# Contribution of innate immune responses to protection induced by inhibition of prolyl hydroxylase in murine colitis

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**Running title**: Innate immune regulation by PHDi

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**Abstract**

Pharmacological stabilization of hypoxia-inducible factor (HIF) through inhibition of prolyl hydroxylation (PHDi) is a new approach to limit damage associated with murine colitis models. Far less is known about how PHDi influences immune function during mucosal inflammation or the relative importance of immunological changes to mucosal protection. We hypothesized that PHDi modulates systemic innate immune responses to colitis-associated bacteremia. Mice with colitis induced by TNBS were administered AKB-4924, a new HIF-1 isoform-predominant PHDi and clinical, immunological and biochemical endpoints were assessed. Administration led to significantly reduced weight loss and disease activity compared to vehicle controls. Treated groups were pyrexic, but did not become hypothermic. AKB-4924 augmented epithelial barrier function and led to an approximately 50-fold reduction in serum endotoxin during colitis. The inhibitor also decreased cytokines involved in pyrogenesis and cryogenesis, significantly reducing serum levels of IL-1β, IL-6 and TNF-α, while increasing IL-10. In parallel, AKB-4924 induced increased neutrophil and macrophage phagocytotic capacity and reduced TNF-α expression ex vivo. AKB-4924 offered no protection against colitis in epithelial-specific HIF-1α deficient mice, strongly implicating epithelial HIF-1α in protection. Taken together, these results indicate that PHDi enhances innate immunity at multiple levels and that the epithelium is a central site of inflammatory protection 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 may lead 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]. Inhibition of prolyl hydroxylase (PHD) 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 phagocytotic 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, though suppression of inflammatory cytokines and enhanced IL-10 production [16]. Thus, it is likely that PHD treatment studies in murine models of colitis also involve an innate cell response, driven by HIF stabilization in neutrophils and macrophages but the importance of these processes have not yet been defined. 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. For this reason, therapeutic strategies employing PHDi in mouse models have favoured systemic administration of the drug, usually interperitoneal injection [6, 9], likely to affect other tissues.

Here, we hypothesized that subcutaneous administration of AKB-4924 affects innate cell responses in a mouse colitis model. Employing the chemically-induced trinitrobenzene sulfonic acid (TNBS) murine model of colitis, we focused on the pyrexic response to bacteremia associated with intestinal inflammation. We examined inflammatory signaling and phagocytotic activity in neutrophils and phagocytes and with the use of 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, increases innate cell phagocytosis and suppresses inflammatory signaling, and that epithelial HIF-regulation is critical for the mucosal protection in mouse models of colitis.

## Results

### AKB-4924 reduces TNBS disease pathology

We first hypothesised that PHDi treatment would influence systemic responses to inflammation, particularly those associated with epithelial barrier dysfunction in a TNBS model of colitis. Initially, we examined the pyrogenic response in TNBS colitic mice that had been treated with AKB-4924, a new HIF-1 predominant PHDi [18]. Animals treated with AKB-4924 showed reduced weight loss **(Figure 1A**, $p<0.01$**)**, attenuated colon shortening **(Figure 1B**, $p<0.05$**)** and decreased disease activity **(Figure 1C**, $p<0.01$**)** at all doses tested, with as little as 0.3mg/Kg AKB-4924 showing marked improvements in each of these endpoints. Interestingly, there was no difference in the early pyrogenic response (+1.1±0.56ºC at 6 hours post TNBS induction, $p<0.001$ compared to vehicle control) to TNBS colitis induction between AKB-4924-treated and untreated animals **(Figure 1D).** However, the subsequent hypothermic response observed in untreated TNBS mice (-1.65±0.58ºC at 168 hours post TNBS induction, $p<0.001$ compared to EtOH control) was not observed in the AKB-4924-treated groups (-0.03±0.50ºC, 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 progression of TNBS colitis. At the peak of acute disease (measured by weight loss, day 2 post TNBS), TNBS-treated animals showed significantly elevated levels of IL-1β (21±9.1-fold**, Figure 2A**, $p<0.001$). At day 2, there was no significant reduction of IL-1β in PHDi-treated animals. However, by day 3 and progressing to day 7, PHDi strongly reduced IL-1β expression ($p<0.01$) compared to untreated colitic mice. Similarly, TNBS colitis animals showed a significant increase in IL-6

