective role in bacterial infections. J Biol Chem. 2007;282(29):20809-15.

275. Shin K, Watts GF, Oettgen HC, Friend DS, Pemberton AD, Gurish MF, et al. Mouse mast cell tryptase mMCP-6 is a critical link between adaptive and innate immunity in the chronic phase of Trichinella spiralis infection. J Immunol. 2008;180(7):4885-91.

276. Carlos D, de Souza Junior DA, de Paula L, Jamur MC, Oliver C, Ramos SG, et al. Mast cells modulate pulmonary acute inflammation and host defense in a murine model of tuberculosis. J Infect Dis. 2007;196(9):1361-8.

277. Vancheri C, Mastruzzo C, Armato F, Tomaselli V, Magri S, Pistorio MP, et al. Intranasal heparin reduces eosinophil recruitment after nasal allergen challenge in patients with allergic rhinitis. J Allergy Clin Immunol. 2001;108(5):703-8.

278. Teixeira MM, Hellewell PG. Suppression by intradermal administration of heparin of eosinophil accumulation but not oedema formation in inflammatory reactions in guinea-pig skin. Br J Pharmacol. 1993;110(4):1496-500.

279. Bulfone-Paus S, Bahri R. Mast Cells as Regulators of T Cell Responses. Front Immunol. 2015;6:394.

280. Malaviya R, Twesten NJ, Ross EA, Abraham SN, Pfeifer JD. Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. J Immunol. 1996;156(4):1490-6.

281. Dudeck J, Ghouse SM, Lehmann CH, Hoppe A, Schubert N, Nedospasov SA, et al. Mast-Cell-Derived TNF Amplifies CD8(+) Dendritic Cell Functionality and CD8(+) T Cell Priming. Cell Rep. 2015;13(2):399-411.

282. Dawicki W, Jawdat DW, Xu N, Marshall JS. Mast cells, histamine, and IL-6 regulate the selective influx of dendritic cell subsets into an inflamed lymph node. J Immunol. 2010;184(4):2116-23.

283. Mazzoni A, Young HA, Spitzer JH, Visintin A, Segal DM. Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. J Clin Invest. 2001;108(12):1865-73.

284. Mazzoni A, Siraganian RP, Leifer CA, Segal DM. Dendritic cell modulation by mast cells controls the Th1/Th2 balance in responding T cells. J Immunol. 2006;177(6):3577-81.

285. Kitawaki T, Kadowaki N, Sugimoto N, Kambe N, Hori T, Miyachi Y, et al. IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells. Int Immunol. 2006;18(12):1789-99.

286. Caron G, Delneste Y, Roelandts E, Duez C, Bonnefoy JY, Pestel J, et al. Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. J Immunol. 2001;167(7):3682-6.

287. Napolitani G, Acosta-Rodriguez EV, Lanzavecchia A, Sallusto F. Prostaglandin E2 enhances Th17 responses via modulation of IL-17 and IFN-gamma production by memory CD4+ T cells. Eur J Immunol. 2009;39(5):1301-12.

288. Mommert S, Gschwandtner M, Koether B, Gutzmer R, Werfel T. Human memory Th17 cells express a functional histamine H4 receptor. Am J Pathol. 2012;180(1):177-85.

289. Palm AK, Garcia-Faroldi G, Lundberg M, Pejler G, Kleinau S. Activated mast cells promote differentiation of B cells into effector cells. Sci Rep. 2016;6:20531.

290. Merluzzi S, Frossi B, Gri G, Parusso S, Tripodo C, Pucillo C. Mast cells enhance proliferation of B lymphocytes and drive their differentiation toward IgA-secreting plasma cells. Blood. 2010;115(14):2810-7.

291. Di Nardo A, Yamasaki K, Dorschner RA, Lai Y, Gallo RL. Mast cell cathelicidin antimicrobial peptide prevents invasive group A Streptococcus infection of the skin. J Immunol. 2008;180(11):7565-73.

292. Malaviya R, Ross EA, MacGregor JI, Ikeda T, Little JR, Jakschik BA, et al. Mast cell phagocytosis of FimH-expressing enterobacteria. J Immunol. 1994;152(4):1907-14.

293. Brooks AC, Whelan CJ, Purcell WM. Reactive oxygen species generation and histamine release by activated mast cells: modulation by nitric oxide synthase inhibition. Br J Pharmacol. 1999;128(3):585-90.

294. von Kockritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, et al. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood. 2008;111(6):3070-80.

295. Hsieh JT, Rathore APS, Soundarajan G, St John AL. Japanese encephalitis virus neuropenetrance is driven by mast cell chymase. Nat Commun. 2019;10(1):706.

296. Bankova LG, Lezcano C, Pejler G, Stevens RL, Murphy GF, Austen KF, et al. Mouse mast cell proteases 4 and 5 mediate epidermal injury through disruption of tight junctions. J Immunol. 2014;192(6):2812-20.

297. Younan G, Suber F, Xing W, Shi T, Kunori Y, Abrink M, et al. The inflammatory response after an epidermal burn depends on the activities of mouse mast cell proteases 4 and 5. J Immunol. 2010;185(12):7681-90.

298. Guttman JA, Finlay BB. Tight junctions as targets of infectious agents. Biochim Biophys Acta. 2009;1788(4):832-41.

299. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. Nat Rev Mol Cell Biol. 2016;17(9):564-80.

300. Knight PA, Wright SH, Lawrence CE, Paterson YY, Miller HR. Delayed expulsion of the nematode Trichinella spiralis in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. J Exp Med. 2000;192(12):1849-56.

301. Lawrence CE, Paterson YY, Wright SH, Knight PA, Miller HR. Mouse mast cell protease-1 is required for the enteropathy induced by gastrointestinal helminth infection in the mouse. Gastroenterology. 2004;127(1):155-65.

302. Deane A. The Role of Mast Cell Proteases in Respiratory Diseases. Newcastle (Australia): University of Newcastle (Australia); 2016.

303. Prieto-Garcia A, Zheng D, Adachi R, Xing W, Lane WS, Chung K, et al. Mast cell restricted mouse and human tryptase.heparin complexes hinder thrombin-induced coagulation of plasma and the generation of fibrin by proteolytically destroying fibrinogen. J Biol Chem. 2012;287(11):7834-44.

304. Adair-Kirk TL, Senior RM. Fragments of extracellular matrix as mediators of inflammation. Int J Biochem Cell Biol. 2008;40(6-7):1101-10.

305. Gendrin C, Shubin NJ, Boldenow E, Merillat S, Clauson M, Power D, et al. Mast cell chymase decreases the severity of group B Streptococcus infections. J Allergy Clin Immunol. 2018;142(1):120-9 e6.

306. Beghdadi W, Madjene LC, Claver J, Pejler G, Beaudoin L, Lehuen A, et al. Mast cell chymase protects against renal fibrosis in murine unilateral ureteral obstruction. Kidney Int. 2013;84(2):317-26.

