

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

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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 trinitrobenzene sulfonic acid 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 with 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 interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  while increasing IL-10. Treatment offered no protection against colitis in epithelial-specific HIF-1 $\alpha$ -deficient mice, strongly implicating epithelial HIF-1 $\alpha$  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.<sup>1</sup> 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.<sup>2–4</sup> Prolyl hydroxylase (PHD) inhibition has been shown to reduce disease severity in murine models of colitis on several levels of clinical scoring.<sup>5–9</sup> The observed mucosal protection is a consequence of PHD-2 sensitive hypoxia-inducible factor (HIF) stabilization<sup>10</sup> and PHD-1 sensitive nuclear factor- $\kappa$ B activation,<sup>11</sup> 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 the roles for compensatory epithelial barrier pathways,<sup>6</sup> anti-apoptotic regulation,<sup>8</sup> and the promotion of restitution and wound healing<sup>7</sup> 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 display increased survival times and enhanced phagocytic capacity.<sup>12</sup> *In vitro* studies have demonstrated that stabilization of HIF with DMOG prolonged neutrophil survival<sup>13</sup> and HIF stabilization by hypoxic incubation enhanced bacterial phagocytosis by neutrophils<sup>14</sup> and macrophages.<sup>15</sup> Further, DMOG treatment ameliorated disease in a murine model of endotoxic shock, through suppression of inflammatory cytokines and enhanced IL-10

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production.<sup>16</sup> More recently, a predominantly HIF-1-specific PHD inhibitor (PHDi), AKB-4924, has been developed.<sup>17</sup> Treatment with AKB-4924 enhanced the bactericidal capacity of keratinocytes against a range of skin pathogens in mouse models of infection.<sup>17</sup> Importantly, the concentrations that were effective were orders of magnitude less than those previously observed with other PHDis (DMOG and FG (functional group)-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 PHDis in colitis have focused primarily on epithelial cells,<sup>6,8,9</sup> the importance of these processes have not yet been defined.

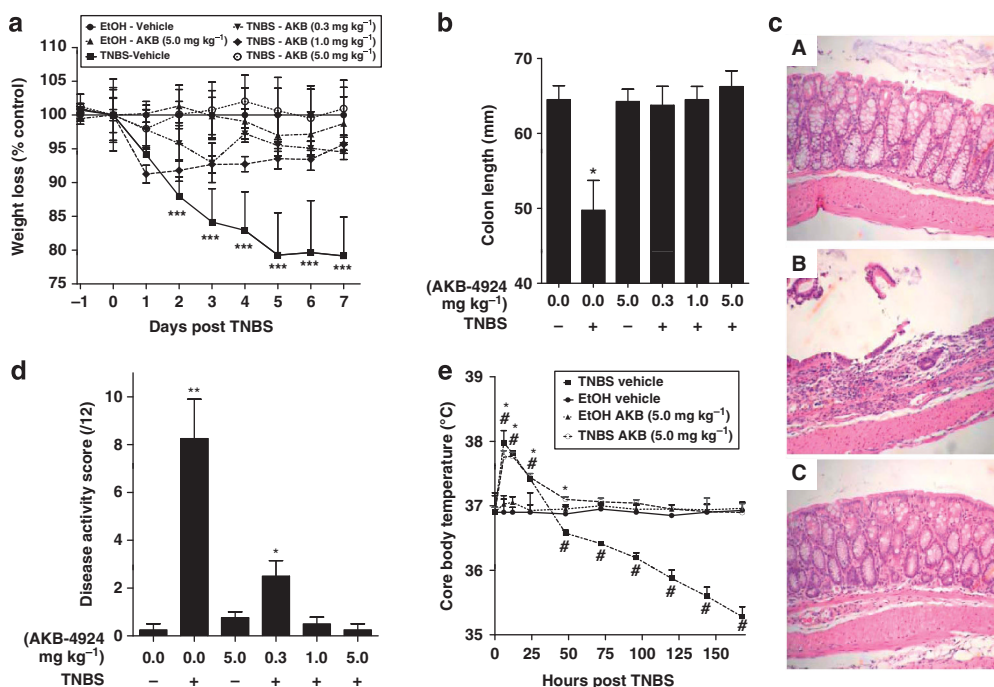
Here, we hypothesized that subcutaneous administration of AKB-4924 enhances innate cell responses in a mouse colitis model. Employing the chemical induction of colitis using trinitrobenzene sulfonic acid (TNBS), we assessed the systemic inflammatory response to bacteremia associated with intestinal inflammation. We examined inflammatory signaling in innate barrier cells and epithelial-specific HIF-1 $\alpha$ -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 by an epithelial mechanism that is critical for the mucosal protection in models of colitis.