(33±11.2-fold, **Figure 2B**) compared to controls. By day 3, there was a significant 2-fold reduction in IL-6 and by day 7 PHDi-treated animals had serum-IL-6 levels comparable to controls (27±3.6-fold reduction). TNF-α was not significantly increased in TNBS-animal serum by day 2, but was significantly elevated in TNBS colitis animals at day 3 (4±1.2-fold) and day 7 (12±5.4-fold) compared to controls **(Figure 2C)**. PHDi-treated TNBS animals showed a significant reduction in serum TNF-α compared to TNBS animals by day 3 and was reduced 4-fold by day 7. As IL-10 is an anti-inflammatory cytokine and may suppress the hypothermic response [19], 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 samples for cytokine levels at day 7 and found significant increases in IL-1β and IL-6 in TNBS animals, which were again ameliorated in PHDi-treated animals in a concentration dependent manner **(Figure 2E and F**, $p<0.01$**)**.

**Innate immune cell responses to AKB-4924**

We next investigated whether PHDi-treatment directly suppressed innate immune cell pyrogen activity *in vitro*. Neutrophils were stimulated with LPS and PHDi treatment led to significant accumulation of nuclear HIF-1α at a level comparable to hypoxia **(Figure 3A)**, suggesting that subcutaneous treatment with PHDi may lead to HIF stabilization in innate immune cells. Furthermore, treatment significantly reduced the induction of TNF-α, IL-1β and IL-6 transcript **(Figure 3B**, $p<0.05$**)** in both mouse neutrophils and macrophages. In addition, PHDi treatment increased phagocytosis of heat-inactivated *E. coli* by both neutrophils and macrophages **(Figure 3C**, $p<0.05$**)**. To examine human relevance, we next examined pyrogen transcript levels in LPS-stimulated human neutrophils and peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood. In support of our results in mice,

PHDi-treatment significantly reduced LPS-induced TNF-α, IL-1β and IL-6 transcripts **(Figure 3D)** in human neutrophils and PBMCs.

We then extended our studies to examine the role of PHDi-mediated HIF activation in neutrophils during TNBS colitis. Mice were depleted of neutrophils and pre-treated with AKB-4924 before inducing colitis with TNBS. While AKB-4924 ameliorated disease in neutrophil depleted mice, colitis was significantly worse than in TNBS mice with neutrophils, as measured by colon shortening **(Figure 3E)**. Reductions in mucosal neutrophils were confirmed by myeloperoxidase assay **(Figure 3F)**. Histological examination of control **(Figure 3G)** and colitic **(Figures 3H-K)** mucosae showed that neutrophil depletion **(Figure 3I)** promoted colitis **(Figure 3H)**. AKB-4924 treatment led to reduced pathology in neutrophil-depleted TNBS mice **(Figure 3J)** but was enhanced in neutrophil-replete animals which had a complete reversal of pathology. Taken together these data suggest that the reduced fever and inflammatory responses following PHDi treatment may, in part, be due to the suppression of leukocyte pyrogenic cytokines, that treatment may enhance innate responses against bacteria by increasing the phagocytotic capacity of innate cells and that the presence of neutrophils is required for maximal therapeutic benefit.

**AKB-4924 promotes remission in established TNBS colitis**

Given that PHDi-treatment acted to enhance innate immune cell phagocytosis and suppressed inflammatory cytokine signalling, we hypothesised that treatment could promote remission of active colitis. TNBS colitic mice were treated with PHDi or vehicle at the peak of acute disease (day 2). PHDi-treated animals recovered significant weight within 24 hours **(Figure 4A)** and had comparable weight, colon length **(Figure 4B**, $p<0.05$**)** and lymph node cellularity **(Figure 4C**, $p<0.05$**)** to controls within 5 days post treatment. PHDi treatment also significantly reduced disease activity score compared to untreated animals **(Figure 4D**,

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, which developed hypothermia as disease progressed **(Figure 4E**, p<0.05**)**. Upon examination of serum cytokine levels, the major pyrogens TNF-α, IL-1β and IL-6 were found to be elevated in AKB-4924 treated TNBS mice, compared to non-TNBS controls, but significantly reduced compared to vehicle treated TNBS animals **(Figure 5**, p<0.01**)**. These data demonstrate that PHDi-treatment is capable of reversing acute inflammatory disease in the colon.

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

We next assessed the effects of AKB-4924 on epithelial barrier function. As PHDi pre-treatment did not prevent pyrexia 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. Initially we 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 6A**, p<0.01**)**. PHDi-treatment significantly reduced both initial (day 2) and later (days 3 and 7) 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 6B**, p<0.01**)**. Serum LPS levels were significantly reduced in colitic animals administered AKB-4924 at all periods tested (p<0.01). These findings suggest that although PHDi treatment does not completely prevent systemic endotoxin exposure, it does augment and increase restitution of intestinal barrier function, thereby significantly limiting the passage of bacterial products across the mucosa.

