Contribution of epithelial innate immunity to systemic protection afforded by prolyl hydroxylase inhibition in murine colitis

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Abstract

Pharmacological stabilization of hypoxia-inducible factor (HIF) through prolyl hydroxylase (PHD) inhibition limits mucosal damage associated with models of murine colitis. However, little is known about how PHD inhibitors (PHDi) influence systemic immune function during mucosal inflammation or the relative importance of immunological changes to mucosal protection. We hypothesized that PHDi enhances systemic innate immune responses to colitis-associated bacteremia. Mice with colitis induced by TNBS were treated with AKB-4924, a new HIF-1 isoform-predominant PHDi and clinical, immunological and biochemical endpoints were assessed. Administration of AKB-4924 led to significantly reduced weight loss and disease activity compared to vehicle controls. Treated groups were pyrexic, but did not become subsequently hypothermic. PHDi treatment augmented epithelial barrier function and led to an approximately 50-fold reduction in serum endotoxin during colitis. AKB-4924 also decreased cytokines involved in pyrogenesis and hypothermia, significantly reducing serum levels of IL-1β, IL-6 and TNF-α, while increasing IL-10. Treatment offered no protection against colitis in epithelial-specific HIF-1α deficient mice, strongly implicating epithelial HIF-1α as the tissue target for AKB-4924-mediated protection. Taken together, these results indicate that inhibition of prolyl hydroxylase with AKB-4924 enhances innate immunity and identifies that the epithelium is a central site of inflammatory protection afforded by PHDi in murine colitis.

Introduction

Inflammatory bowel diseases (IBD), are characterized by repeated wounding of the mucosa and loss of the intestinal epithelial barrier function [1]. This leads to the passage of bacteria or bacterial products from the lumen to the serosa and into the blood, resulting in systemic bacteremia and endotoxemia, which are both common features of IBD [2-4]. Prolyl hydroxylase (PHD) inhibition has been shown to reduce disease severity in murine models of colitis on several levels of clinical scoring [5-9]. The observed mucosal protection is a consequence of PHD-2 sensitive HIF stabilization [10] and PHD-1 sensitive NF-κB activation [11], as the pan-prolyl hydroxylase inhibitors employed in these studies, such as dimethyloxallyl glycine (DMOG) activate both pathways. This mucosal protection in murine colitis models is multi-factorial, and roles for compensatory epithelial barrier pathways [6], anti-apoptotic regulation [8] and the promotion of restitution and wound healing [7] have been demonstrated.

Recent studies have demonstrated the importance of HIF in immune cell responses to infection. Neutrophils obtained from patients with heterozygous germline mutations in the von Hippel Lindau protein (pVHL) display increased survival times and enhanced phagocytic capacity [12]. *In vitro* studies have demonstrated that stabilization of HIF with DMOG prolonged neutrophil survival [13] and HIF stabilization by hypoxic incubation enhanced bacterial phagocytosis by neutrophils [14] and macrophages[15]. Further, DMOG treatment ameliorated disease in a murine model of endotoxic shock, through suppression of inflammatory cytokines and enhanced IL-10 production [16]. More recently, a predominantly HIF-1-specific prolyl hydroxylase inhibitor (PHDi), AKB-4924, has been developed [17]. Treatment with AKB-4924 enhanced the bactericidal capacity of keratinocytes against a range of skin pathogens in mouse models of infection [17]. Importantly, the concentrations that were effective were orders of magnitude less than those previously observed with other PHDi's (DMOG and FG-class compounds), which typically suffer from poor solubility. *Thus, it is*

likely that PHDi treatment in murine models of colitis also drive an innate cell response, driven by HIF stabilization in immune cells. However, as previous studies into mucosal protection by PHD inhibitors in colitis have focused primarily on epithelial cells[6, 8, 9], the importance of these processes have not yet been defined.

Here, we hypothesized that subcutaneous administration of AKB-4924 enhances innate cell responses in a mouse colitis model. Employing the chemical induction of colitis using trinitrobenzene sulfonic acid (TNBS), we assessed the systemic inflammatory response to bacteremia associated with intestinal inflammation. We examined inflammatory signaling in innate barrier cells and epithelial-specific HIF-1α deficient mice. We also compared the relative importance of HIF-mediated epithelial barrier responses and HIF-driven innate cell activity. Our results suggest that PHDi treatment stabilizes HIF and suppresses inflammatory signaling through an epithelial mechanism that is critical for the mucosal protection in models of colitis.

Results

AKB-4924 reduces TNBS disease pathology

We first hypothesised that PHDi treatment would enhance systemic immune cell responses to inflammation, particularly those associated with intestinal epithelial barrier dysfunction in a TNBS model of colitis. Initially, we examined the pyrogenic response in TNBS colitic mice treated with AKB-4924, a new HIF-1 predominant PHDi [17]. Animals treated with AKB-4924 showed reduced weight loss **(Figure 1A**, $p<0.01$**)**, attenuated colon shortening **(Figure 1B**, $p<0.05$**)** at all doses tested (0.3, 1, and 5 mg/Kg), with as little as 0.3mg/Kg AKB-4924 showing marked improvements in each of these endpoints. *Histologically, AKB-4924 treated colitic animals showed quite significantly reduced tissue damage compared to vehicle and decreased disease activity indices* ***(Figure 1C)*** *and resulted in significantly decreased disease activity scores at all doses tested (****Figure 1D,*** *$p<0.01$).*