## RESULTS

### AKB-4924 reduces TNBS disease pathology

We first hypothesized 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 isoform-predominant PHDi. Animals treated with AKB-4924 showed reduced weight loss (**Figures 1a**,  $P < 0.01$ ), attenuated colon shortening (**Figures 1b**,  $P < 0.05$ ) at all the doses tested (0.3, 1, and 5 mg kg<sup>-1</sup>), with as little as 0.3 mg kg<sup>-1</sup> AKB-4924 showing marked improvements in each of these end points. Histologically, AKB-4924-treated colitic animals showed quite significantly reduced tissue damage compared with vehicle and decreased disease activity indices (**Figure 1c**) and resulted in significantly decreased disease activity scores at all the doses tested (**Figures 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 ( $+1.1 \pm 0.56$  °C at 6 h post TNBS induction,  $P < 0.001$  compared with vehicle control) to TNBS administration between AKB-4924-treated and untreated animals (**Figure 1e**). However, the subsequent hypothermic response observed in untreated TNBS mice ( $-1.65 \pm 0.58$  °C at 168 h post TNBS induction,  $P < 0.001$  compared



**Figure 1** Influence of AKB-4924 treatment on disease activity in trinitrobenzene sulfonic acid (TNBS) colitis. AKB-4924 (0, 0.3, 1.0 or 5.0 mg kg<sup>-1</sup> 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 normalized relative to initial weight and control animals. On day 7, animals were killed and colons were excised and measured to assess (b) colon length. Panel (c) represents H&E staining of (A) tissue isolated from control animals, (B) TNBS animals treated with vehicle, and (C) TNBS animal treated with AKB-4924. (d) Disease activity was assessed as sum of scoring (0–3) for colon thickening (colon weight/length), occult blood, fecal pellet consistency, and percentage of weight loss. (e) Temperature was measured by infrared (IR) thermometer at  $t = 0, 6, 12,$  and 24 h and then at 24-h intervals until killing.  $N = 6$ , \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , # $P < 0.05$ , analysis of variance (a, e), two-tailed, Student's  $t$ -test (b, d).

with EtOH control) was not observed in the AKB-4924-treated groups ( $-0.03 \pm 0.50^\circ\text{C}$ , non-significant compared with EtOH control), suggesting that PHDi influences the systemic inflammatory response in TNBS colitis.

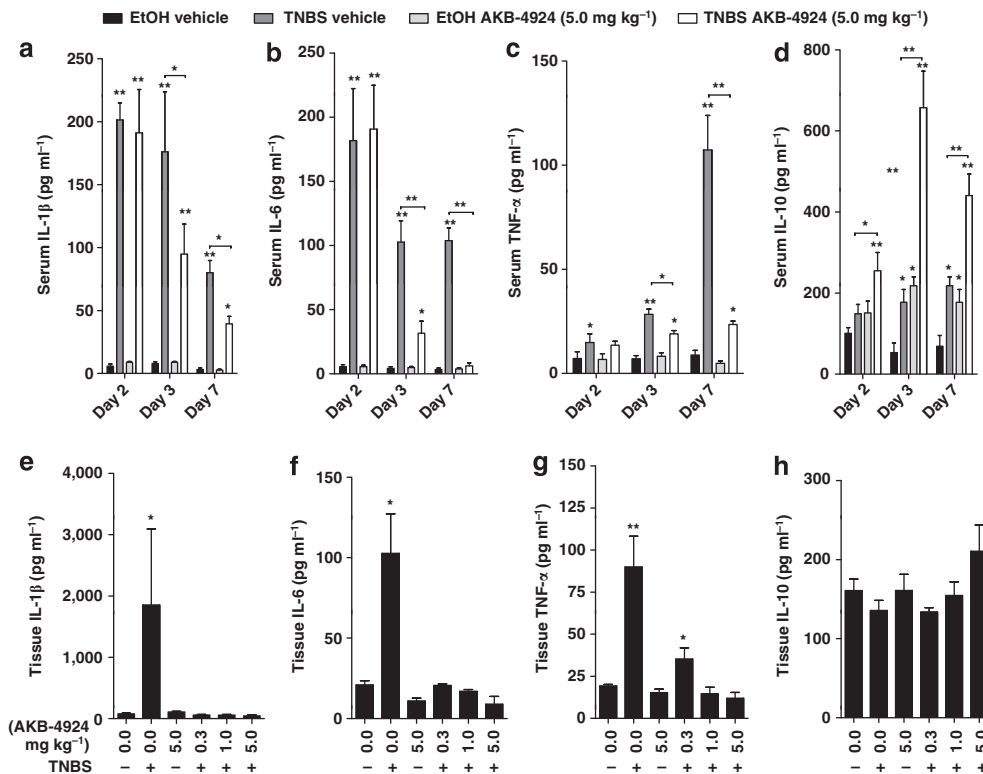
**AKB-4924 alters endogenous pyrogen responses in TNBS colitis**