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

Guided by our results that both barrier function and innate immune cell responses are enhanced by PHDi, we examined the relative importance of intestinal epithelial HIF-1α. To do this, we examined whether PHDi-treatment protected intestinal epithelium-specific HIF-1α-deficient mice from TNBS colitis. Consistent with previous studies [20], intestinal epithelial-specific HIF-1α-deficient mice developed more severe colitis than wildtype animals measured by weight loss **(Figure 7A**, $p<0.01$**)** and colon shortening **(Figure 7B**, $p<0.025$**)**. Importantly, AKB-4924 did not significantly influence the course of disease in epithelial-specific HIF-1α-null mice (p=not significant for weight loss or colon length).

To further investigate the importance of epithelial HIF-1α (**Figure 7A and B**), we examined enriched intestinal epithelial cell preparations for AKB-4924-induced transcription of the HIF-1 gene targets ITF [21] and CD73 [22]. PHDi-treatment induced significant increases in both ITF ($p<0.05$) and CD73 ($p<0.05$) in colitic animals at day 7 compared to untreated controls **(Figure 7C)**. Likewise, examination of the HIF target gene ITGB1 [7] revealed an early increased in response to AKB-4924 (**Figure 7D,** day 3, $p<0.05$ compared to vehicle control) which had resolved by day 7 (p=not significant compared to vehicle). Together these data suggest that epithelial HIF-1α-mediated effects are central to the protective actions of PHDi treatment in colitis.

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

We next examined whether protection afforded by AKB-4924 was specific for TNBS colitis. To do this we assessed the ant-inflammatory potential of AKB-4924 in the TNFΔARE mouse model[23]. 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 judged by quantitative histologic examination, treatment of these animals with AKB-4924 revealed significant decreases in histopathology (**Fig. 8A**), acute (**Fig. 8B**) and chronic inflammation (**Fig. 8C**), villus distortion (**Fig. 8D**) and overall inflammatory indices (**Fig. 8E**). Such findings suggest that attenuation of inflammation by PHDi is not limited to chemically-induced colitis models and is broadly protective against inflammatory bowel diseases.

## Discussion

This study aimed to clarify the relative roles of innate immune cell responses to mucosal protection afforded by a new HIF-1 isoform-predominant PHDi (AKB-4924) in mouse models of colitis. Previous work has demonstrated that pharmacological HIF stabilization offers protection to the mucosal barrier [6, 9], and that HIF promotes barrier restitution [22], cell survival [8] and accelerates the healing process [7] in models of murine colitis. Here, we extended these studies by demonstrating that of PHDi's suppress innate immune, inflammatory and pyrexic responses in vivo during colitis. We show that pharmacologic HIF-1 stabilization enhances epithelial barrier function and phagocytic responses to bacterermia in colitis. We also identified an important role for epithelial HIF-1α in mucosal protection induced by PHDi based therapeutic strategies.

Inflammatory bowel diseases (IBD) are characterised by repeated wounding of the mucosa and loss of the intestinal epithelial barrier, inflammation and ultimately bacteremia. Indeed, IBD patients have increased intestinal permeability [24-26] and show increased levels of serum endotoxin [3, 27, 28]. This is mirrored in experimental models of colitis, where serum endotoxin drives the secretion of pyrogens [29] leading to early pyrexia (fever) and later onset of hypothermia [19]. We observed an early pyrogenic response in animals with TNBS colitis,

coupled with increased serum levels of classical pyrogens TNF-α, IL-1β and IL-6. 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, threated 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 downregulation 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 [30-32]. 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, 33] and supports the innate immune functions of dendritic cells [34], mast cells [35] and epithelial cells [7, 36]. 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 [37]. 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. AKB-4924 treatment led to a suppression of LPS-driven expression of TNF-α, IL-1β and IL-6 in both human and murine neutrophils and serum and increased serum levels of IL-10. The elevated systemic levels of IL-10 did not appear to originate from HIF stabilisation in neutrophils or PBMCs and recent evidence suggests that IL-10 may be secreted by PHDi activated B1 cells [16]. In addition, PHDi-treatment enhanced

phagocytosis by mouse neutrophils and macrophages and human neutrophils, but not PBMC's providing further evidence of an innate element to the mucosal protection afforded by PHDi treatment. Depletion of neutrophils led to a reduction in the efficacy of AKB-4924, suggesting that neutrophils play an important role in the therapeutic benefit of PHD inhibition. However AKB-4924 treated PMN-depleted colitic mice still displayed a significantly reduced pathology over untreated mice, suggesting that other compensatory pathways are also at play.