307. Piliponsky AM, Chen CC, Rios EJ, Treuting PM, Lahiri A, Abrink M, et al. The chymase mouse mast cell protease 4 degrades TNF, limits inflammation, and promotes survival in a model of sepsis. Am J Pathol. 2012;181(3):875-86.

308. Omoto Y, Tokime K, Yamanaka K, Habe K, Morioka T, Kurokawa I, et al. Human mast cell chymase cleaves pro-IL-18 and generates a novel and biologically active IL-18 fragment. J Immunol. 2006;177(12):8315-9.

309. Waern I, Lundequist A, Pejler G, Wernersson S. Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation. Mucosal Immunol. 2013;6(5):911-20.

310. Nelissen S, Vangansewinkel T, Geurts N, Geboes L, Lemmens E, Vidal PM, et al. Mast cells protect from post-traumatic spinal cord damage in mice by degrading inflammation-associated cytokines via mouse mast cell protease 4. Neurobiol Dis. 2014;62:260-72.

311. Desbiens L, Lapointe C, Gendron L, Gharagozloo M, Vincent L, Pejler G, et al. Experimental autoimmune encephalomyelitis potentiates mouse mast cell protease 4-dependent pressor responses to centrally or systemically administered big endothelin-1. J Pharmacol Exp Ther. 2019.

312. Choi HW, Bowen SE, Miao Y, Chan CY, Miao EA, Abrink M, et al. Loss of Bladder Epithelium Induced by Cytolytic Mast Cell Granules. Immunity. 2016;45(6):1258-69.

313. Tchougounova E, Lundequist A, Fajardo I, Winberg JO, Abrink M, Pejler G. A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2. J Biol Chem. 2005;280(10):9291-6.

314. Magarinos NJ, Bryant KJ, Fosang AJ, Adachi R, Stevens RL, McNeil HP. Mast cell-restricted, tetramer-forming tryptases induce aggrecanolysis in articular cartilage by activating matrix metalloproteinase-3 and -13 zymogens. J Immunol. 2013;191(3):1404-12.

315. Lin L, Bankaitis E, Heimbach L, Li N, Abrink M, Pejler G, et al. Dual targets for mouse mast cell protease-4 in mediating tissue damage in experimental bullous pemphigoid. J Biol Chem. 2011;286(43):37358-67.

316. Akahoshi M, Song CH, Piliponsky AM, Metz M, Guzzetta A, Abrink M, et al. Mast cell chymase reduces the toxicity of Gila monster venom, scorpion venom, and vasoactive intestinal polypeptide in mice. J Clin Invest. 2011;121(10):4180-91.

317. Schneider LA, Schlenner SM, Feyerabend TB, Wunderlin M, Rodewald HR. Molecular mechanism of mast cell mediated innate defense against endothelin and snake venom sarafotoxin. J Exp Med. 2007;204(11):2629-39.

318. De Leo B, Esnal-Zufiaurre A, Collins F, Critchley HOD, Saunders PTK. Immunoprofiling of human uterine mast cells identifies three phenotypes and expression of ERbeta and glucocorticoid receptor. F1000Res. 2017;6:667.

319. Drudy L, Sheppard BL, Bonnar J. The ultrastructure of mast cells in the uterus throughout the normal menstrual cycle and the postmenopause. J Anat. 1991;175:51-63.

320. Mori A, Zhai YL, Toki T, Nikaido T, Fujii S. Distribution and heterogeneity of mast cells in the human uterus. Hum Reprod. 1997;12(2):368-72.

321. Kalyani R, Rajeshwari G. Significance of Mast cells in Non-neoplastic and Neoplastic lesions of Uterine Cervix. Biomed Res Ther. 2016;3(1):469-75.

322. Cabanillas-Saez A, Schalper JA, Nicovani SM, Rudolph MI. Characterization of mast cells according to their content of tryptase and chymase in normal and neoplastic human uterine cervix. Int J Gynecol Cancer. 2002;12(1):92-8.

323. Valle GR, Castro AS, Nogueira JC, Caliari MV, Graça DS, Nascimento EF. Eosinophils and mast cells in the oviduct of heifers under natural and superovulated estrous cycles. Anim Reprod. 2009;6(2):386-91.

324. Walter J, Klein C, Wehrend A. Distribution of mast cells in vaginal, cervical and uterine tissue of non-pregnant mares: investigations on correlations with ovarian steroids. Reprod Domest Anim. 2012;47(2):e29-31.

325. Karaca T, Arikan S, Kalender H, Yoruk M. Distribution and heterogeneity of mast cells in female reproductive tract and ovary on different days of the oestrus cycle in Angora goats. Reprod Domest Anim. 2008;43(4):451-6.

326. Karaca T, Yoruk M, Uslu S, Cetin Y, Uslu BA. Distribution of eosinophil granulocytes and mast cells in the reproductive tract of female goats in the preimplantation phase. Vet Res Commun. 2009;33(6):545-54.

327. Brandon JM, Evans JE. Changes in uterine mast cells during the estrous cycle in the Syrian hamster. Am J Anat. 1983;167(2):241-7.

328. Salamonsen LA, Jeziorska M, Newlands GF, Dey SK, Woolley DE. Evidence against a significant role for mast cells in blastocyst implantation in the rat and mouse. Reprod Fertil Dev. 1996;8(8):1157-64.

329. Woidacki K, Popovic M, Metz M, Schumacher A, Linzke N, Teles A, et al. Mast cells rescue implantation defects caused by c-kit deficiency. Cell Death Dis. 2013;4:e462.

330. Woidacki K, Meyer N, Schumacher A, Goldschmidt A, Maurer M, Zenclussen AC. Transfer of regulatory T cells into abortion-prone mice promotes the expansion of uterine mast cells and normalizes early pregnancy angiogenesis. Sci Rep. 2015;5:13938.

331. Meyer N, Woidacki K, Maurer M, Zenclussen AC. Safeguarding of Fetal Growth by Mast Cells and Natural Killer Cells: Deficiency of One Is Counterbalanced by the Other. Front Immunol. 2017;8:711.

332. Jensen F, Woudwyk M, Teles A, Woidacki K, Taran F, Costa S, et al. Estradiol and progesterone regulate the migration of mast cells from the periphery to the uterus and induce their maturation and degranulation. PLoS One. 2010;5(12):e14409.

333. Zaitsu M, Narita S, Lambert KC, Grady JJ, Estes DM, Curran EM, et al. Estradiol activates mast cells via a non-genomic estrogen receptor-alpha and calcium influx. Mol Immunol. 2007;44(8):1977-85.

334. Engemise SL, Willets JM, Emembolu JO, Konje JC. The effect of the levonorgestrel-releasing intrauterine system, Mirena(R) on mast cell numbers in women with endometriosis undergoing symptomatic treatment. Eur J Obstet Gynecol Reprod Biol. 2011;159(2):439-42.

335. Aydin Y, Tuncel N, Gurer F, Tuncel M, Kosar M, Oflaz G. Ovarian, uterine and brain mast cells in female rats: cyclic changes and contribution to tissue histamine. Comp Biochem Physiol A Mol Integr Physiol. 1998;120(2):255-62.