Guided by these altered hypothermic responses in PHDi-treated animals, we investigated serum levels of endogenous pyrogens over the course of 1-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-1 $\beta$  ( $21 \pm 9$ -fold, **Figure 2a**,  $P < 0.01$ ). At day 2, there was no significant reduction of IL-1 $\beta$  in PHDi-treated animals. However, at days 3 and 7, PHDi strongly reduced IL-1 $\beta$  expression ( $P < 0.01$ ) compared with placebo-exposed colitic mice. Similarly, TNBS colitis animals had significant increases in IL-6 ( $33 \pm 11$ -fold, **Figure 2b**) compared with controls. By day 3, PHDi treatment significantly reduced IL-6 ( $61 \pm 5\%$  reduction,  $P < 0.01$ ), and by day 7, levels were back to baseline and were comparable with non-TNBS controls ( $97 \pm 3\%$  reduction,  $P < 0.01$ ). TNF- $\alpha$  was significantly increased in TNBS-treated animals by day 2, and increased at day 3 ( $4 \pm 1$ -fold,  $P < 0.05$ ) and day 7 ( $12 \pm 5$ -fold,  $P < 0.01$ ) compared with controls (**Figure 2c**). PHDi treatment resulted in a significant reduction in TNF- $\alpha$  by day 3 and was reduced by day 7 ( $76 \pm 8\%$

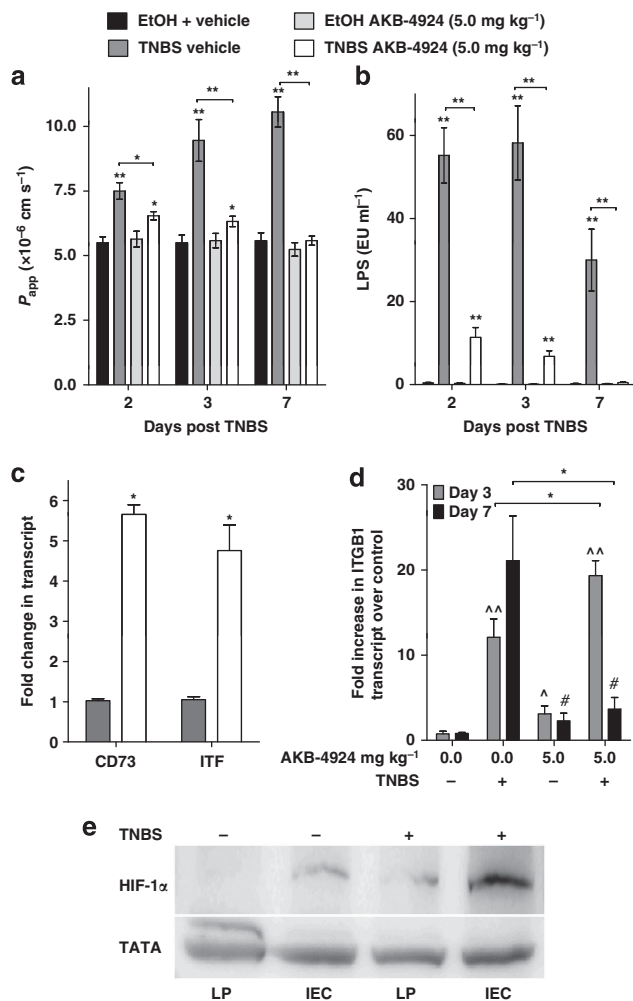
reduction,  $P < 0.01$ ). As IL-10 is an anti-inflammatory cytokine and may suppress the hypothermic response,<sup>18</sup> we investigated serum levels and found that they were significantly increased in PHDi-treated TNBS animals compared with untreated controls from days 2–7 (**Figure 2d**). In addition, we examined whole-colon homogenates for cytokine levels at day 7 and found significant increases in IL-1 $\beta$ , IL-6, and TNF- $\alpha$  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 hypothesized that the pyrexia observed was due to lipopolysaccharide (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 with untreated controls ( $P < 0.01$ ), and permeability continued to increase up to 7 days (**Figures 3a**,  $P < 0.01$ ). PHDi



**Figure 2** Influence of AKB-4924 treatment on pyrogen levels in trinitrobenzene sulfonic acid (TNBS) colitis. AKB-4924 ( $5.0 \text{ mg kg}^{-1}$  in cyclodextrin vehicle) or vehicle-treated animals were killed 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 enzyme-linked immunosorbent assay (ELISA) for (a) interleukin (IL)-1 $\beta$ , (b) IL-6, (c) tumor necrosis factor (TNF)- $\alpha$ , and (d) IL-10. On day 7, whole-colon tissue was homogenized in lysis buffer and assayed by ELISA for (e) IL-1 $\beta$ , (f) IL-6, (g) TNF- $\alpha$ , and (h) IL-10.  $N = 6$ ,  $*P < 0.05$ ,  $**P < 0.01$  two-tailed Student's  $t$ -test.



