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Acute colitis drives tolerance by persistently altering the epithelial barrier and innate and adaptive immunity

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TITLE PAGE

Title: Acute colitis persistently alters the epithelial barrier and innate and adaptive immunity driving tolerance to colitogenic microbiota.

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4861 words so far..

Abstract

Inflammatory Bowel Disease (IBD) is a chronic idiopathic disease of the lower gastrointestinal tract. IBD has a remitting and relapsing disease course, however relatively little is understood regarding how inflammatory damage in acute colitis influences the microbiota, epithelial barrier and immune function in subsequent colitis. 28 days after mice had acute and self-limiting tri-nitro benzene sulphonic acid (TNBS) induced colitis we observed that the distal colon had healed, mucosa was restored, and innate immune response had subsided but colonic trans-epithelial permeability and T_{REG} infiltration were increased relative to health. Furthermore, the adherent microbiota composition and responsiveness of stimulated innate immune bone marrow cells differed from health. Subsequent instillation of TNBS resulted in reduced effects on inflammatory damage, paracellular permeability and innate immune infiltration relative to acute colitis. However, T_{REG} infiltration was increased and stimulated T-cells in the mesenteric lymph nodes responsiveness shifted from pro-inflammatory in acute colitis to immune-suppressive in reactivated colitis. These effects were observed despite acute and reactivated colitis having a similar colonic microbiota composition and effect on the mucosal layer. Collectively, these results indicate that tolerance to colitogenic microbiota is promoted by chronic changes to the epithelial barrier and innate immune response in addition to T_{REG}.

Introduction

Inflammatory Bowel Disease (IBD) is a chronic and debilitating disease that is characterized by gross ulceration of the lower gastrointestinal tract. The causes of IBD remain unknown, however are currently thought to involve a complex genetic disposition resulting in aberrant immune responses to environmental stimuli that are normally tolerated. Animal models of colitis have provided fundamental advances in our understanding of how colitis is initiated and maintained, implicating epithelial defects and the microbiota¹. However, IBD has a remitting and relapsing disease course and relatively little is understood regarding how the inflammatory damage associated with acute colitis shapes responses in relapsing colitis. This paucity in our understanding of IBD is due to the majority of animal models progressing directly from acute to fulminant colitis, preventing investigations of remission and reactivation of colitis, modelling relapse.

Damage to the epithelial barrier precedes ulceration and impaired trans- and para-cellular permeability increases the access that luminal antigens, including the microbiota, have to the immune system in the colon^{2,3}. Primary genetic defects in the colonic epithelial barrier are not sufficient to cause ulceration but can increase susceptibility to colitis^{4,5}.

Furthermore, colitogenic microbiota are horizontally transferrable and dysbiotic microbiota and their metabolites influence inflammation in colitis⁶⁻⁸. However, and somewhat counter-intuitively, damage to the epithelial barrier has been also shown to induce the development immune suppressive mechanisms associated with oral tolerance which protect against colitis^{5,9}.

The Tri-Nitro Benzene Sulphonic acid (TNBS) model of colitis is one of only few models of intestinal inflammation that are self-limiting, permitting investigations of remission and

reactivation. Acute TNBS colitis is associated with impaired colonic epithelial barrier integrity, decreased microbiota diversity and innate immune infiltration¹⁰⁻¹³. Oral administration of haptenized colonic homogenates protects against TNBS colitis by inducing immune tolerance, and we recently demonstrated that a single TNBS enema caused a persistent increase in T_{HELPER} cell infiltration into the colon and reduced inflammatory damage upon colitis reactivation^{11, 14}. Numerous studies now demonstrate that acute colitis chronically alters nerve function in the colon modelling symptoms associated with altered motility and visceral pain experienced by people with remissive IBD and Irritable Bowel Syndrome^{15, 16}. However, it remains unclear whether the immunoregulatory effects observed involve altered epithelial barrier permeability to luminal antigen, altered microbiota composition, or both.

We aimed to determine the chronic effects that acute TNBS colitis has on colonic epithelial permeability, microbiota composition and immune tolerance and the influence these changes have on reactivated colitis. We observed that acute TNBS colitis persistently altered epithelial permeability, colonic microbiota composition and immune profile long after healing. However, both epithelial permeability and colonic microbiota composition were similarly affected in acute and reactivated colitis. Our results indicate that the reduction in inflammatory damage that occurs in reactivated TNBS colitis is consistent with an upregulation of immune tolerance pathways.

Results

Epithelial permeability and secretory responses.