336. Jeziorska M, Salamonsen LA, Woolley DE. Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. Biol Reprod. 1995;53(2):312-20.

337. Milne SA, Rakhyoot A, Drudy TA, Brechin S, Riley SC, Critchley HO. Co-localization of matrix metalloproteinase-1 and mast cell tryptase in the human uterus. Mol Hum Reprod. 2001;7(6):559-65.

338. Sivridis E, Giatromanolaki A, Agnantis N, Anastasiadis P. Mast cell distribution and density in the normal uterus--metachromatic staining using lectins. Eur J Obstet Gynecol Reprod Biol. 2001;98(1):109-13.

339. Garfield RE, Irani AM, Schwartz LB, Bytautiene E, Romero R. Structural and functional comparison of mast cells in the pregnant versus nonpregnant human uterus. Am J Obstet Gynecol. 2006;194(1):261-7.

340. Menzies FM, Shepherd MC, Nibbs RJ, Nelson SM. The role of mast cells and their mediators in reproduction, pregnancy and labour. Hum Reprod Update. 2011;17(3):383-96.

341. Nakamura T, Chihara M, Ichii O, Otsuka-Kanazawa S, Nagasaki KI, Elewa YHA, et al. Ovarian mast cells migrate toward ovary-fimbria connection in neonatal MRL/MpJ mice. PLoS One. 2018;13(4).

342. Nakamura T, Otsuka S, Ichii O, Sakata Y, Nagasaki K, Hashimoto Y, et al. Relationship between numerous mast cells and early follicular development in neonatal MRL/MpJ mouse ovaries. PLoS One. 2013;8(10):e77246.

343. Szukiewicz D, Pyzlak M, Klimkiewicz J, Szewczyk G, Maslinska D. Mast cell-derived interleukin-8 may be involved in the ovarian mechanisms of follicle growth and ovulation. Inflammation Research. 2007;56(1):S35-S6.

344. Milne SA, Rakhyoot A, Drudy TA, Brechin S, Riley SC, Critchley HO. s. Mol Hum Reprod. 2001;7(6):559-65.

345. Zhang J, Nie G, Jian W, Woolley DE, Salamonsen LA. Mast Cell Regulation of Human Endometrial Matrix Metalloproteinases: A Mechanism Underlying Menstruation1. Biology of Reproduction. 1998;59(3):693-703.

346. Zhao X, Ma W, Das SK, Dey SK, Paria BC. Blastocyst H(2) receptor is the target for uterine histamine in implantation in the mouse. Development. 2000;127(12):2643-51.

347. Varayoud J, Ramos JG, Bosquiazzo VL, Munoz-de-Toro M, Luque EH. Mast cells degranulation affects angiogenesis in the rat uterine cervix during pregnancy. Reproduction. 2004;127(3):379-87.

348. Bosquiazzo VL, Ramos JG, Varayoud J, Munoz-de-Toro M, Luque EH. Mast cell degranulation in rat uterine cervix during pregnancy correlates with expression of vascular endothelial growth factor mRNA and angiogenesis. Reproduction. 2007;133(5):1045-55.

349. Meyer N, Schuler T, Zenclussen AC. Simultaneous Ablation of Uterine Natural Killer Cells and Uterine Mast Cells in Mice Leads to Poor Vascularization and Abnormal Doppler Measurements That Compromise Fetal Well-being. Front Immunol. 2017;8:1913.

350. Bytautiene E, Vedernikov YP, Saade GR, Romero R, Garfield RE. Endogenous mast cell degranulation modulates cervical contractility in the guinea pig. Am J Obstet Gynecol. 2002;186(3):438-45.

351. Bytautiene E, Vedernikov YP, Saade GR, Romero R, Garfield RE. IgE-independent mast cell activation augments contractility of nonpregnant and pregnant guinea pig myometrium. Int Arch Allergy Immunol. 2008;147(2):140-6.

352. Needham K, Fadia M, Dahlstrom JE, Harrington K, Shadbolt B, Robson SJ. Significance of mast cell distribution in placental tissue and membranes in spontaneous preterm birth. Journal of inflammation research. 2016;9:141-5.

353. Chatterjea D, Martinov T. Mast cells: versatile gatekeepers of pain. Mol Immunol. 2015;63(1):38-44.

354. Zhu TH, Ding SJ, Li TT, Zhu LB, Huang XF, Zhang XM. Estrogen is an important mediator of mast cell activation in ovarian endometriomas. Reproduction. 2018;155(1):73-83.

355. Kempuraj D, Papadopoulou N, Stanford EJ, Christodoulou S, Madhappan B, Sant GR, et al. Increased numbers of activated mast cells in endometriosis lesions positive for corticotropin-releasing hormone and urocortin. Am J Reprod Immunol. 2004;52(4):267-75.

356. Sugamata M, Ihara T, Uchiide I. Increase of activated mast cells in human endometriosis. Am J Reprod Immunol. 2005;53(3):120-5.

357. Anaf V, Chapron C, El Nakadi I, De Moor V, Simonart T, Noel JC. Pain, mast cells, and nerves in peritoneal, ovarian, and deep infiltrating endometriosis. Fertil Steril. 2006;86(5):1336-43.

358. Bornstein J, Goldschmid N, Sabo E. Hyperinnervation and mast cell activation may be used as histopathologic diagnostic criteria for vulvar vestibulitis. Gynecol Obstet Invest. 2004;58(3):171-8.

359. Afrin LB, Dempsey TT, Rosenthal LS, Dorff SR. Successful mast-cell-targeted treatment of chronic dyspareunia, vaginitis, and dysfunctional uterine bleeding. J Obstet Gynaecol. 2019:1-6.

360. Guimaraes JV, Costa FB, Andrade WM, Vencio EF, Salge AK, Siqueira KM, et al. Quantification of mast cells in the uterine cervix of women infected with human immunodeficiency virus. Ann Diagn Pathol. 2011;15(5):318-22.

361. Sundstrom JB, Little DM, Villinger F, Ellis JE, Ansari AA. Signaling through Toll-like receptors triggers HIV-1 replication in latently infected mast cells. J Immunol. 2004;172(7):4391-401.

362. Sundstrom JB, Ellis JE, Hair GA, Kirshenbaum AS, Metcalfe DD, Yi H, et al. Human tissue mast cells are an inducible reservoir of persistent HIV infection. Blood. 2007;109(12):5293-300.

363. Im SJ, Ahn MH, Han IH, Song HO, Kim YS, Kim HM, et al. Histamine and TNF-alpha release by rat peritoneal mast cells stimulated with Trichomonas vaginalis. Parasite. 2011;18(1):49-55.

364. Han IH, Park SJ, Ahn MH, Ryu JS. Involvement of mast cells in inflammation induced by Trichomonas vaginalis via crosstalk with vaginal epithelial cells. Parasite Immunol. 2012;34(1):8-14.

365. Nam YH, Min D, Kim HP, Song KJ, Kim KA, Lee YA, et al. Leukotriene B4 receptor BLT-mediated phosphorylation of NF-kappaB and CREB is involved in IL-8 production in human mast cells induced by Trichomonas vaginalis-derived secretory products. Microbes Infect. 2011;13(14-15):1211-20.