**Figure 3** Influence of AKB-4924 on intestinal epithelial barrier function and hypoxia-inducible factor (HIF) target gene expression. AKB-4924 (0 or 5.0 mg kg<sup>-1</sup> in cyclodextrin vehicle) was administered subcutaneously on day -1 relative to trinitrobenzene sulfonic acid (TNBS) or EtOH intrarectal lavage. Animals were killed on days 2, 3, or 7, and colons were excised and tied off by suture into intestinal sacs. Sacs were loaded with fluorescein isothiocyanate-dextran 4400 (FD-4; 500  $\mu$ g ml<sup>-1</sup>) and (a) the apparent permeability ( $P_{app}$ ) was assessed. Blood was collected by sterile cardiac puncture and (b) serum assayed for lipopolysaccharide (LPS) content. Colon epithelial mRNA was screened by quantitative PCR for induction of the HIF-target genes CD73 and (c) intestinal trefoil factor (ITF) or (d)  $\beta$ 1 integrin (ITGB1). Panel (e) represents western blot analysis for HIF-1 $\alpha$  in nuclear isolates from intestinal epithelial cells (IEC) and lamina propria (LP) cells in control and TNBS colitis animals in the presence of AKB-4924. TATA-binding protein was employed as a housekeeper.  $N=6$ , \*,  $\wedge$ , # $P<0.05$ ; \*\*,  $\wedge$ , ## $P<0.01$ ; analysis of variance (a, b, d), two-tailed, Student's  $t$ -test (c).

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 (Figures 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 by enhanced mucosal wound healing through mechanisms including induction of the HIF-1 target genes intestinal trefoil factor (ITF)<sup>19</sup> and 5'ecto-nucleotidase (CD73).<sup>20</sup> 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 with untreated controls (Figure 3c). Likewise, examination of the wound healing-associated HIF target gene,  $\beta$ 1 integrin (ITGB1),<sup>7</sup> revealed an early increase in response to AKB-4924 (Figure 3d, day 3,  $P<0.05$  compared with vehicle control), which had resolved by day 7 ( $P$ -value not significant compared with 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-1 $\alpha$  by western blot and revealed a preferential stabilization of HIF-1 $\alpha$  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, 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 with 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,<sup>12</sup> we investigated whether AKB-4924 influenced the capacity of leukocytes to phagocytose bacteria. Isolated mouse neutrophils and macrophages demonstrated an increased phagocytosis of heat-inactivated *Escherichia coli* (Figure 4b,  $P<0.05$ ). Together, these data suggest that the mucosal protection afforded by treatment is multi-factorial, enhancing both epithelial barrier function and leukocyte phagocytosis.

### Epithelial HIF-1 $\alpha$ 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-1 $\alpha$ . To achieve this, we utilized intestinal epithelial-specific Hif1a-deficient mice.<sup>21</sup> As shown in Figure 5a, and as we have shown in the past,<sup>21</sup> HIF-1 $\alpha$  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.

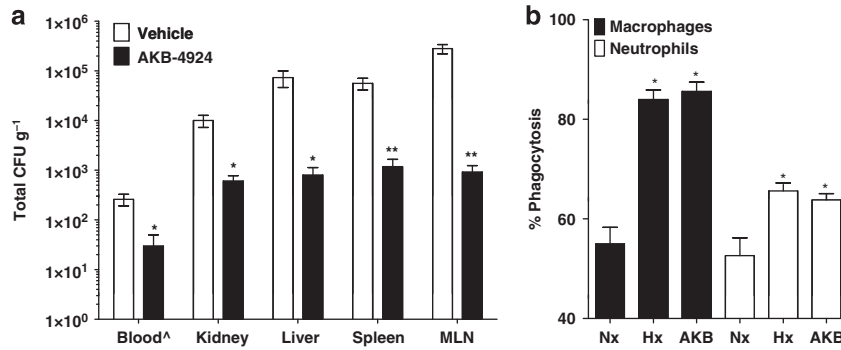


To define the functional contribution of epithelial HIF-1 $\alpha$ , we examined whether PHDi treatment protected intestinal epithelial-specific Hif1a-deficient mice from TNBS colitis. Consistent with previous studies,<sup>21</sup> intestinal epithelial-specific HIF-1 $\alpha$ -deficient mice developed more severe colitis than wild-type animals as measured by weight loss (Figures 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-1 $\alpha$ -deficient mice ( $P$ -value not significant for weight loss or colon length). Together, these data suggest that