Acute TNBS colitis caused a reduction in colon length and luminal mucous (**Fig 1A, B**) relative to healthy controls, as expected, and each of these parameters normalized in

Remission. Colon length shortening did not occur to the same extent in Reactivated colitis as in Acute colitis, confirming our previous findings with this model ¹¹. However, mucous was depleted to a similar extent in Reactivated as in Acute colitis.

In Ussing chamber experiments, changes in ~~altered~~ trans-epithelial electrical resistance (R_{TE}) and conductance (G) are interpreted as altered para-cellular permeability, while changes in I_{SC} are interpreted as altered trans-cellular ~~permeability-ion transport~~ ¹⁷. Acute colitis was associated with an increase in conductance and a reduction in R_{TE} as expected with this model ^{10, 13} (**Fig 1Ci, ii**). Epithelial conductance and R_{TE} normalized in Remission but were not affected to the same extent in Reactivated as occurred in Acute colitis. Baseline epithelial short circuit current (I_{SC}) was decreased in Acute colitis relative to Healthy mice, as expected, however it increased in Remission to levels higher than in Healthy mice, suggesting overcompensation (**Fig 1Ciii**). Baseline I_{SC} decreased to a similar extent in Reactivated as in Acute colitis. In light of these observations we next investigated the chloride secretory response by epithelial cells, and observed that the ~~change in muted baseline I_{SC} caused caused by TNBS was underpinned by dysregulated by chloride movement transport, with in response to addition of forskolin- (CFTR agonist) and carbachol- (CaCC agonist) induced responses! -was~~ significantly blunted in Acute colitis relative to Healthy mice (**Fig 1Di-iii**). Interestingly, I_{SC} responses to these secretagogues remained blunted in Remission when compared those from Healthy mice, and responses in Reactivated colitis did not differ from those in Acute colitis. However, summation of baseline I_{SC} with secretagogue elicited change in I_{SC} did not reveal any differences between Health and Remission or Acute and Reactivated mice (**Supplementary Figure 1**). These results indicate that the chloride (Cl^-) secretory capacity of the colonic epithelium is persistently blunted following a single episode of

colitis, despite normalization of gross damage and baseline electrophysiological parameters.

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Colonic microbiota composition.

The composition of the colonic adherent microbiota differed between treatment groups as indicated by permutational-based ANOVA (PERMANOVA, $p=0.0001$, square root estimated components of variation (ECV) = 0.123, 9910 permutations) and consistent with the non-metric multidimensional scaling (NMDS) plot (**Fig 2 and Supplementary Figure 2**). Specifically, pairwise comparisons indicated that the colonic adherent microbiota composition differed between Healthy mice and Acute colitis and between Healthy and Remission mice (**Table 1**). Interestingly, the composition of colonic adherent microbiota did not differ between Acute and Reactivated colitis. A similar pattern of differences in microbiota composition was observed in luminal faecal samples from the treatment groups ($P(\text{perm}) = 0.0001$, square root ECV = 0.083, 9903 permutations). However, NMDS analysis indicated that the differences in faecal microbiota are of a lower magnitude than those observed with adherent colonic microbiota (**Table 1 and Supplementary Figure 3**). No differences were observed between treatment groups in the alpha diversity of bacterial richness, evenness and diversity in colonic adherent or faecal microbiota (**Supplementary Figure 4A and B respectively**), indicating that the broad microbial community structure remained unaltered between treatment groups.

The relative abundance of nine bacterial taxa in the adherent colonic microbiota were observed to differ between treatment groups. The median relative abundance of microbiota of the genera *Bacteroides* and *Parabacteroides* of the Bacteroidaceae group, *Escherichia-Shigella* and *Enterococcus* were increased in Acute colitis relative to Healthy mice (**Fig 3A-D respectively**). While levels of both *Bacteroides* and *Parabacteroides* also

increased in Reactivated colitis relative to Remission, the levels of *Escheichia-Shigella* and *Enterococcus* did not. Interestingly, the relative abundances of *Lactobacillus*, *Erysipelatoclostridium* and RC9 gut group did not differ between Health and Acute colitis, but were increased in Reactivated colitis relative to Remission (**Fig 3E-G respectively**). For *Erysipelatoclostridium* and RC9 gut group these changes may be due to differences in their relative abundances between Health and Remission. The relative abundances of *Tyzerella* was decreased in Acute colitis relative to Health, and in Reactivated colitis relative to Remission but did not differ between Health and Remission (**Fig 3H**). *Helicobacter* abundance was not altered in Acute colitis relative to Health, however it was substantially increased in Remission relative to Health followed by a decrease in Remission relative to Reactivated colitis (**Fig 3I**). Fewer bacterial taxa were found to be altered in relative abundance in luminal faecal samples, in keeping with our findings that the differences in composition of luminal faecal microbiota were of a lower magnitude than in adherent colonic microbiota. Only *Bacteroides* and *Lactobacillus* were observed to differ between groups, with a similar pattern to that observed in adherent colonic microbiota (**Supplementary Figure 4**).