In the course of these studies, we examined core body temperature as an indicator of disease severity. Interestingly, there was no difference in the early pyrogenic response (+1.1±0.56^{o}C at 6 hours post TNBS induction, $p<0.001$ compared to vehicle control) to TNBS administration between AKB-4924-treated and untreated animals ***(Figure 1E).*** *However, the subsequent hypothermic response observed in untreated TNBS mice (-1.65±0.58^{o}C at 168 hours post TNBS induction, $p<0.001$ compared to EtOH control) was not observed in the AKB-4924-treated groups (-0.03±0.50^{o}C, non significant compared to EtOH control) suggesting that PHDi influences the systemic inflammatory response in TNBS colitis.*

AKB-4924 alters endogenous pyrogen responses in TNBS colitis

Guided by these altered hypothermic responses in PHDi-treated animals, we investigated serum levels of endogenous pyrogens over the course of one week TNBS colitis progression. At the peak of acute disease (measured by weight loss, day 2 post-TNBS), colitic animals had

significantly elevated levels of IL-1β (21±9-fold**, Figure 2A**, p<0.01). At day 2, there was no significant reduction of IL-1β in PHDi-treated animals. However, at days 3 and 7, PHDi strongly reduced IL-1β expression (p<0.01) compared to placebo exposed colitic mice. Similarly, TNBS colitis animals had significant increases in IL-6 (33±11-fold, **Figure 2B**) compared to controls. B*y day 3, PHDi treatment significantly reduced IL-6 (61±5% reduction, p<0.01) and by day 7, levels were back to baseline and were comparable to non-TNBS controls (97±3% reduction, p<0.01).* TNF-α was significantly increased in TNBS-treated animals by day 2, and increased at day 3 (4±1-fold, p<0.05) and day 7 (12±5-fold, p<0.01) compared to controls **(Figure 2C)**. PHDi-treatment resulted in a significant reduction in TNF-α by day 3 and was reduced by day 7 (76±8% reduction, p<0.01). As IL-10 is an anti-inflammatory cytokine and may suppress the hypothermic response [18], we investigated serum levels and found that they were significantly increased in PHDi-treated TNBS animals compared to untreated controls from day 2 to 7 **(Figure 2D)**. In addition, we examined whole colon homogenates for cytokine levels at day 7 and found significant increases in IL-1β, IL-6 and TNF-α in TNBS animals, which were again ameliorated by PHDi-treatment in a concentration dependent manner **(Figure 2E-G**, p<0.01**)**. IL-10 was unchanged in colon homogenates **(Figure 2H)**. Such findings reveal that PHDi controls systemic pyrogenic responses in murine colitis.

AKB-4924 significantly reduces intestinal permeability and endotoxemia in TNBS colitis

We next assessed the influence of AKB-4924 on epithelial barrier function. As PHDi pre-treatment did not prevent pyrexia (fever) in TNBS colitic mice, we hypothesised that the pyrexia observed was due to LPS exposure that occurred as a result of increased intestinal epithelial permeability associated with acute colitis. To test this, we first examined intestinal permeability in untreated and AKB-4924 treated TNBS and control animals. TNBS animals

had increased intestinal permeability 2 days after administration compared to untreated controls ($p<0.01$), and permeability continued to increase up to 7 days **(Figure 3A**, $p<0.01$**)**. PHDi treatment significantly reduced both initial (day 2, $p<0.05$) and later (days 3 and 7, $p<0.01$) increases in permeability. To investigate whether such increases in permeability could result in endotoxemia, we examined serum LPS levels. Untreated TNBS colitic animals had significantly higher levels of serum LPS than healthy control groups throughout the time course **(Figure 3B**, $p<0.01$**)**. Serum LPS levels were significantly reduced (>80%) in colitic animals administered AKB-4924 at all time points tested ($p<0.01$).

We have previously demonstrated that HIF stabilization promotes barrier function through enhanced mucosal wound healing through mechanisms including induction of the HIF-1 target genes intestinal trefoil factor (ITF) [19] and 5'ecto-nucleotidase (CD73) [20]. As shown in **Figure 3C**, enriched intestinal epithelial cell preparations from AKB-4924-treated animals revealed that PHDi-treatment induced significant increases in both ITF ($p<0.05$) and CD73 ($p<0.05$) at day 7 compared to untreated controls **(Figure 3C)**. *Likewise, examination of the wound healing-associated HIF target gene, β1 integrin (ITGB1)[7], revealed an early increase in response to AKB-4924* (**Figure 3D,** day 3, $p<0.05$ compared to vehicle control) which had resolved by day 7 (p=not significant compared to vehicle). We next assessed the relative expression of HIF in epithelial and lamina propria cells from control and TNBS colitis animals in the presence AKB-4924. Nuclear isolates from enriched intestinal epithelial cells and epithelial denuded lamina propria tissue were examined for HIF-1α by Western blot and revealed a preferential stabilization of HIF-1α in intestinal epithelial cells of animals treated with AKB-4924 **(Figure 3E)**.