366. Min A, Lee YA, Kim KA, Shin MH. BLT1-mediated O-GlcNAcylation is required for NOX2-dependent migration, exocytotic degranulation and IL-8 release of human mast cell induced by Trichomonas vaginalis-secreted LTB4. Microbes Infect. 2018;20(6):376-84.

367. Gendrin C, Vornhagen J, Ngo L, Whidbey C, Boldenow E, Santana-Ufret V, et al. Mast cell degranulation by a hemolytic lipid toxin decreases GBS colonization and infection. Sci Adv. 2015;1(6):e1400225.

368. Shantz E. The Role of Mast Cells ant Toll-Like Receptor 2 (TLR2) in Host Defence and Pathology Following *Chlamydia* Infection. Halifax (Canada): Dalhousie University; 2016.

369. World Health Organization. Report on global sexually transmitted infection surveillance (2015). 2015 2015.

370. Horne AW, Stock SJ, King AE. Innate immunity and disorders of the female reproductive tract. Reproduction. 2008;135(6):739-49.

371. Shaw JH, Behar AR, Snider TA, Allen NA, Lutter EI. Comparison of Murine Cervicovaginal Infection by Chlamydial Strains: Identification of Extrusions Shed In vivo. Front Cell Infect Microbiol. 2017;7:18.

372. Sturdevant GL, Caldwell HD. Innate immunity is sufficient for the clearance of Chlamydia trachomatis from the female mouse genital tract. Pathog Dis. 2014;72(1):70-3.

373. Galli SJ. Mast cells and basophils. Curr Opin Hematol. 2000;7(1):32-9.

374. Mukai K, Tsai M, Saito H, Galli SJ. Mast cells as sources of cytokines, chemokines, and growth factors. Immunol Rev. 2018;282(1):121-50.

375. Wong GW, Zhuo L, Kimata K, Lam BK, Satoh N, Stevens RL. Ancient origin of mast cells. Biochem Biophys Res Commun. 2014;451(2):314-8.

376. Malaviya R, Ikeda T, Abraham SN, Malaviya R. Contribution of mast cells to bacterial clearance and their proliferation during experimental cystitis induced by type 1 fimbriated E. coli. Immunol Lett. 2004;91(2-3):103-11.

377. Carlos D, Frantz FG, Souza-Junior DA, Jamur MC, Oliver C, Ramos SG, et al. TLR2-dependent mast cell activation contributes to the control of Mycobacterium tuberculosis infection. Microbes Infect. 2009;11(8-9):770-8.

378. Gekara NO, Weiss S. Mast cells initiate early anti-Listeria host defences. Cell Microbiol. 2008;10(1):225-36.

379. Arifuzzaman M, Mobley YR, Choi HW, Bist P, Salinas CA, Brown ZD, et al. MRGPR-mediated activation of local mast cells clears cutaneous bacterial infection and protects against reinfection. Sci Adv. 2019;5(1):eaav0216.

380. Salamonsen LA, Woolley DE. Menstruation: induction by matrix metalloproteinases and inflammatory cells. J Reprod Immunol. 1999;44(1-2):1-27.

381. Narita S, Goldblum RM, Watson CS, Brooks EG, Estes DM, Curran EM, et al. Environmental estrogens induce mast cell degranulation and enhance IgE-mediated release of allergic mediators. Environ Health Perspect. 2007;115(1):48-52.

382. Zhang J, Nie G, Jian W, Woolley DE, Salamonsen LA. Mast cell regulation of human endometrial matrix metalloproteinases: A mechanism underlying menstruation. Biol Reprod. 1998;59(3):693-703.

383. Spanggaard H, Knudsen UB, Uldbjerg N, Jeziorska M, Woolley DE, Danielsen CC. Mast cells in cervical ripening--an immunohistochemical and biomechanical study in rats. Eur J Obstet Gynecol Reprod Biol. 1997;73(1):91-7.

384. Bytautiene E, Vedernikov YP, Saade GR, Romero R, Garfield RE. The effect of a mast cell degranulating agent on vascular resistance in the human placental vascular bed and on the tone of isolated placental vessels. Reprod Sci. 2008;15(1):26-32.

385. Bytautiene E, Vedernikov YP, Saade GR, Romero R, Garfield RE. Degranulation of uterine mast cell modifies contractility of isolated myometrium from pregnant women. Am J Obstet Gynecol. 2004;191(5):1705-10.

386. Molderings GJ, Zienkiewicz T, Homann J, Menzen M, Afrin LB. Risk of solid cancer in patients with mast cell activation syndrome: Results from Germany and USA. F1000Res. 2017;6:1889.

387. Nyirjesy P, Sobel JD, Weitz MV, Leaman DJ, Small MJ, Gelone SP. Cromolyn cream for recalcitrant idiopathic vulvar vestibulitis: results of a placebo controlled study. Sex Transm Infect. 2001;77(1):53-7.

388. Kaushic C, Zhou F, Murdin AD, Wira CR. Effects of estradiol and progesterone on susceptibility and early immune responses to Chlamydia trachomatis infection in the female reproductive tract. Infect Immun. 2000;68(7):4207-16.

389. Fung KY, Mangan NE, Cumming H, Horvat JC, Mayall JR, Stifter SA, et al. Interferon-epsilon protects the female reproductive tract from viral and bacterial infection. Science. 2013;339(6123):1088-92.

390. Ribatti D. The Staining of Mast Cells: A Historical Overview. Int Arch Allergy Immunol. 2018;176(1):55-60.

391. Eckelhart E, Warsch W, Zebedin E, Simma O, Stoiber D, Kolbe T, et al. A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. Blood. 2011;117(5):1565-73.

392. Lin TJ, Garduno R, Boudreau RT, Issekutz AC. Pseudomonas aeruginosa activates human mast cells to induce neutrophil transendothelial migration via mast cell-derived IL-1 alpha and beta. J Immunol. 2002;169(8):4522-30.

393. Hepworth MR, Danilowicz-Luebert E, Rausch S, Metz M, Klotz C, Maurer M, et al. Mast cells orchestrate type 2 immunity to helminths through regulation of tissue-derived cytokines. Proc Natl Acad Sci U S A. 2012;109(17):6644-9.

394. Zhu J, Paul WE. Heterogeneity and plasticity of T helper cells. Cell Res. 2010;20(1):4-12.

395. De Clercq E, Devriendt B, Yin L, Chiers K, Cox E, Vanrompay D. The immune response against Chlamydia suis genital tract infection partially protects against re-infection. Vet Res. 2014;45:95.

396. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. J Leukoc Biol. 1997;61(3):233-45.

397. Pennock JL, Grencis RK. In vivo exit of c-kit+/CD49d(hi)/beta7+ mucosal mast cell precursors from the bone marrow following infection with the intestinal nematode Trichinella spiralis. Blood. 2004;103(7):2655-60.