Adaptive immune responses.

We, and others, have previously demonstrated that TNBS colitis induces CD4+ T_{HELPER} (T_H) infiltration in Reactivated colitis and induces immune tolerance^{11, 14}. We expanded on these findings to investigate infiltration by regulatory T cells (T_{REG}), the key tolerance cytokine TGF-β₁, and the stimulatory capacity of T cells in the mesenteric lymph nodes (MLN). T_{REG} infiltration in the colon was not altered in Acute colitis relative to Health, but increased substantially in Remission compared to Health and remained much higher in Reactivated colitis compared to Acute colitis (**Fig 4Ai**). Colonic TGF-β₁ concentrations

Commented [HRW2]: i am wondering if the results would benefit from a table showing all endpoints and whether they were changed relative to health; just to have a single point for comparison of all markers. Could also be demonstrated in a figure? I would be happy to create if you want?

decreased in Acute colitis relative to Health, normalized in Remission and did not decrease to the same extent in Reactivated compared to Acute colitis (**Fig 4B**). T_{REG} proportions in the MLN were unchanged at any stage of colitis (**Fig 4Aii**), however the stimulatory capacity of T cells differed substantially. CD3/CD28 stimulated MLN cell secretion of IFN- γ and IL-10 was increased in Acute colitis relative to health, and both normalized in Remission (**Fig 5A, B**). However, while secretion of IFN- γ was decreased in Reactivated relative to Acute colitis, secretion of IL-10 increased.

Innate immune responses.

We previously demonstrated that colonic IL-1 β and IL-6 concentrations and F4/80+ macrophage infiltration are lower in Reactivated colitis compared to Acute colitis¹¹. We expanded on these findings to investigate cytokines and chemokines involved in innate immune activation (IL-1 β , GM-CSF, IL-10) and chemotaxis (CXCL2 and CCL2) into the colon, infiltration by dendritic cells and neutrophils and the stimulatory capacity of innate immune cells. Colonic IL-1 β , GMCSF and CXCL2 concentrations were increased in colons in Acute colitis relative to Health, normalized in remission and were lower in Reactivated relative to Acute colitis (**Fig 6Ai, Aii and Aiv respectively**). Colonic IL-10 concentrations did not differ between Health and Acute colitis, or between Health and Remission or Acute and Reactivated colitis, although there was a modest decrease in Reactivated colitis compared to Remission (**Fig 6Aiii**). Colonic CCL2 concentrations were not affected by Acute or Reactivated colitis (**Fig 6Av**). These changes in cytokine and chemokine secretion were associated with altered innate immune cell infiltration into the colon. We applied the Ly6G/C axis to differentiate between neutrophils (Ly6G+ Ly6C-) and monocyte / macrophages (Ly6G- Ly6C+)¹⁸ and also investigated CD11c+ dendritic cells. Acute colitis increased colonic infiltration of Ly6C+ Ly6G- monocyte / macrophages, Ly6C-

Ly6G⁺ neutrophils and CD11b⁺ CD11c⁺ dendritic cells (**Fig 6B-D**). Ly6C⁺ Ly6G⁻ monocyte / macrophage infiltration was lower in Remission relative to Health, and remained low in Reactivated colitis when compared to infiltration in Acute colitis, similar to our previous results investigating F4/80⁺ macrophages. Colonic infiltration of Ly6c⁻ Ly6G⁺ neutrophils normalized in Remission, but increased in Reactivated relative to Acute colitis, contrasting starkly against CD11b⁺ CD11c⁺ dendritic cells and Ly6C⁺ Ly6G⁻ monocyte / macrophages. CD11b⁺ CD11c⁺ dendritic cell infiltration normalized in Remission, but was lower in Reactivated relative to Acute colitis.