In order to assess whether the serum LPS was a result of bacterial translocation across the epithelium, we isolated and cultured total colony forming units (CFU) of aerobic bacterial populations from the blood, mesenteric lymph nodes (MLN), liver, spleen and

kidneys of TNBS colitis animals treated with PHDi or vehicle. There was a significant decrease in recovered viable bacteria in the blood and organs of TNBS colitis animals treated with AKB-4924, compared to vehicle control ***(Figure 4A)****. Non-colitic animals demonstrated negligible bacterial translocation to extra-intestinal organs (data not shown).*

Given the level of translocated viable bacteria entering the circulation in colitic animals and based on previous studies demonstrating that another PHDi, DMOG, increased the phagocytic capacity of neutrophils[12]*, we investigated whether AKB-4924 influenced the capacity of leukocytes to phagoctose bacteria. Isolated mouse neutrophils and macrophages demonstrated an increased phagocytosis of heat-inactivated E. coli* ***(Figure 4B****, p<0.05****)****. Together these data suggest that the mucosal protection of afforded by treatment is multi-factorial, enhancing both epithelial barrier function and leukocyte phagocytosis.*

Epithelial HIF-1α is required for PHDi-induced mucosal protection in TNBS colitis

Guided by our results that PHDi preferentially stabilized epithelial HIF and barrier function is enhanced by PHDi, we examined the relative importance of intestinal epithelial HIF-1α. To achieve this, we utilized intestinal epithelial-specific Hif1a-deficient mice[21]*. As shown in* ***Figure 5A****, and as we have shown in the past*[21]*, HIF-1α is stabilized by colitis induction using TNBS. The addition of AKB-4924 significantly enhanced such induction (****Figure 5A)*** *with essentially undetectable levels in intestinal epithelial-specific Hif1a-deficient mice.*

To define the functional contribution of epithelial HIF-1α, we examined whether PHDi-treatment protected intestinal epithelial-specific Hif1a-deficient mice from TNBS colitis. Consistent with previous studies[21]*, intestinal epithelial-specific HIF-1α-deficient mice developed more severe colitis than wildtype animals as measured by weight loss* ***(Figure 5B****, p<0.01****)*** *and colon shortening* ***(Figure 5C****, p<0.025****)****. Importantly, AKB-4924 did not significantly influence the course of disease in epithelial-specific HIF-1α-deficient mice*

(p=not significant for weight loss or colon length). Together these data suggest that epithelial HIF-1α-mediated effects are central to the protective actions of PHDi treatment in colitis. These findings suggest that PHDi treatment potently augments and increases restitution of intestinal barrier function, thereby significantly limiting the passage of bacterial products across the mucosa.

AKB-4924 promotes remission in established TNBS colitis

Given that PHDi-treatment enhanced suppressed inflammatory cytokine activity, we hypothesised that treatment could promote remission of active murine colitis. Colitic mice were treated with PHDi or vehicle at the peak of acute disease (day 2). PHDi-treated animals showed significant weight gain within 48 hours ***(Figure 6A)*** *and had comparable weight, colon length* ***(Figure 6B****, p<0.05****)*** *and lymph node cellularity* ***(Figure 6C****, p<0.05****)*** *to controls 5 days post treatment. PHDi treatment also significantly reduced disease activity score compared to untreated animals* ***(Figure 6D****, p<0.05****)****. In addition, PHDi-treated animals did not develop hypothermia and temperatures returned to baseline over the course of treatment, in contrast to vehicle treated animals that developed hypothermia as disease progressed* ***(Figure 6E****, p<0.05****)****. Upon examination of serum cytokine levels, AKB-4924 suppressed the levels of the major pyrogens IL-1β (p<0.01****)****, IL-6* ***(*** *p<0.01****)*** *and TNF-α* ***(****p<0.01****)*** *(data not shown) compared to vehicle treated TNBS animals. These data demonstrate that PHDi-treatment can reverse acute inflammatory colitis.*

AKB-4924 is protective in a spontaneous TNF-α mediated transgenic mouse model of ileitis

To determine the universality of our observed protection afforded by AKB-4924, we extended these studies to a genetic mouse model of spontaneous intestinal inflammation. To do this, we assessed the therapeutic potential of AKB-4924 in the TNFΔARE mouse model[22]*.*

*These mice spontaneously develop transmural Crohn's disease-like chronic inflammation in the terminal ileum. Ten-week old TNFΔARE mice were treated every second day for 10 days with AKB-4924 (0.5mg/mouse/dose i.p.). As determined by quantitative histologic examination, treatment of these animals with AKB-4924 revealed significant decreases in overall histopathology (**Figure 7A**), acute (**Figure 7B**) and chronic inflammation (**Figure 7C**), villus distortion (**Figure 7D**) and overall inflammatory indices (**Figure 7E**). These findings suggest that attenuation of inflammation by PHDi is not limited to chemically-induced colitis models and is broadly protective in different models of intestinal inflammation.*

Discussion

This study aimed to clarify the relative role of innate immunity to mucosal protection afforded by a new HIF-1α isoform-predominant PHDi, AKB-4924, in mouse models of intestinal inflammation. Previous work has demonstrated that pharmacological HIF stabilization offers protection to the mucosal barrier [6, 9], and that HIF promotes barrier restitution [20], cell survival [8] and accelerates the healing process [7] in models of murine colitis. Here, we extended these studies by demonstrating that a PHDi promotes innate immunity, suppresses the pyrexic response *in vivo* during colitis. *We show that pharmacologic HIF-1 stabilization enhances epithelial barrier function and diminishes endotoxemia associated with increased bacterial translocation in colitis.* Studies in conditional *Hif1a*-null animals revealed a central role for intestinal epithelial HIF-1α in mucosal protection associated with PHDi-based therapeutic strategies.