398. Stevens RL, Friend DS, McNeil HP, Schiller V, Ghildyal N, Austen KF. Strain-specific and tissue-specific expression of mouse mast cell secretory granule proteases. Proc Natl Acad Sci U S A. 1994;91(1):128-32.

399. Gurish MF, Pear WS, Stevens RL, Scott ML, Sokol K, Ghildyal N, et al. Tissue-regulated differentiation and maturation of a v-abl-immortalized mast cell-committed progenitor. Immunity. 1995;3(2):175-86.

400. Reynolds DS, Serafin WE, Faller DV, Wall DA, Abbas AK, Dvorak AM, et al. Immortalization of murine connective tissue-type mast cells at multiple stages of their differentiation by coculture of splenocytes with fibroblasts that produce Kirsten sarcoma virus. J Biol Chem. 1988;263(25):12783-91.

401. Mokhtar NM, Cheng CW, Cook E, Bielby H, Smith SK, Charnock-Jones DS. Progestin regulates chemokine (C-X-C motif) ligand 14 transcript level in human endometrium. Mol Hum Reprod. 2010;16(3):170-7.

402. Dominguez F, Galan A, Martin JJ, Remohi J, Pellicer A, Simon C. Hormonal and embryonic regulation of chemokine receptors CXCR1, CXCR4, CCR5 and CCR2B in the human endometrium and the human blastocyst. Mol Hum Reprod. 2003;9(4):189-98.

403. Park DW, Yang KM. Hormonal regulation of uterine chemokines and immune cells. Clin Exp Reprod Med. 2011;38(4):179-85.

404. Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol Reprod. 1999;60(5):1158-65.

405. Marshall JS, Ford GP, Bell EB. Formalin sensitivity and differential staining of mast cells in human dermis. Br J Dermatol. 1987;117(1):29-36.

406. Nakamura A, Kawabori S, Unno T. Study on staining methods for mast cells in the nasal mucosa. Auris Nasus Larynx. 1991;18(4):359-66.

407. Broome M, Villarreal B. Differential staining of mast cells with toluidine blue. Journal of Histotechnology. 2012;35(1):27-30.

408. Oksaharju A, Lappalainen J, Tuomainen AM, Pussinen PJ, Puolakkainen M, Kovanen PT, et al. Pro-atherogenic lung and oral pathogens induce an inflammatory response in human and mouse mast cells. J Cell Mol Med. 2009;13(1):103-13.

409. Vliagoftis H, Lacy P, Luy B, Adamko D, Hollenberg M, Befus D, et al. Mast cell tryptase activates peripheral blood eosinophils to release granule-associated enzymes. Int Arch Allergy Immunol. 2004;135(3):196-204.

410. O'Reilly M, Alpert R, Jenkinson S, Gladue RP, Foo S, Trim S, et al. Identification of a histamine H4 receptor on human eosinophils--role in eosinophil chemotaxis. J Recept Signal Transduct Res. 2002;22(1-4):431-48.

411. Raible DG, Schulman ES, DiMuzio J, Cardillo R, Post TJ. Mast cell mediators prostaglandin-D2 and histamine activate human eosinophils. J Immunol. 1992;148(11):3536-42.

412. Schirmer B, Bringmann L, Seifert R, Neumann D. In vivo Evidence for Partial Activation of Eosinophils via the Histamine H4-Receptor: Adoptive Transfer Experiments Using Eosinophils From H4R(-/-) and H4R(+/+) Mice. Front Immunol. 2018;9:2119.

413. Nagarajan UM, Tripathy M, Kollipara A, Allen Jt, Goodwin A, Whittimore J, et al. Differential signaling pathways are initiated in macrophages during infection depending on the intracellular fate of Chlamydia spp. Immunol Cell Biol. 2018;96(3):246-56.

414. Chen SC, Mehrad B, Deng JC, Vassileva G, Manfra DJ, Cook DN, et al. Impaired pulmonary host defense in mice lacking expression of the CXC chemokine lungkine. J Immunol. 2001;166(5):3362-8.

415. Zhao J, Zhu H, Wong CH, Leung KY, Wong WS. Increased lungkine and chitinase levels in allergic airway inflammation: a proteomics approach. Proteomics. 2005;5(11):2799-807.

416. Rossi DL, Hurst SD, Xu Y, Wang W, Menon S, Coffman RL, et al. Lungkine, a novel CXC chemokine, specifically expressed by lung bronchoepithelial cells. J Immunol. 1999;162(9):5490-7.

417. Schmitz JM, McCracken VJ, Dimmitt RA, Lorenz RG. Expression of CXCL15 (Lungkine) in murine gastrointestinal, urogenital, and endocrine organs. J Histochem Cytochem. 2007;55(5):515-24.

418. Velin D, Narayan S, Bernasconi E, Busso N, Ramelli G, Maillard MH, et al. PAR2 promotes vaccine-induced protection against Helicobacter infection in mice. Gastroenterology. 2011;141(4):1273-82, 82 e1.

419. Roman K, Done JD, Schaeffer AJ, Murphy SF, Thumbikat P. Tryptase-PAR2 axis in experimental autoimmune prostatitis, a model for chronic pelvic pain syndrome. Pain. 2014;155(7):1328-38.

420. Huang C, Friend DS, Qiu WT, Wong GW, Morales G, Hunt J, et al. Induction of a selective and persistent extravasation of neutrophils into the peritoneal cavity by tryptase mouse mast cell protease 6. J Immunol. 1998;160(4):1910-9.

421. Kang BN, Ha SG, Bahaie NS, Hosseinkhani MR, Ge XN, Blumenthal MN, et al. Regulation of serotonin-induced trafficking and migration of eosinophils. PLoS One. 2013;8(1):e54840.

422. Patnode ML, Bando JK, Krummel MF, Locksley RM, Rosen SD. Leukotriene B4 amplifies eosinophil accumulation in response to nematodes. J Exp Med. 2014;211(7):1281-8.

423. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, et al. Bone marrow and the control of immunity. Cell Mol Immunol. 2012;9(1):11-9.

424. Shakoory B, Fitzgerald SM, Lee SA, Chi DS, Krishnaswamy G. The role of human mast cell-derived cytokines in eosinophil biology. J Interferon Cytokine Res. 2004;24(5):271-81.

425. Wong CK, Ng SS, Lun SW, Cao J, Lam CW. Signalling mechanisms regulating the activation of human eosinophils by mast-cell-derived chymase: implications for mast cell-eosinophil interaction in allergic inflammation. Immunology. 2009;126(4):579-87.

426. Galdiero MR, Varricchi G, Seaf M, Marone G, Levi-Schaffer F, Marone G. Bidirectional Mast Cell-Eosinophil Interactions in Inflammatory Disorders and Cancer. Front Med (Lausanne). 2017;4:103.

427. Gao Y, Williams AP. Role of Innate T Cells in Anti-Bacterial Immunity. Front Immunol. 2015;6:302.

428. Su H, Feilzer K, Caldwell HD, Morrison RP. Chlamydia trachomatis genital tract infection of antibody-deficient gene knockout mice. Infect Immun. 1997;65(6):1993-9.