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 [21], CD73 [22], and the adenosine A2B receptor [38]. Stabilization of HIF by inhibition of PHD has been shown to drive the expression of these barrier protective genes [6, 39] and also to promote restitution of the damaged mucosa through induction of the $\beta_1$ integrin ITGB1 [7]. In addition PHDi-driven NFκB activation is demonstrated to promote antiapoptotic 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[39]. In our studies 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 [23] that are important in the maintenance of GI immunological homeostasis.

In summary, we have demonstrated that systemic administration of PHDi suppresses inflammatory signaling and promotes increased phagocytosis of bacterial products, protecting against colitis-induced bacteremia. Furthermore, treatment increased epithelial barrier function and accelerated wound healing that limits exposure to the luminal contents that is central to the protection by PHDi observed in murine models of colitis. Targeted delivery to the mucosal epithelium may further increase the therapeutic efficacy of PHDi-mediated treatment 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 HIF-1α expression[20] were used to determine HIF isoform specificity. TNFΔARE mice were housed for 10 weeks under specific pathogen free conditions as previously described [40].

The PHDi AKB-4924 (Aerpio Therapeutics, Cincinnati, OH) was administered daily via subcutaneous injection to the scruff. Animals were treated at either 0.3, 1, or 5 mg/Kg in or 100 μL cyclodextrin vehicle. Mice were monitored daily and all protocols were performed in strict adherence to 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. Neutrophil depletion was achieved by administration of Ly6G antibody as previously described [41, 42].

### 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 [43]. Blood

was collected by cardiac puncture. Serum, mRNA and protein samples were stored at -80ºC until analysis. Protein analysis of serum and colon tissue was carried out by Mesoscale high-sensitivity or conventional ELISA. mRNA analysis was carried out by real-time-PCR analysis using previously validated primers for CD73[20], ITF[20] and ITGB1[7]. Serum endotoxin levels were assessed by colorimetric assays (QCL-1000, Lonza).

**Leukocyte isolation**

To isolate murine neutrophils and macrophages, C57BL/6 mice were administered 1 ml of 3% sterile thioglycolate (Sigma) intraperitoneally as previously described [44-46]. 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. Human neutrophils and peripheral blood monocytes (PBMCs) were isolated from venous blood from healthy volunteers, using Histopaque 1077 and 1119 (Sigma) [47].

The transcriptional profiles of neutrophils and monocytes subjected to normoxia ($pO_2$ 147 torr) or hypoxia ($pO_2$ 20 torr) or AKB-4924 (0.1 mM), with and without LPS stimulation (lipopolysaccharide from Escherichia coli Serotype 0111:B4, Sigma) were assessed in isolated RNA by quantitative real-time PCR. Protein expression was assessed by western blot analysis. Nuclear protein extracts isolated from neutrophils were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with anti-HIF-1α (Novus Biosciences). TATA binding protein was used as a reference housekeeping protein.

**Phagocytosis assays**

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, normoxia or with 0.1 mM 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. Fluoresence was assayed in a 96 well fluorometic plate reader and the percentage phagocytosis calculated, relative to the innoculum.

**Permeability assays**

Permeability assays were conducted with intestinal tissue sacs based on previously described assays. Briefly, mouse colons were excised and flushed with oxygenated TC199 medium. The sacs 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. 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 tube 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.* (). 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. The apparent permeability ($P_{app}$) for FD-4 was calculated from the following equation: $P_{app}$ (cm/s) = (dQ/dt)/(A · Co), where dQ/dt is the transport rate (mol/s), A is the surface area of the monolayer or sac ($cm^2$), and Co is the initial concentration in the donor compartment (mol/mL).

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**Figure Legends:**

**Figure 1**: **Effect of AKB-4924 treatment on disease activity in TNBS colitis**. AKB-4924 (0, 0.3, 1.0 or 5.0 mg/Kg) was administered subcutaneously on day -1 followed by 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 colons were excised and measured to assess (B) Colon shortening. (C) Disease activity was as assessed as the sum of scoring (0-3) for colon thickening (colon weight/length), occult blood, faecal pellet consistency and % weight loss. Temperature was measured by intrarectal 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, D), two-tailed, Students T-test (B, C)

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**Figure 2: Effect of AKB-4924 treatment on pyrogen levels in TNBS colitis**. AKB-4924 (0 or 5.0 mg/Kg) 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. N=6, *$p<0.05$, **$p<0.0$ two-tailed Students T-test.