Inflammatory bowel diseases (IBD) are characterised by repeated wounding of the mucosa, loss of the intestinal epithelial barrier, inflammation and ultimately bacteremia. Indeed, IBD patients have increased intestinal permeability [23-25] and elevated levels of serum endotoxin [3, 26, 27]. This is mirrored in experimental models of colitis, where serum endotoxin drives the secretion of pyrogens [28] leading to early pyrexia and later onset of hypothermia [18]. We observed an early pyrogenic response in animals with TNBS colitis, coupled with increased serum levels of classical pyrogens IL-1β, IL-6 and TNF-α. This is likely to be driven by significantly increased intestinal permeability leading to elevated serum LPS. PHDi treatment of mice with TNBS-induced colitis led to reduced intestinal permeability and significantly lower levels of serum LPS. Interestingly, treated animals did develop some pyrexia and exhibited early increases in pyrogens, although this was significantly lower than in untreated controls, suggesting that sufficient endotoxin exposure occurred to induce fever. However, treatment prevented the development of hypothermia, which was concurrent with

the suppression of serum pyrogens and secretion of IL-10. This led us to investigate whether HIF activation directly regulated innate responses that were driven by LPS exposure.

Hypoxia occurs concurrently with inflammatory responses in IBD and colitis in mice, most likely through the combination of increased metabolic demands of inflamed tissue and altered blood flow that results from tissue damage [29-31]. It is now accepted that HIF plays an important role in regulating immune cell function and suppressing inflammation. Furthermore, the induction of HIF promotes the bactericidal activities of phagocytic cells [13, 32] and supports the innate immune functions of dendritic cells [33], mast cells [34] and epithelial cells [7, 35]. The role of HIF in immune cell regulation is unsurprising, given the steep oxygen gradient between healthy and inflamed tissue. The oxygen content of healthy tissues typically range from 2.5 to 9% oxygen, while markedly lower levels (less than 1% oxygen) occur in inflamed sites [36]. In order to function in these hypoxic environments, immune cells can adapt to hypoxia and indeed, hypoxia driven HIF signaling appears to be an important input signal for innate immune defense.

It is now accepted that HIF is the major transcriptional regulator of epithelial compensatory pathways in hypoxia, including the induction of HIF-target genes ITF [19], CD73 [20], and the adenosine A2B receptor [37]. Stabilization of HIF by inhibition of PHD has been shown to drive the expression of these barrier protective genes [6, 38] and also to promote restitution of the damaged mucosa through induction of the β_1 integrin ITGB1 [7]. In addition PHDi-driven NFκB activation has been demonstrated to promote anti-apoptotic gene expression in epithelial cells [8] and it is proposed that the culmination of these pathways leads to the mucosal protection promoted by PHDi in models of IBD [38]. In our studies AKB-4924 preferentially stabilized HIF-1α in intestinal epithelial cells. *In addition, AKB-4924 offered no protection to TNBS colitis in a mouse strain with a targeted epithelial deletion of HIF-1α. In wildtype TNBS mice treated with AKB-4924, isolated epithelial cells showed induction of ITF*

and CD73, while the kinetics of ITGB1 expression were accelerated, suggesting earlier epithelial restitution. Thus it appears that while HIF induction in leukocytes may augment innate responses to bacteremia during mucosal inflammation, the loss of barrier function, or perhaps critically, the loss of reparative pathways, will eventually overwhelm the innate immune system. The observation that AKB-4924 reduced mucosal inflammation in TNFΔARE mice supports these findings. These animals display a translational dysregulation of TNF message, resulting in TNF-α overproduction and loss of TNF-driven modulation of hemopoietic cells [22] that are important in the maintenance of gastrointestinal immunological homeostasis.

In summary, we have demonstrated that systemic administration of PHDi suppresses inflammatory signaling and promotes increased phagocytosis of bacteria, protecting against colitis-induced bacteremia. It is likely that the prevention of exposure to the luminal contents through increased epithelial barrier function and accelerated wound healing that is central to the protection by PHDi observed in murine models of colitis.

Methods

Animal models of colitis

Age-matched (6 week old), female C57BL/6 mice were housed for one week to allow microflora equilibration. Mice were anesthetized with isoflourane, shaved, and sensitized by epicutaneous application of 1% TNBS (Sigma Chemical, St. Louis, MO) in 100% ethanol. After 7 days, mice were again anesthetized with isoflourane and intrarectally administered 5 μl/g body weight of a 2.5% TNBS solution as previously described [6]. Vehicle treated control animals received an equivalent volume of 50% ethanol alone. Mice were monitored for development of disease over 7 days. In some experiments, mice lacking intestinal epithelial *Hif1a* expression [21] were used to determine HIF isoform specificity. TNFΔARE mice were housed for 10 weeks under specific pathogen free conditions as previously described [39].

The PHDi AKB-4924 (Aerpio Therapeutics, Cincinnati, OH, [0.3, 1, or 5 mg/Kg]) was administered daily via 100μL subcutaneous injection to the scruff. Control was 100 μL cyclodextrin vehicle. Mice were monitored daily and all protocols were performed in strict adherence with institutional animal ethics guidelines. Animals' weights were monitored every 24 hours over the course of the experiment from day -1 relative to TNBS induction. Temperature was measured via infrared thermometer during the course of disease and treatment.