429. van Panhuys N, Prout M, Forbes E, Min B, Paul WE, Le Gros G. Basophils are the major producers of IL-4 during primary helminth infection. J Immunol. 2011;186(5):2719-28.

430. de Paulis A, Prevete N, Fiorentino I, Walls AF, Curto M, Petraroli A, et al. Basophils infiltrate human gastric mucosa at sites of Helicobacter pylori infection, and exhibit chemotaxis in response to H. pylori-derived peptide Hp(2-20). J Immunol. 2004;172(12):7734-43.

431. Nigrovic PA, Gray DH, Jones T, Hallgren J, Kuo FC, Chaletzky B, et al. Genetic inversion in mast cell-deficient (Wsh) mice interrupts corin and manifests as hematopoietic and cardiac aberrancy. Am J Pathol. 2008;173(6):1693-701.

432. Piliponsky AM, Chen CC, Grimbaldeston MA, Burns-Guydish SM, Hardy J, Kalesnikoff J, et al. Mast cell-derived TNF can exacerbate mortality during severe bacterial infections in C57BL/6-KitW-sh/W-sh mice. Am J Pathol. 2010;176(2):926-38.

433. Michel A, Schuler A, Friedrich P, Doner F, Bopp T, Radsak M, et al. Mast cell-deficient Kit(W-sh) "Sash" mutant mice display aberrant myelopoiesis leading to the accumulation of splenocytes that act as myeloid-derived suppressor cells. J Immunol. 2013;190(11):5534-44.

434. Zhou JS, Xing W, Friend DS, Austen KF, Katz HR. Mast cell deficiency in Kit(W-sh) mice does not impair antibody-mediated arthritis. J Exp Med. 2007;204(12):2797-802.

435. Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. Am J Pathol. 2005;167(3):835-48.

436. Voehringer D. The role of basophils in helminth infection. Trends Parasitol. 2009;25(12):551-6.

437. Ohnmacht C, Schwartz C, Panzer M, Schiedewitz I, Naumann R, Voehringer D. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. Immunity. 2010;33(3):364-74.

438. Bjork J, Hedqvist P, Arfors KE. Increase in vascular permeability induced by leukotriene B4 and the role of polymorphonuclear leukocytes. Inflammation. 1982;6(2):189-200.

439. Vangansewinkel T, Geurts N, Quanten K, Nelissen S, Lemmens S, Geboes L, et al. Mast cells promote scar remodeling and functional recovery after spinal cord injury via mouse mast cell protease 6. FASEB J. 2016;30(5):2040-57.

440. Seeds EA, Page CP. Heparin inhibits allergen-induced eosinophil infiltration into guinea-pig lung via a mechanism unrelated to its anticoagulant activity. Pulm Pharmacol Ther. 2001;14(2):111-9.

441. De Filippo K, Dudeck A, Hasenberg M, Nye E, van Rooijen N, Hartmann K, et al. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood. 2013;121(24):4930-7.

442. Shin K, Nigrovic PA, Crish J, Boilard E, McNeil HP, Larabee KS, et al. Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes. J Immunol. 2009;182(1):647-56.

443. Carlos D, Machado ER, De Paula L, Sa-Nunes A, Sorgi CA, Jamur MC, et al. Evidence for eosinophil recruitment, leukotriene B4 production and mast cell hyperplasia following Toxocara canis infection in rats. Braz J Med Biol Res. 2011;44(4):319-26.

444. Liu ZQ, Song JP, Liu X, Jiang J, Chen X, Yang L, et al. Mast cell-derived serine proteinase regulates T helper 2 polarization. Sci Rep. 2014;4:4649.

445. Stevens RL, Fox CC, Lichtenstein LM, Austen KF. Identification of chondroitin sulfate E proteoglycans and heparin proteoglycans in the secretory granules of human lung mast cells. Proc Natl Acad Sci U S A. 1988;85(7):2284-7.

446. Sali A, Matsumoto R, McNeil HP, Karplus M, Stevens RL. Three-dimensional models of four mouse mast cell chymases. Identification of proteoglycan binding regions and protease-specific antigenic epitopes. J Biol Chem. 1993;268(12):9023-34.

447. Wong GW, Yasuda S, Morokawa N, Li L, Stevens RL. Mouse chromosome 17A3.3 contains 13 genes that encode functional tryptic-like serine proteases with distinct tissue and cell expression patterns. J Biol Chem. 2004;279(4):2438-52.

448. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev. 2007;217:141-54.

449. Ghildyal N, Friend DS, Freelund R, Austen KF, McNeil HP, Schiller V, et al. Lack of expression of the tryptase mouse mast cell protease 7 in mast cells of the C57BL/6J mouse. J Immunol. 1994;153(6):2624-30.

450. Huang C, Morales G, Vagi A, Chanasyk K, Ferrazzi M, Burklow C, et al. Formation of enzymatically active, homotypic, and heterotypic tetramers of mouse mast cell tryptases. Dependence on a conserved Trp-rich domain on the surface. J Biol Chem. 2000;275(1):351-8.

451. de Souza Junior DA, Santana AC, da Silva EZ, Oliver C, Jamur MC. The Role of Mast Cell Specific Chymases and Tryptases in Tumor Angiogenesis. Biomed Res Int. 2015;2015:142359.

452. Huang H, Reed CP, Zhang JS, Shridhar V, Wang L, Smith DI. Carboxypeptidase A3 (CPA3): a novel gene highly induced by histone deacetylase inhibitors during differentiation of prostate epithelial cancer cells. Cancer Res. 1999;59(12):2981-8.

453. Tanco S, Lorenzo J, Garcia-Pardo J, Degroeve S, Martens L, Aviles FX, et al. Proteome-derived peptide libraries to study the substrate specificity profiles of carboxypeptidases. Mol Cell Proteomics. 2013;12(8):2096-110.

454. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol. 2012;34(1):43-62.

455. Luyendyk JP, Schoenecker JG, Flick MJ. The multifaceted role of fibrinogen in tissue injury and inflammation. Blood. 2019;133(6):511-20.

456. Fu Z, Thorpe M, Alemayehu R, Roy A, Kervinen J, de Garavilla L, et al. Highly Selective Cleavage of Cytokines and Chemokines by the Human Mast Cell Chymase and Neutrophil Cathepsin G. J Immunol. 2017;198(4):1474-83.

457. Abonia JP, Friend DS, Austen WG, Jr., Moore FD, Jr., Carroll MC, Chan R, et al. Mast cell protease 5 mediates ischemia-reperfusion injury of mouse skeletal muscle. J Immunol. 2005;174(11):7285-91.

458. Hamilton MJ, Sinnamon MJ, Lyng GD, Glickman JN, Wang X, Xing W, et al. Essential role for mast cell tryptase in acute experimental colitis. Proc Natl Acad Sci U S A. 2011;108(1):290-5.

459. Hickey DK, Fahey JV, Wira CR. Mouse estrous cycle regulation of vaginal versus uterine cytokines, chemokines, alpha-/beta-defensins and TLRs. Innate Immun. 2013;19(2):121-31.