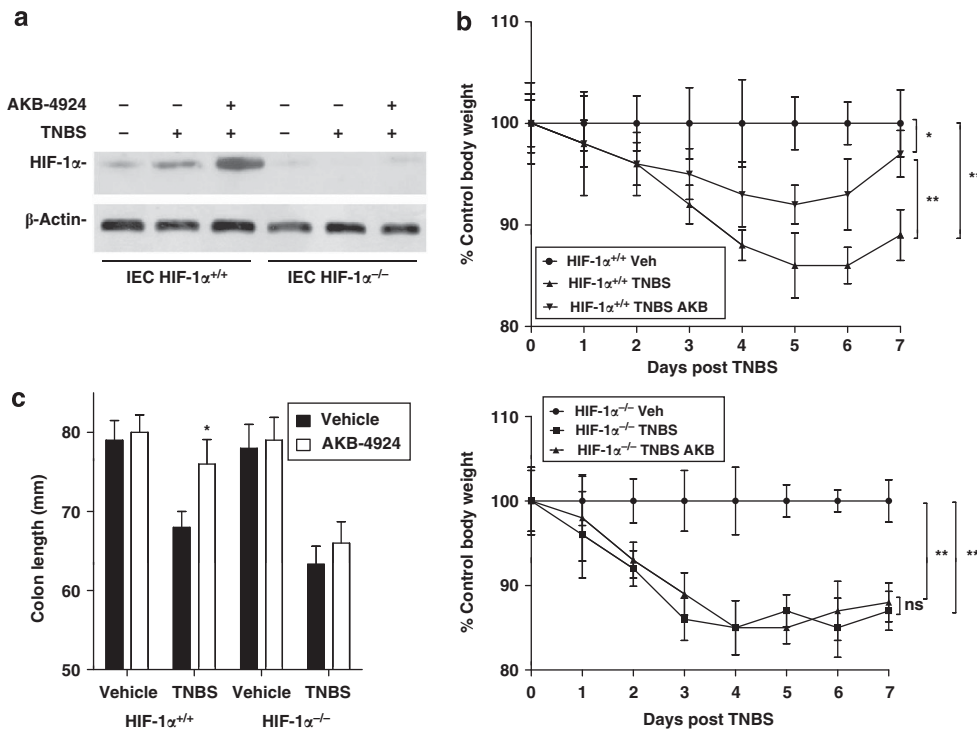
epithelial HIF-1 $\alpha$ -mediated effects are central to the protective actions of PHDi treatment in colitis. These findings suggest that PHDi treatment potently augments and increases restitution of intestinal barrier function, thereby significantly limiting the passage of bacterial products across the mucosa.

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

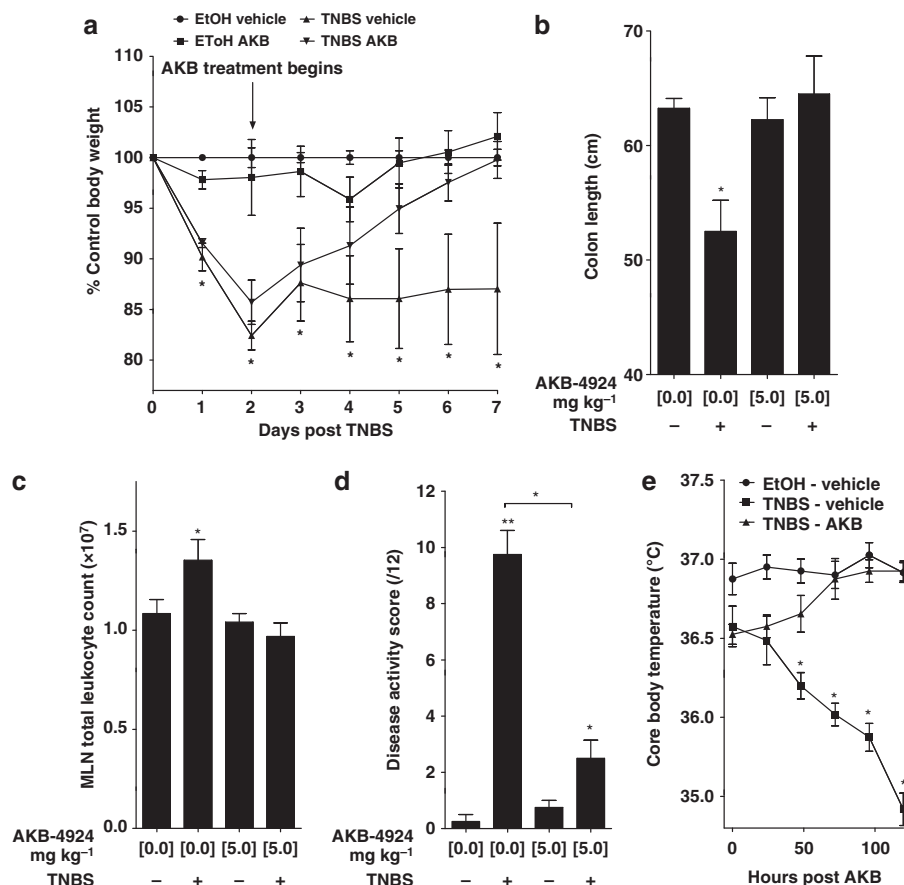
Given that PHDi treatment suppressed inflammatory cytokine activity, we hypothesized that treatment could promote remission of active murine colitis. Colitic mice were treated