Sample analysis

Colons were excised and divided for protein, mRNA and histological analysis. Samples for protein analysis were stored in Tris-lysis buffer, mRNA was isolated by Trizol mRNA isolation and histological samples were fixed in 4% formalin. In subsets of experiments, intestinal epithelial cells were isolated for mRNA analysis as previously described[40]. *Western blotting for HIF-1α was performed as previously described*[21]. Blood was collected by cardiac

puncture. Serum, mRNA and protein samples were stored at -80^{o}C until analysis. Protein analysis of serum and colon tissue was carried out by Mesoscale high-sensitivity or conventional ELISA. *mRNA analysis was performed by real-time-PCR asaays using previously validated primers for CD73* [21], *ITF* [21] *and ITGB1* [7]. Serum endotoxin levels were assessed using colorimetric assays according to the manufacturer's instructions (QCL-1000, Lonza).

Permeability assays

Permeability assays were conducted with intestinal tissue sacs based on previously described assays [41]. *Briefly, at each endpoint, animals were euthanized and mouse colons were excised and flushed with oxygenated TC199 medium. The colons were tied tightly at one end with silk suture and a small animal vascular catheter (Data Sciences International Physiocath 277-1-002) was tied in to the other end to form an intestinal sac. Each colon yielded two sacs, 2 cm long. A 1 ml syringe with a sterile 26 gauge micro lance was fixed to the catheter and 250 µL of FD-4 (1.0 mg/mL) was injected into each sac lumen. Each sac was placed into separate 50 mL conical tubes containing 15 ml of oxygenated TC-199 medium on a shaking water bath for 30 min at 37°C, according to the method of Barthe et al.* [42]. *Samples (50 µL) were collected from the bath every 15 min and replaced with fresh medium. After 120 min, the sacs were cut open and the contents sampled. FD-4 concentration was calculated from fluorescent standard curves (Spectramax m5e, Molecular Devices, USA). The velocity of FD-4 across the intestinal barrier; the apparent permeability (P_{app}) for FD-4, was calculated from the following equation:*

$$P_{app}\ (cm/s) = (dQ/dt)/(A \cdot Co),$$

where dQ/dt is the transport rate (mol/s of FD-4), A is the surface area of the monolayer or sac (cm^2), and Co is the initial concentration in the donor compartment (mol/mL)[41, 43, 44].

Leukocyte isolation phagocytosis assays

To isolate murine neutrophils and macrophages, C57BL/6 mice were administered 1 ml of 3% sterile thioglycolate (Sigma) intraperitoneally as previously described [45-47]. For neutrophil isolation, animals were sacrificed after 6 hours, and peritoneal lavage was performed using 10 ml of endotoxin-free PBS. The lavage fluid was centrifuged at 600 ×g for 10 min. Total cells were enumerated using a hemocytometer and neutrophils were enumerated (>90%) by Wright-Giemsa staining. For macrophage isolation, the assay was performed as above but animals were lavaged 72 hours after thioglycolate administration.

For phagocytosis assays, E. coli were cultured to log phase in LB broth, heat inactivated at 60°C for 1 hour, and labeled with FITC as previously described [12]. Isolated phagocytes were equilibrated in hypoxia or normoxia, with or without 10 μM AKB-4924 for 1 hour prior to the addition of bacteria at a ratio of 1:10 for a further hour. Cells were then pelleted, washed and extracellular FITC was quenched with Trypan blue. Fluorescence was assayed in a 96 well fluorometic plate reader and the percentage phagocytosis calculated, relative to the inoculum.

Microbiological analysis

Detection of viable enteric bacteria in extraintestinal organs was performed by organ culture and CFU count [48, 49]. Samples of MLN, liver, kidney and spleen were removed under sterile conditions immediately upon euthanasia. Tissues were dissected free from fat, weighed, and homogenised in 5 mL of sterile PBS. Serial dilutions of homogenates were plated on MacConkey and blood agar plates and incubated for up to 72 h at 37°C. After incubation, colony forming units (CFU) were counted and adjusted by tissue sample weight (CFU/g). Blood samples were obtained by sterile cardiac puncture, aseptically diluted in blood heart

infusion (BHI) broth, cultured for 48 hours and plated as above. Viable bacteria counts were expressed as CFU/mL blood.

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References

1. Karrasch T, Jobin C. Wound healing responses at the gastrointestinal epithelium: a close look at novel regulatory factors and investigative approaches. *Zeitschrift fur Gastroenterologie* 2009; **47**(12)**:** 1221-1229.

2. Lawrance IC, Radford-Smith GL, Bampton PA, Andrews JM, Tan PK, Croft A *et al.* Serious infections in patients with inflammatory bowel disease receiving anti-tumor-necrosis-factor-alpha therapy: an Australian and New Zealand experience. *J Gastroenterol Hepatol* 2010; **25**(11)**:** 1732-1738.

3. Pastor Rojo O, Lopez San Roman A, Albeniz Arbizu E, de la Hera Martinez A, Ripoll Sevillano E, Albillos Martinez A. Serum lipopolysaccharide-binding protein in endotoxemic patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**(3)**:** 269-277.

4. Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR *et al.* Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 2004; **113**(9)**:** 1296-1306.