LPS stimulated bone marrow cells increased IL-10 secretion in acute colitis relative to health (**Fig 5C**), while secretion IL-1 β , GMCSF, CXCL2 and CCL2 were not affected (**Fig 5A, B, D and E respectively**). This pattern differed substantially in Remission and Reactivated colitis. IL-10 concentrations normalized in Remission and increased to a similar extent in Reactivated colitis as in Acute colitis. CCL2 and IL-1 β secretion increased in Remission relative to Health, and remained increased in Reactivated colitis to a greater extent than in Acute colitis. GMSCF and CXCL2 secretion decreased in Remission relative to Health, and while GMSCF secretion was observed to increase to a greater extent in Reactivated colitis compared to Acute colitis, CXCL2 secretion was lower. These results indicate that acute colitis persistently alters innate immune cell infiltration into the colon, but also chronically alters innate immune cells in the bone marrow, suggesting a memory response.

Discussion

Our findings demonstrate that a transient colitis event chronically alters epithelial permeability, microbiota composition and the immune profile in the colon. Furthermore, these changes profoundly influence epithelial and immune responses to subsequent colitis independently of altered colonic microbiota composition.

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Epithelial chloride secretion is persistently altered by colitis

Restoration of the colonic mucosa is a high priority in IBD management as it leads to symptom resolution and reduced risk of colectomy¹⁹. Tissue morphology appears histologically normal in remissive IBD, however little is understood regarding whether inflammation has persistent effects on epithelial function. Our findings demonstrate that colonic trans-cellular epithelial permeability is persistently altered by acute colitis despite the normalization of gross morphology. We observed that acute colitis impaired basal para- and trans- epithelial permeability, as expected^{10, 13}. Inflammation subsided in remission and basal para-cellular epithelial permeability normalized, however basal trans-cellular permeability was enhanced relative to health. Increased trans-cellular permeability is typically interpreted as the secretion of chloride (Cl⁻) into the lumen, which is followed by passive diffusion of sodium (Na⁺) and water. This predisposes to diarrhea, which is a common symptom experienced by people with IBD in remission and Irritable Bowel Syndrome (IBS)²⁰. Further investigation revealed that both forskolin induced cAMP - dependent and carbachol induced intracellular Ca²⁺ -dependent Cl⁻ secretory pathways were substantially blunted in remission relative to health, and instead more closely resembled Cl⁻ secretion in acute colitis. Interestingly, basal trans-cellular permeability and secretagogue provoked Cl⁻ secretion were impaired in reactivated colitis to a similar extent

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as in acute colitis despite the substantial reduction in gross inflammation and lack of noticeable effect on basal para-cellular permeability. This indicates that the mechanisms underlying trans-cellular permeability are more sensitive to inflammation than those underlying para-cellular permeability. Trans-cellular permeability to ions is normally tightly regulated by ion channels, co-transporters and enzymes including the cystic fibrosis transmembrane conductance regulator (CFTR), epithelial sodium channels (ENaC), the sodium-hydrogen exchanger (NHE), the Na-K-Cl cotransporter (NKCC) and the Na-K-ATPase enzyme. Summation of baseline and secretagogue induced changes in trans-cellular permeability revealed that the maximal secretory Cl⁻ capacity was substantially reduced in acute colitis but did not differ markedly between remission and health, or between acute and reactivated colitis. This indicates that the altered Cl⁻ secretion we observed in remission is most likely due to basal changes in mechanisms responsible for Cl⁻ secretion rather than ongoing tissue damage. ENaC, NHE and Na-K-ATPase are reportedly down-regulated in colonic tissue from active IBD subjects and in acute TNBS colitis and potentially contribute to the differences in baseline trans-cellular permeability that we observed in acute, remissive and reactivated colitis^{21, 22}. However, ENaC and NHE are both blocked by amiloride and are therefore unlikely to explain the differences in secretagogue provoked Cl⁻ secretion we observed. Longitudinal comparisons of trans-cellular ion secretion in colonic tissue from people with naïve, remissive and relapsing IBD are yet to be performed. While studies of this nature are inherently difficult our current results indicate that they would provide important insights into the mechanisms underlying the continuation of symptoms such as diarrhea in people with IBD in remission and potentially also IBS-D.