460. Kim DK, Lee YM. Requirement of c-jun transcription factor on the mouse mast cell protease-6 expression in the mast cells. Arch Biochem Biophys. 2004;431(1):71-8.

461. Morii E, Ogihara H, Oboki K, Kataoka TR, Jippo T, Kitamura Y. Effect of MITF on transcription of transmembrane tryptase gene in cultured mast cells of mice. Biochem Biophys Res Commun. 2001;289(5):1243-6.

462. Li ZL, Ueki K, Kumagai K, Araki R, Otsuki Y. Regulation of bcl-2 transcription by estrogen receptor-alpha and c-Jun in human endometrium. Med Mol Morphol. 2014;47(1):43-53.

463. Chiappetta C, Kirkland JL, Loose-Mitchell DS, Murthy L, Stancel GM. Estrogen regulates expression of the jun family of protooncogenes in the uterus. J Steroid Biochem Mol Biol. 1992;41(2):113-23.

464. Shiozawa T, Miyamoto T, Kashima H, Nakayama K, Nikaido T, Konishi I. Estrogen-induced proliferation of normal endometrial glandular cells is initiated by transcriptional activation of cyclin D1 via binding of c-Jun to an AP-1 sequence. Oncogene. 2004;23(53):8603-10.

465. Meyer N, Woidacki K, Knofler M, Meinhardt G, Nowak D, Velicky P, et al. Chymase-producing cells of the innate immune system are required for decidual vascular remodeling and fetal growth. Sci Rep. 2017;7:45106.

466. Hafner LM, Cunningham K, Beagley KW. Ovarian steroid hormones: effects on immune responses and Chlamydia trachomatis infections of the female genital tract. Mucosal Immunol. 2013;6(5):859-75.

467. Flynn L, Byrne B, Carton J, Kelehan P, O'Herlihy C, O'Farrelly C. Menstrual cycle dependent fluctuations in NK and T-lymphocyte subsets from non-pregnant human endometrium. Am J Reprod Immunol. 2000;43(4):209-17.

468. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. Clin Exp Reprod Med. 2011;38(3):119-25.

469. Dagalv A, Holmborn K, Kjellen L, Abrink M. Lowered expression of heparan sulfate/heparin biosynthesis enzyme N-deacetylase/n-sulfotransferase 1 results in increased sulfation of mast cell heparin. J Biol Chem. 2011;286(52):44433-40.

470. Moelleken K, Hegemann JH. The Chlamydia outer membrane protein OmcB is required for adhesion and exhibits biovar-specific differences in glycosaminoglycan binding. Mol Microbiol. 2008;67(2):403-19.

471. Su H, Raymond L, Rockey DD, Fischer E, Hackstadt T, Caldwell HD. A recombinant Chlamydia trachomatis major outer membrane protein binds to heparan sulfate receptors on epithelial cells. Proc Natl Acad Sci U S A. 1996;93(20):11143-8.

472. Nagarajan UM, Sikes JD, Yeruva L, Prantner D. Significant role of IL-1 signaling, but limited role of inflammasome activation, in oviduct pathology during Chlamydia muridarum genital infection. J Immunol. 2012;188(6):2866-75.

473. McLachlan JB, Hart JP, Pizzo SV, Shelburne CP, Staats HF, Gunn MD, et al. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. Nat Immunol. 2003;4(12):1199-205.

474. Johnson RM, Kerr MS, Slaven JE. An atypical CD8 T-cell response to Chlamydia muridarum genital tract infections includes T cells that produce interleukin-13. Immunology. 2014;142(2):248-57.

475. Jordan SJ, Bakshi RK, Brown LT, Chi X, Geisler WM. Stimulated peripheral blood mononuclear cells from chlamydia-infected women release predominantly Th1-polarizing cytokines. Cytokine. 2019;113:458-61.

476. Andrew DW, Cochrane M, Schripsema JH, Ramsey KH, Dando SJ, O'Meara CP, et al. The duration of Chlamydia muridarum genital tract infection and associated chronic pathological changes are reduced in IL-17 knockout mice but protection is not increased further by immunization. PLoS One. 2013;8(9):e76664.

477. O'Meara CP, Armitage CW, Harvie MC, Andrew DW, Timms P, Lycke NY, et al. Immunity against a Chlamydia infection and disease may be determined by a balance of IL-17 signaling. Immunol Cell Biol. 2014;92(3):287-97.

478. O'Mahony L, Akdis M, Akdis CA. Regulation of the immune response and inflammation by histamine and histamine receptors. J Allergy Clin Immunol. 2011;128(6):1153-62.

479. Cowden JM, Yu F, Banie H, Farahani M, Ling P, Nguyen S, et al. The histamine H4 receptor mediates inflammation and Th17 responses in preclinical models of arthritis. Ann Rheum Dis. 2014;73(3):600-8.

480. Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, et al. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nat Med. 2009;15(6):633-40.

481. Lee HY, Schripsema JH, Sigar IM, Lacy SR, Kasimos JN, Murray CM, et al. A role for CXC chemokine receptor-2 in the pathogenesis of urogenital Chlamydia muridarum infection in mice. FEMS Immunol Med Microbiol. 2010;60(1):49-56.

482. Perfettini JL, Darville T, Gachelin G, Souque P, Huerre M, Dautry-Varsat A, et al. Effect of Chlamydia trachomatis infection and subsequent tumor necrosis factor alpha secretion on apoptosis in the murine genital tract. Infect Immun. 2000;68(4):2237-44.

483. McNeil HP, Adachi R, Stevens RL. Mast cell-restricted tryptases: structure and function in inflammation and pathogen defense. J Biol Chem. 2007;282(29):20785-9.

484. Sun J, Kintner J, Schoborg RV. The host adherens junction molecule nectin-1 is downregulated in Chlamydia trachomatis-infected genital epithelial cells. Microbiology. 2008;154(Pt 5):1290-9.

485. Grujic M, Paivandy A, Gustafson AM, Thomsen AR, Ohrvik H, Pejler G. The combined action of mast cell chymase, tryptase and carboxypeptidase A3 protects against melanoma colonization of the lung. Oncotarget. 2017;8(15):25066-79.

486. Scandiuzzi L, Beghdadi W, Daugas E, Abrink M, Tiwari N, Brochetta C, et al. Mouse mast cell protease-4 deteriorates renal function by contributing to inflammation and fibrosis in immune complex-mediated glomerulonephritis. J Immunol. 2010;185(1):624-33.

487. Vangansewinkel T, Lemmens S, Geurts N, Quanten K, Dooley D, Pejler G, et al. Mouse mast cell protease 4 suppresses scar formation after traumatic spinal cord injury. Sci Rep. 2019;9(1):3715.

488. Wechsler JB, Szabo A, Hsu CL, Krier-Burris RA, Schroeder HA, Wang MY, et al. Histamine drives severity of innate inflammation via histamine 4 receptor in murine experimental colitis. Mucosal Immunol. 2018;11(3):861-70.