5. Ward JB, Lawler K, Amu S, Taylor CT, Fallon PG, Keely SJ. Hydroxylase inhibition attenuates colonic epithelial secretory function and ameliorates experimental diarrhea. *FASEB J* 2011; **25**(2)**:** 535-543.

6. Robinson A, Keely S, Karhausen J, Gerich ME, Furuta GT, Colgan SP. Mucosal protection by hypoxia-inducible factor prolyl hydroxylase inhibition. *Gastroenterology* 2008; **134**(1)**:** 145-155.

7. Keely S, Glover LE, MacManus CF, Campbell EL, Scully MM, Furuta GT *et al.* Selective induction of integrin beta1 by hypoxia-inducible factor: implications for wound healing. *Faseb J* 2009; **23**(5)**:** 1338-1346.

8. Tambuwala MM, Cummins EP, Lenihan CR, Kiss J, Stauch M, Scholz CC *et al.* Loss of prolyl hydroxylase-1 protects against colitis through reduced epithelial cell apoptosis and increased barrier function. *Gastroenterology* 2010; **139**(6)**:** 2093-2101.

9. Cummins EP, Seeballuck F, Keely SJ, Mangan NE, Callanan JJ, Fallon PG *et al.* The hydroxylase inhibitor dimethyloxalylglycine is protective in a murine model of colitis. *Gastroenterology* 2008; **134**(1)**:** 156-165.

10. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. *EMBO J* 2003; **22**(16)**:** 4082-4090.

11. Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F *et al.* Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. *Proc Natl Acad Sci U S A* 2006; **103**(48)**:** 18154-18159.

12. Walmsley SR, Cowburn AS, Clatworthy MR, Morrell NW, Roper EC, Singleton V *et al.* Neutrophils from patients with heterozygous germline mutations in the von Hippel Lindau protein (pVHL) display delayed apoptosis and enhanced bacterial phagocytosis. *Blood* 2006; **108**(9)**:** 3176-3178.

13. Walmsley SR, Print C, Farahi N, Peyssonnaux C, Johnson RS, Cramer T *et al.* Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *J Exp Med* 2005; **201**(1)**:** 105-115.

14. Fritzenwanger M, Jung C, Goebel B, Lauten A, Figulla HR. Impact of short-term systemic hypoxia on phagocytosis, cytokine production, and transcription factor activation in peripheral blood cells. *Mediators Inflamm* 2011; **2011:** 429501.

15. Anand RJ, Gribar SC, Li J, Kohler JW, Branca MF, Dubowski T *et al.* Hypoxia causes an increase in phagocytosis by macrophages in a HIF-1alpha-dependent manner. *J Leukoc Biol* 2007; **82**(5)**:** 1257-1265.

16. Hams E, Saunders SP, Cummins EP, O'Connor A, Tambuwala MT, Gallagher WM *et al.* The hydroxylase inhibitor dimethyloxallyl glycine attenuates endotoxic shock via alternative activation of macrophages and IL-10 production by b1 cells. *Shock* 2011; **36**(3)**:** 295-302.

17. Okumura CY, Hollands A, Tran DN, Olson J, Dahesh S, von Kockritz-Blickwede M *et al.* A new pharmacological agent (AKB-4924) stabilizes hypoxia inducible factor-1 (HIF-1) and increases skin innate defenses against bacterial infection. *J Mol Med (Berl)* 2012.

18. Leon LR. Hypothermia in systemic inflammation: role of cytokines. *Front Biosci* 2004; **9:** 1877-1888.

19. Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S *et al.* Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. *J Exp Med* 2001; **193**(9)**:** 1027-1034.

20. Synnestvedt K, Furuta GT, Comerford KM, Louis N, Karhausen J, Eltzschig HK *et al.* Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J Clin Invest* 2002; **110**(7)**:** 993-1002.

21. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH. Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J Clin Invest* 2004; **114**(8)**:** 1098-1106.

22. Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 1999; **10**(3)**:** 387-398.

23. Welcker K, Martin A, Kolle P, Siebeck M, Gross M. Increased intestinal permeability in patients with inflammatory bowel disease. *Eur J Med Res* 2004; **9**(10)**:** 456-460.

24. Takeuchi K, Maiden L, Bjarnason I. Genetic aspects of intestinal permeability in inflammatory bowel disease. *Novartis Found Symp* 2004; **263:** 151-158; discussion 159-163, 211-158.

25. Oriishi T, Sata M, Toyonaga A, Sasaki E, Tanikawa K. Evaluation of intestinal permeability in patients with inflammatory bowel disease using lactulose and measuring antibodies to lipid A. *Gut* 1995; **36**(6)**:** 891-896.

26. Caradonna L, Amati L, Lella P, Jirillo E, Caccavo D. Phagocytosis, killing, lymphocyte-mediated antibacterial activity, serum autoantibodies, and plasma endotoxins in inflammatory bowel disease. *Am J Gastroenterol* 2000; **95**(6)**:** 1495-1502.

27. Gardiner KR, Halliday MI, Barclay GR, Milne L, Brown D, Stephens S *et al.* Significance of systemic endotoxaemia in inflammatory bowel disease. *Gut* 1995; **36**(6)**:** 897-901.

28. Barbier M, Cherbut C, Aube AC, Blottiere HM, Galmiche JP. Elevated plasma leptin concentrations in early stages of experimental intestinal inflammation in rats. *Gut* 1998; **43**(6)**:** 783-790.