**Figure 4** Influence of AKB-4924 on systemic bacteremia in trinitrobenzene sulfonic acid (TNBS) colitis. (a) Recovery of viable bacteria translocated to blood and extra-intestinal organs (liver, kidney, spleen, and mesenteric lymph nodes (MLN) in TNBS colitis animals after treatment with vehicle or AKB-4924 (5 mg kg<sup>-1</sup>). Bacterial counts are expressed in colony-forming units per gram tissue (CFU g<sup>-1</sup>), except for blood where counts are expressed as CFU ml<sup>-1</sup>. (b) Murine neutrophils and macrophages were isolated by peritoneal lavage and incubated with 10  $\mu$ M AKB-4924 (AKB) or incubated in hypoxia (Hx; pO<sub>2</sub>, 20 torr for 6 h) or normoxia (Nx) and assessed for the ability to phagocytose fluorescein isothiocyanate-labelled *Escherichia Coli*.  $N = 5$ , \* $P < 0.05$ , \*\* $P < 0.01$  two-tailed, Student's  $t$ -test.



**Figure 5** Functional epithelial hypoxia-inducible factor (HIF)-1 $\alpha$  is critical for AKB-4924-induced mucosal protection. (a) Western blot analysis for HIF-1 $\alpha$  from epithelial scrapings from intestinal epithelial cells (IEC) HIF-1 $\alpha$ <sup>+/+</sup> and IEC HIF-1 $\alpha$ <sup>-/-</sup> animals subjected to trinitrobenzene sulfonic acid (TNBS) colitis in the presence and absence of AKB-4924 (0 or 5.0 mg kg<sup>-1</sup>), 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 killed, and colons were excised and measured to assess (c) colon shortening.  $N = 5$ , \* $P < 0.05$ , \*\* $P < 0.01$ , analysis of variance (b), two-tailed, Student's  $t$ -test (c). Veh, vehicle.



**Figure 6** Influence of AKB-4924 on animals with active trinitrobenzene sulfonic acid (TNBS) colitis. AKB-4924 (0 or 5.0 mg kg<sup>-1</sup>) 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 killed 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 assessed as sum of scoring (0–3) for colon thickening (colon weight/length), occult blood, fecal pellet consistency, and percentage of weight loss. **(e)** Temperature was measured by IR thermometer at 24-h intervals from first AKB-4924 administration until killing.  $N=6$ , \* $P<0.05$ , \*\* $P<0.01$ , analysis of variance (**a**, **e**), two-tailed, Student's  $t$ -test (**b**, **c**, **d**).

with PHDi or vehicle at the peak of acute disease (day 2). PHDi-treated animals showed significant weight gain within 48 h (**Figure 6a**) and had comparable weight, colon length (**Figures 6b**,  $P<0.05$ ) and lymph node cellularity (**Figure 6c**,  $P<0.05$ ) than controls 5 days post treatment. PHDi treatment also significantly reduced disease activity score compared with 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-1 $\beta$  ( $P<0.01$ ), IL-6 ( $P<0.01$ ), and TNF- $\alpha$  ( $P<0.01$ ) (data not shown) compared with vehicle-treated TNBS animals. These data demonstrate that PHDi treatment can reverse acute inflammatory colitis.