**Figure 3**: **Effects of AKB-4924 treatment on leukocyte responses and the role of these cells in treatment of TNBS colitis**. Murine neutrophils and macrophages were isolated by peritoneal lavage and treated with AKB-4924 (AKB) or incubated in hypoxia (Hx; pO2, 20 torr for 6-h) or normoxia (Nx) in the presence or absence of LPS. (A) Murine neutrophil nuclear extracts were examined for nuclear HIF-1α by western blot. (B) AKB-4924- and/or LPS-treated murine neutrophil or macrophage mRNA was assayed by qPCR for expression of

IL-1β, IL-6 and TNF-α, relative to untreated control. (C) murine neutrophils or macrophages were assessed for the ability to phagocytose FITC-labelled LPS in the presence of AKB-4924. (D) AKB-4924- and/or LPS-treated human blood neutrophil or peripheral blood monocyte (PBMC) mRNA was assayed by qPCR for expression of IL-1β, IL-6 and TNF-α, relative to untreated control. TNBS colitic, neutrophil-depleted animals were treated with AKB-4924 (0 or 5mg/Kg) and colitis was assessed by (E) colon shortening, relative to control and neutrophil-replete TNBS animals. Mucosal neutrophil infiltration was assessed by (F) myleoperoxidase assay. Histological images of the colonic mucosa in (G) untreated, (H) TNBS-colitis, (I) neutrophil-depleted TNBS colitis, (J) AKB-4924-treated, neutrophil-depleted TNBS colitis and (K) AKB-4924-treated TNBS colitis animals. N=6, *$p<0.05$, **$p<0.01$, ANOVA (A, D), two-tailed, Students T-test (B, C, E, F).

**Figure 4: Effects of AKB-4924 on established TNBS colitis.** AKB-4924 (0 or 5.0 mg/Kg) was administered subcutaneously on the 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 measured to assess colon shortening, and total cell populations in mesenteric lymph nodes (MLN) were enumerated (C) using a hemocytometer. (D) Disease activity was as assessed as the sum of scoring (0-3) for colon thickening (colon weight/length), occult blood, faecal pellet consistency and % weight loss. Temperature was measured by intrarectal thermometer at 24 hr 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).

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**Figure 5: Influence of post-colitis treatment with AKB-4924 on serum pyrogen levels.** Animals at peak of acute colitis as assessed by weight loss (day 2 post TNBS induction) were treated with AKB-4924 (0 or 5.0 mg/Kg) every second day. Animals were sacrificed on day 7 relative to TNBS or EtOH rectal lavage. Blood was taken by cardiac puncture and serum assayed in triplicate for each animal by multiplex ELISA for A) IL-1β, B) IL-6 or C) TNF-α. N=6, *p<0.05, **p<0.0 two-tailed Students T-test.

**Figure 6: Effect of AKB-4924 on intestinal epithelial barrier function in TNBS colitis.** AKB-4924 (0 or 5.0 mg/Kg) was administered subcutaneously on day -1 relative to TNBS or EtOH intrarectal lavage. Cyclodextrin was administered as a control. 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; 500ug/mL) and (A) the apparent permeability assessed. Blood was collected by cardiac puncture and (B) serum assayed for LPS. N=6, *p<0.05, **p<0.01, ANOVA (A), two-tailed, Students T-test (B).

**Figure 7: Functional epithelial HIF-1α is critical for AKB-4924-induced protection.** AKB-4924 (0 or 5.0 mg/Kg) was administered subcutaneously on day -1 relative to TNBS or EtOH by intrarectal lavage. Cyclodextrin was administered as a control. (A) Weight was measured daily as an indicator of disease severity. On day 7 animals were sacrificed and colons were excised and measured to assess (B) colon shortening. Colon mRNA was screened by qPCR for induction of (C) CD73 and ITF or (D) ITGB. N=5, *p<0.05, **p<0.01, ANOVA (A, D), two-tailed, Students T-test (B, C).

**Figure 8: Effect of AKB-4924 on ileitis in TNFΔARE mice.** 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 for (B) acute inflammatory index, (C) chronic inflammatory index, (D) villus distortion and (E) total inflammatory score. N=5, *p<0.05, **p<0.0 two-tailed, Students T-test.