29. Kokura S, Yoshida N, Yoshikawa T. Anoxia/reoxygenation-induced leukocyte-endothelial cell interactions. *Free Radic Biol Med* 2002; **33:** 427-432.

30. Haddad JJ. Science review: redox and oxygen-sensitive transcription factors in the regulation of oxidant-mediated lung injury: role for hypoxia-inducible factor-1alpha. *Crit Care* 2003; **7:** 47-54.

31. Saadi S, Wrenshall LE, Platt JL. Regional manifestations and control of the immune system. *Faseb J* 2003; **16:** 849-856.

32. Elks PM, van Eeden FJ, Dixon G, Wang X, Reyes-Aldasoro CC, Ingham PW *et al.* Activation of hypoxia-inducible factor-1alpha (Hif-1alpha) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model. *Blood* 2011; **118**(3)**:** 712-722.

33. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG *et al.* Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. *J Immunol* 2008; **180**(7)**:** 4697-4705.

34. Jeong HJ, Moon PD, Kim SJ, Seo JU, Kang TH, Kim JJ *et al.* Activation of hypoxia-inducible factor-1 regulates human histidine decarboxylase expression. *Cell Mol Life Sci* 2009; **66**(7)**:** 1309-1319.

35. MacManus CF, Campbell EL, Keely S, Burgess A, Kominsky DJ, Colgan SP. Anti-inflammatory actions of adrenomedullin through fine tuning of HIF stabilization. *FASEB J* 2011; **25**(6)**:** 1856-1864.

36. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. *Nat Rev Immunol* 2009; **9**(9)**:** 609-617.

37. Frick JS, MacManus CF, Scully M, Glover LE, Eltzschig HK, Colgan SP. Contribution of adenosine A2B receptors to inflammatory parameters of experimental colitis. *J Immunol* 2009; **182**(8)**:** 4957-4964.

38. Taylor CT, Colgan SP. Hypoxia and gastrointestinal disease. *J Mol Med (Berl)* 2007; **85**(12)**:** 1295-1300.

39. Collins CB, Aherne CM, McNamee EN, Lebsack MD, Eltzschig H, Jedlicka P *et al.* Flt3 ligand expands CD103+ dendritic cells and FoxP3+ T regulatory cells, and attenuates Crohn's-like murine ileitis. *Gut* 2012; **61**(8)**:** 1154-1162.

40. Campbell EL, MacManus CF, Kominsky DJ, Keely S, Glover LE, Bowers BE *et al.* Resolvin E1-induced intestinal alkaline phosphatase promotes resolution of inflammation through LPS detoxification. *Proc Natl Acad Sci U S A* 2010; **107**(32)**:** 14298-14303.

41. Keely S, Rullay A, Wilson C, Carmichael A, Carrington S, Corfield A *et al.* In vitro and ex vivo intestinal tissue models to measure mucoadhesion of poly (methacrylate) and N-trimethylated chitosan polymers. *Pharm Res* 2005; **22**(1)**:** 38-49.

42. Barthe L, Woodley J, Houin G. Gastrointestinal absorption of drugs: methods and studies. *Fundam Clin Pharmacol* 1999; **13**(2)**:** 154-168.

43. Keely S, Feighery L, Campion DP, O'Brien L, Brayden DJ, Baird AW. Chloride-led disruption of the intestinal mucous layer impedes Salmonella invasion: evidence for an 'enteric tear' mechanism. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2011; **28**(4)**:** 743-752.

44. Keely S, Ryan SM, Haddleton DM, Limer A, Mantovani G, Murphy EP *et al.* Dexamethasone-pDMAEMA polymeric conjugates reduce inflammatory biomarkers in human intestinal epithelial monolayers. *Journal of controlled release : official journal of the Controlled Release Society* 2009; **135**(1)**:** 35-43.

45. Coxon A, Rieu P, Barkalow FJ, Askari S, Sharpe AH, von Andrian UH *et al.* A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity* 1996; **5**(6)**:** 653-666.

46. Mizgerd JP, Quinlan WM, LeBlanc BW, Kutkoski GJ, Bullard DC, Beaudet AL *et al.* Combinatorial requirements for adhesion molecules in mediating neutrophil emigration during bacterial peritonitis in mice. *J Leukoc Biol* 1998; **64**(3)**:** 291-297.

47. Fortier AH, Falk LA. Isolation of murine macrophages. *Curr Protoc Immunol* 2001; **Chapter 14.1:** 1-9.

48. Asfaha S, MacNaughton WK, Appleyard CB, Chadee K, Wallace JL. Persistent epithelial dysfunction and bacterial translocation after resolution of intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**(3)**:** G635-644.

49. Llopis M, Antolin M, Guarner F, Salas A, Malagelada JR. Mucosal colonisation with Lactobacillus casei mitigates barrier injury induced by exposure to trinitronbenzene sulphonic acid. *Gut* 2005; **54**(7)**:** 955-959.