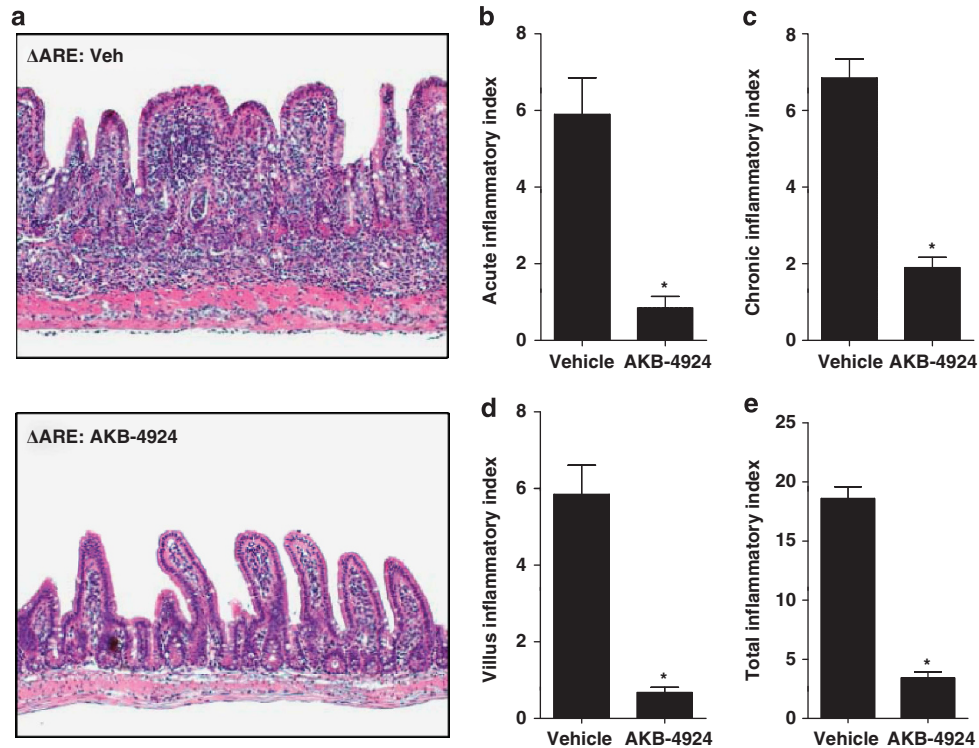
#### AKB-4924 is protective in a spontaneous TNF- $\alpha$ -mediated transgenic mouse model of ileitis

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 TNF $\Delta$ ARE mouse model.<sup>22</sup> These mice spontaneously develop transmural Crohn's disease-like chronic inflammation in the terminal ileum. Ten-week-old TNF $\Delta$ ARE mice were treated every second day for 10 days with AKB-4924 (0.5 mg per mouse per dose intraperitoneally). As determined by quantitative histological 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-1 $\alpha$  isoform-predominant PHDi, AKB-4924, in mouse models of intestinal inflammation. Previous work has demonstrated that



**Figure 7** Influence of AKB-4924 on TNF $\Delta$ ARE ileitis. AKB-4924 (0 or 5.0 mg kg<sup>-1</sup>) was administered to 10-week-old TNF $\Delta$ ARE 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, Student's  $t$ -test. Veh, vehicle.

pharmacological HIF stabilization offers protection to the mucosal barrier<sup>6,9</sup> and that HIF promotes barrier restitution,<sup>20</sup> cell survival,<sup>8</sup> and accelerates the healing process<sup>7</sup> in models of murine colitis. Here, we extended these studies by demonstrating that a PHDi promotes innate immunity, and suppresses the pyrexia response *in vivo* during colitis. We show that pharmacological 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-1 $\alpha$  in mucosal protection associated with PHDi-based therapeutic strategies.

IBD are characterized by repeated wounding of the mucosa, loss of the intestinal epithelial barrier, inflammation and ultimately bacteremia. Indeed, IBD patients have increased intestinal permeability<sup>23–25</sup> and elevated levels of serum endotoxin.<sup>3,26,27</sup> This is mirrored in experimental models of colitis, where serum endotoxin drives the secretion of pyrogens<sup>28</sup> leading to early pyrexia and later onset of hypothermia.<sup>18</sup> We observed an early pyrogenic response in animals with TNBS colitis, coupled with increased serum levels of classical pyrogens IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . 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.<sup>29–31</sup> It is now accepted that HIF has an important role in regulating immune cell function and suppressing inflammation. Furthermore, the induction of HIF promotes the bactericidal activities of phagocytic cells<sup>13,32</sup> and supports the innate immune functions of dendritic cells,<sup>33</sup> mast cells,<sup>34</sup> and epithelial cells.<sup>7,35</sup> 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 (<1% oxygen) occur in inflamed sites.<sup>36</sup> 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,<sup>19</sup> CD73,<sup>20</sup> and the adenosine A2B receptor.<sup>37</sup> Stabilization of HIF by inhibition of PHD has been shown to drive the expression