489. Metz M, Doyle E, Bindslev-Jensen C, Watanabe T, Zuberbier T, Maurer M. Effects of antihistamines on innate immune responses to severe bacterial infection in mice. Int Arch Allergy Immunol. 2011;155(4):355-60.

490. Li H, Yang T, Ning Q, Li F, Chen T, Yao Y, et al. Cigarette smoke extract-treated mast cells promote alveolar macrophage infiltration and polarization in experimental chronic obstructive pulmonary disease. Inhal Toxicol. 2015;27(14):822-31.

491. Hansbro PM, Hamilton MJ, Fricker M, Gellatly SL, Jarnicki AG, Zheng D, et al. Importance of mast cell Prss31/transmembrane tryptase/tryptase-gamma in lung function and experimental chronic obstructive pulmonary disease and colitis. J Biol Chem. 2014;289(26):18214-27.

492. Gensel JC, Kopper TJ, Zhang B, Orr MB, Bailey WM. Predictive screening of M1 and M2 macrophages reveals the immunomodulatory effectiveness of post spinal cord injury azithromycin treatment. Sci Rep. 2017;7:40144.

493. Nie MF, Xie Q, Wu YH, He H, Zou LJ, She XL, et al. Serum and Ectopic Endometrium from Women with Endometriosis Modulate Macrophage M1/M2 Polarization via the Smad2/Smad3 Pathway. J Immunol Res. 2018;2018:6285813.

494. Yao Y, Xu XH, Jin L. Macrophage Polarization in Physiological and Pathological Pregnancy. Front Immunol. 2019;10:792.

495. The Human Protein Atlas. TPSG1 [Available from: https://www.proteinatlas.org/ENSG00000116176-TPSG1/tissue.

496. Fagerberg L, Hallstrom BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics. 2014;13(2):397-406.

497. Cheng W, Shivshankar P, Li Z, Chen L, Yeh IT, Zhong G. Caspase-1 contributes to Chlamydia trachomatis-induced upper urogenital tract inflammatory pathologies without affecting the course of infection. Infect Immun. 2008;76(2):515-22.

498. Yang Z, Tang L, Zhou Z, Zhong G. Neutralizing antichlamydial activity of complement by chlamydia-secreted protease CPAF. Microbes Infect. 2016;18(11):669-74.

499. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014;6:13.

500. Menon S, Timms P, Allan JA, Alexander K, Rombauts L, Horner P, et al. Human and Pathogen Factors Associated with Chlamydia trachomatis-Related Infertility in Women. Clin Microbiol Rev. 2015;28(4):969-85.

501. Okayama Y, Kawakami T. Development, migration, and survival of mast cells. Immunol Res. 2006;34(2):97-115.

502. Woidacki K, Jensen F, Zenclussen AC. Mast cells as novel mediators of reproductive processes. Front Immunol. 2013;4:29.

503. Woidacki K, Zenclussen AC, Siebenhaar F. Mast cell-mediated and associated disorders in pregnancy: a risky game with an uncertain outcome? Front Immunol. 2014;5:231.

504. Woodbury RG, Miller HR. Quantitative analysis of mucosal mast cell protease in the intestines of Nippostrongylus-infected rats. Immunology. 1982;46(3):487-95.

505. Zarnegar B, Westin A, Evangelidou S, Hallgren J. Innate Immunity Induces the Accumulation of Lung Mast Cells During Influenza Infection. Front Immunol. 2018;9:2288.

506. Rose SB, Garrett SM, Stanley J, Pullon SRH. Retesting and repeat positivity following diagnosis of Chlamydia trachomatis and Neisseria gonorrhoea in New Zealand: a retrospective cohort study. BMC Infect Dis. 2017;17(1):526.

507. Gaydos CA, Wright C, Wood BJ, Waterfield G, Hobson S, Quinn TC. Chlamydia trachomatis reinfection rates among female adolescents seeking rescreening in school-based health centers. Sex Transm Dis. 2008;35(3):233-7.

508. Carlos D, Fremond C, Samarina A, Vasseur V, Maillet I, Ramos SG, et al. Histamine plays an essential regulatory role in lung inflammation and protective immunity in the acute phase of Mycobacterium tuberculosis infection. Infect Immun. 2009;77(12):5359-68.

509. Dunford PJ, O'Donnell N, Riley JP, Williams KN, Karlsson L, Thurmond RL. The histamine H4 receptor mediates allergic airway inflammation by regulating the activation of CD4+ T cells. J Immunol. 2006;176(11):7062-70.

510. Rice PA, Shafer WM, Ram S, Jerse AE. Neisseria gonorrhoeae: Drug Resistance, Mouse Models, and Vaccine Development. Annu Rev Microbiol. 2017;71:665-86.

511. Unemo M, Del Rio C, Shafer WM. Antimicrobial Resistance Expressed by Neisseria gonorrhoeae: A Major Global Public Health Problem in the 21st Century. Microbiol Spectr. 2016;4(3).

512. Aich A, Afrin LB, Gupta K. Mast Cell-Mediated Mechanisms of Nociception. Int J Mol Sci. 2015;16(12):29069-92.

513. Heron A, Dubayle D. A focus on mast cells and pain. J Neuroimmunol. 2013;264(1-2):1-7.

514. Rudick CN, Bryce PJ, Guichelaar LA, Berry RE, Klumpp DJ. Mast cell-derived histamine mediates cystitis pain. PLoS One. 2008;3(5):e2096.

515. Binda MM, Donnez J, Dolmans MM. Targeting mast cells: a new way to treat endometriosis. Expert Opin Ther Targets. 2017;21(1):67-75.

516. Rudolph M, Docke WD, Muller A, Menning A, Rose L, Zollner TM, et al. Induction of overt menstruation in intact mice. PLoS One. 2012;7(3):e32922.

517. Yang C, Whitmire WM, Sturdevant GL, Bock K, Moore I, Caldwell HD. Infection of Hysterectomized Mice with Chlamydia muridarum and Chlamydia trachomatis. Infect Immun. 2017;85(7).

518. Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Kohler A, et al. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. Immunity. 2011;34(6):973-84.

519. Otsuka A, Kubo M, Honda T, Egawa G, Nakajima S, Tanizaki H, et al. Requirement of interaction between mast cells and skin dendritic cells to establish contact hypersensitivity. PLoS One. 2011;6(9):e25538.

520. Sarett SM, Nelson CE, Duvall CL. Technologies for controlled, local delivery of siRNA. J Control Release. 2015;218:94-113.

521. Hori Y, Nihei Y, Kurokawa Y, Kuramasu A, Makabe-Kobayashi Y, Terui T, et al. Accelerated clearance of Escherichia coli in experimental peritonitis of histamine-deficient mice. J Immunol. 2002;169(4):1978-83.

522. Rajasekaran N, Solomon S, Watanabe T, Ohtsu H, Gajda M, Brauer R, et al. Histidine decarboxylase but not histamine receptor 1 or 2 deficiency protects from K/BxN serum-induced arthritis. Int Immunol. 2009;21(11):1263-8.