Figure Legends:

***Figure 1**: **Influence of AKB-4924 treatment on disease activity in TNBS colitis**. AKB-4924 (0, 0.3, 1.0 or 5.0 mg/Kg in cyclodextrin vehicle) was administered subcutaneously on day -1 relative to TNBS or EtOH intrarectal lavage. (A) Weight was monitored daily as an indicator of disease severity and normalised relative to initial weight and control animals. On day 7 animals were sacrificed and colons were excised and measured to assess (B) colon length. Panel C represents H&E staining of tissue isolated from control animals (i), TNBS animals treated with vehicle (ii), and TNBS animal treated with AKB-4924 (iii). (D) Disease activity was as assessed as sum of scoring (0-3) for colon thickening (colon weight/length), occult blood, fecal pellet consistency and % weight loss. (E) Temperature was measured by infrared (IR) thermometer at t=0, 6, 12 and 24 hrs and then at 24 hr intervals until sacrifice. N=6, *p<0.05, **p<0.01, ANOVA (A, E), two-tailed, Students T-test (B, D).*

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***Figure 2: Influence of AKB-4924 treatment on pyrogen levels in TNBS colitis**. AKB-4924 (5.0 mg/Kg in cyclodextrin vehicle) or vehicle-treated animals were sacrificed on day 2, 3 or 7 relative to TNBS or EtOH rectal lavage. Blood was collected by cardiac puncture and serum assayed in triplicate for each animal by multiplex ELISA for (A) IL-1β, (B) IL-6, (C) TNF-α, (D) IL-10. On day 7, whole colon tissue was homogenised in lysis buffer and assayed by ELISA for (E) IL-1β, (F) IL-6, (G) TNF-α, (H) IL-10. N=6, *p<0.05, **p<0.01 two-tailed Students T-test.*

***Figure 3: Influence of AKB-4924 on intestinal epithelial barrier function and HIF target gene expression.** AKB-4924 (0 or 5.0 mg/Kg in cyclodextrin vehicle) was administered subcutaneously on day -1 relative to TNBS or EtOH intrarectal lavage. Animals were sacrificed on day 2, 3 or 7 and colons were excised and tied off by suture into intestinal sacs.*

*Sacs were loaded with FITC-dextran 4400 (FD-4; 500 μg/mL) and (A) the apparent permeability (P_{app}) was assessed. Blood was collected by sterile cardiac puncture and (B) serum assayed for LPS content. Colon epithelial mRNA was screened by qPCR for induction of the HIF target genes CD73 and ITF (C) or ITGB1 (D). Panel E represents western blot analysis for HIF-1α in nuclear isolates from intestinal epithelial cells (IEC) and lamina propria cells (LP) in control and TNBS colitis animals in the presence of AKB-4924. TATA binding protein was employed as a housekeeper. N=6, *, ^, # p<0.05, **, ^^, ## p<0.01, ANOVA (A, B, D), two-tailed, Students T-test (C).*

Figure 4 Influence of AKB-4924 on systemic bacteremia in TNBS colitis

*Panel A shows recovery of viable bacteria translocated to blood and extraintestinal organs (liver, kidney, spleen and MLN; mesenteric lymph nodes in TNBS colitis animals after treatment with vehicle or AKB-4924 (5 mg/Kg). Bacterial counts are expressed in colony forming units, per gram tissue (CFU/g), except for ^ (blood) where counts are expressed as CFU/mL. (B) Murine neutrophils and macrophages were isolated by peritoneal lavage and incubated with 10μM AKB-4924 (AKB) or incubated in hypoxia (Hx; pO_2, 20 torr for 6h) or normoxia (Nx) and assessed for the ability to phagocytose FITC-labelled E. Coli. N=5, *p<0.05, **p<0.01 two-tailed, Students T-test.*

Figure 5: Functional epithelial HIF-1α is critical for AKB-4924-induced mucosal protection. *Panel A represents Western blot analysis for HIF-1α from epithelial scrapings from IEC HIF-1$\alpha^{+/+}$ and IEC HIF-1$\alpha^{-/-}$ animals subjected to TNBS colitis in the presence and absence of AKB-4924 (0 or 5.0 mg/Kg), which was administered subcutaneously on day -1 relative to TNBS or EtOH by intrarectal lavage. Cyclodextrin was administered as a control. (B) Weight was measured daily as an indicator of disease severity. On day 7 animals were*

*sacrificed and colons were excised and measured to assess (C) colon shortening. N=5, *p<0.05, **p<0.01, ANOVA (B), two-tailed, Students T-test (C).*

Figure 6: Influence of AKB-4924 on animals with active TNBS colitis. *AKB-4924 (0 or 5.0 mg/Kg) was administered subcutaneously on day of peak weight loss (day 2) relative to TNBS or EtOH intrarectal lavage. Cyclodextrin was administered as a control. (A) Weight was measured daily as an indicator of disease severity and normalized relative to initial weight and control animals. On day 7 animals were sacrificed and (B) colons were excised and assessed for shortening, and (C) total cell populations in mesenteric lymph nodes (MLN) were counted using a haemocytometer. (D) Disease activity was as assessed as sum of scoring (0-3) for colon thickening (colon weight/length), occult blood, faecal pellet consistency and % weight loss. (E) Temperature was measured by IR thermometer at 24hr intervals from first AKB-4924 administration until sacrifice. N=6, *p<0.05, **p<0.01, ANOVA (A, E), two-tailed, Students T-test (B, C, D).*

Figure 7: Influence of AKB-4924 on TNFΔARE ileitis. *AKB-4924 (0 or 5.0 mg/Kg) was administered to 10-week old TNFΔARE mice every second day over a 10 day period. Animals were assessed (A) histologically, and scored for (B) acute inflammatory index, (C) chronic inflammatory index, (D) villus inflammatory index and (E) total inflammatory index. N=5, *p<0.05, two-tailed, Students T-test.*