of these barrier-protective genes<sup>6,38</sup> and also to promote restitution of the damaged mucosa through induction of the ITGB1.<sup>7</sup> In addition, PHDi-driven nuclear factor- $\kappa$ B activation has been demonstrated to promote anti-apoptotic gene expression in epithelial cells,<sup>8</sup> and it is proposed that the culmination of these pathways leads to the mucosal protection promoted by PHDi in models of IBD.<sup>38</sup> In our studies, AKB-4924 preferentially stabilized HIF-1 $\alpha$  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-1 $\alpha$ . 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 TNF $\Delta$ ARE mice supports these findings. These animals display a translational dysregulation of TNF message, resulting in TNF- $\alpha$  overproduction and loss of TNF-driven modulation of hemopoietic cells<sup>22</sup> 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 serosal exposure to the luminal contents, through increased epithelial barrier function and accelerated wound healing, 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 1 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  $\mu$ l per g body weight of a 2.5% TNBS solution as previously described.<sup>6</sup> 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<sup>21</sup> were used to determine HIF isoform specificity. TNF $\Delta$ ARE mice were housed for 10 weeks under specific pathogen-free conditions as previously described.<sup>39</sup>

The PHDi AKB-4924 (Aerpio Therapeutics, Cincinnati, OH, (0.3, 1, or 5 mg kg<sup>-1</sup>)) was administered daily via 100- $\mu$ l subcutaneous injection to the scruff. Control was 100- $\mu$ l 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 h 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.<sup>40</sup> Western blotting for HIF-1 $\alpha$  was performed as previously described.<sup>21</sup> Blood was collected by cardiac puncture. Serum, mRNA, and protein samples were stored at - 80 °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 assays using previously validated primers for CD73,<sup>21</sup> ITF,<sup>21</sup> and ITGB1.<sup>7</sup> Serum endotoxin levels were assessed using colorimetric assays according to the manufacturer's instructions (QCL-1000, Lonza Inc., Walkersville, MD).

**Permeability assays.** Permeability assays were conducted with intestinal tissue sacs based on previously described assays.<sup>41</sup> Briefly, at each end point, 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, New Brighton, MN) 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  $\mu$ l of FD-4 (1.0 mg ml<sup>-1</sup>) 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 120 min at 37 °C, according to the method of Barthe *et al.*<sup>42</sup> Samples (120  $\mu$ l) 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 Inc., Sunnyvale, CA). The velocity of FD-4 across the intestinal barrier; the apparent permeability ( $P_{app}$ ) for FD-4, was calculated from the following equation:

$$P_{app} (cm s^{-1}) = (dQ/dt)/(A C_0),$$

where  $dQ/dt$  is the transport rate (mol s<sup>-1</sup> of FD-4),  $A$  is the surface area of the monolayer or sac (cm<sup>2</sup>), and  $C_0$  is the initial concentration in the donor compartment (mol ml<sup>-1</sup>).<sup>41,43,44</sup>

**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.<sup>45-47</sup> For neutrophil isolation, animals were killed after 6 h, and peritoneal lavage was performed using 10 ml of endotoxin-free phosphate-buffered saline. The lavage fluid was centrifuged at 600  $\times$  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 h after thioglycolate administration.

For phagocytosis assays, *E. coli* were cultured to log phase in LB (lysogeny broth) broth, heat inactivated at 60 °C for 1 h, and labeled with fluorescein isothiocyanate as previously described.<sup>12</sup> Isolated phagocytes were equilibrated in hypoxia or normoxia, with or without 10  $\mu$ M AKB-4924 for 1 h before the addition of bacteria at a ratio of 1:10 for a further hour. Cells were then pelleted, washed, and extracellular fluorescein isothiocyanate was quenched with Trypan blue. Fluorescence was assayed in a 96-well fluorometric plate reader and the percentage phagocytosis calculated, relative to the inoculum.

**Microbiological analysis.** Detection of viable enteric bacteria in extra-intestinal organs was performed by organ culture and CFU count.<sup>48,49</sup> Samples of mesenteric lymph nodes, liver, kidney, and spleen were removed under sterile conditions immediately upon euthanasia. Tissues were dissected free from fat, weighed, and homogenized in 5 ml of sterile phosphate-buffered saline. Serial dilutions of homogenates were plated on MacConkey and blood agar plates and incubated for up to 72 h at 37 °C. After incubation, CFU were counted and adjusted by tissue sample weight (CFU g<sup>-1</sup>). Blood samples were obtained by sterile cardiac puncture, aseptically diluted in brain heart infusion



broth, cultured for 48 h, and plated as above. Viable bacteria counts were expressed as CFU per ml blood.

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#### DISCLOSURE

The authors declare no conflict of interest.

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