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Microbiota composition in acute colitis is comparable to reactivated colitis

Epithelial barrier defects increase the exposure that the immune system has to luminal contents, including gut microbes, and drive the development of immune responses. IBD in humans and animal models is consistently associated with altered microbiota, although it remains unclear whether dysbiosis is causative or reflects the disease process²³⁻²⁵. We observed that while colitis altered the microbiota profile in both adherent and luminal populations, the effects were more substantial on adherent microbiota. This indicates that colitis predominately alters microbiota that are in closest proximity to the host epithelium and immune system. Acute colitis was associated with increased abundance of *Bacteroides*, *Parabacteroides*, *Escherichia-shigella* and *Enterococcus* and decreased abundance of *Tyzzarella* in our model. This effect was largely similar in reactivated colitis, where we also observed an increase in *Lactobacillus* after a strong trend toward an increase in acute colitis. The association of taxa within the Bacteroidetes phylum, as well as *Escherichia* and *Enterococcus* in colitis are supported by their increased abundance in ulcerative colitis patients and monoculture and antibiotic ablation approaches in mouse models of colitis²⁶⁻²⁸. However, monocolonization of the intestine with *Bacteroides* has previously shown to promote T_{REG} development and protect against TNBS colitis²⁹. This potential discrepancy may be explained by findings that *Bacteroides*, a commensal bacterium of the gut, influences gut function and host immune responses in a host-dependent manner²⁷. The abundance of *Helicobacter* was not altered in our model of acute colitis but was substantially increased in remission when the abundances of other microbiota had normalized. Colonization of the intestine with *Helicobacter* has previously shown to protect against TNBS and chronic dextran sodium sulphate induced colitis via induction of immune suppressive mechanisms including T_{REG} promotion^{30,31}. *Helicobacter* colonization of the stomach causes inflammation and ulceration in approximately 20% of cases, however the links between *Helicobacter* and symptoms associated with remissive

IBD and IBS in the lower GI tract are more tenuous³². Our results indicate that assessment of adherent microbiota is crucial to determine the effect colitis has on host factors involved in inflammatory damage in colitis. While levels of *Lactobacillus* increased in this study, previous studies indicate that the abundance of fecal *Lactobacillus* is decreased in TNBS colitis, and that prophylactic treatment with *Lactobacillus* suggest that it has a protective effect against TNBS colitis^{12,33}. Furthermore, we observed that that colonic adherent microbiota profiles were similar in acute and reactivated colitis despite the lack of inflammatory damage in the latter. This finding indicates that changes in microbiota profile do not necessarily correlate with inflammation, but instead that immune responsiveness to microbiota is adaptable and inflammatory responsiveness damage is dependent on prior signals from the local environment.

Acute colitis induces immune suppression

We observed that acute colitis induces long lasting immune tolerance that protects against subsequent bouts of colitis, confirming our previous findings that innate immune responses dominate in acute colitis while adaptive responses dominate in remission and reactivated colitis¹¹. Here, we identified the adaptive immune population as T_{REG}, which have a central role in oral tolerance pathways³⁴. Our results build on the seminal findings from Neurath *et. al.*¹⁴ which revealed that feeding mice with TNBS treated colonic proteins promotes oral tolerance and protects against TNBS colitis. Furthermore, we demonstrated that T_{REG} infiltration is increased during remission, even though the immune response induced by haptenized proteins has cleared and the mucous layer was restored. T_{REG} proportions in the mesenteric lymph nodes were not altered at any stage of colitis, however the phenotype of stimulated T cells in the lymph nodes shifts from pro-inflammatory IFN- γ dominant in acute colitis to immune-suppressive IL-10 dominant in reactivated colitis.

Recent studies indicate that T cell differentiation is not terminal but instead is responsive to environmental signals ^{35, 36}, and our results suggest that T cells in the mesenteric lymph nodes are skewed toward an immune-suppressive profile in reactivated colitis but that T_{REG} proliferate only after trafficking to the colon and receiving appropriate environmental signals. Notably, we observed that TGF- β_1 was reduced in the colon in acute colitis, and while it increased in reactivated relative to acute colitis it did not differ substantially from health. TGF- β_1 has a prominent role in oral tolerance, however it is secreted by a wide range of cell types including epithelial cells and fibroblasts and its decrease most likely reflects damage to epithelial cells rather than a loss of T_{REG}. Alternatively decreased TGF- β_1 concentrations may result from alterations in the signaling pathways involved in tolerance such as SMAD7, as has been observed in intestinal tissue from people with Crohn's disease ³⁷.

Tolerance is associated with downregulation of innate immune responses

In contrast to T_{REG}, colonic infiltration by Ly6G⁺ neutrophils normalized in remission, while infiltration by Ly6C⁺ monocyte / macrophages decreased relative to health, a trend we also observed with CD11c⁺ dendritic cells. Colonic infiltration by both Ly6C⁺ monocyte / macrophages and CD11c dendritic cells did not increase to the same extent in reactivated colitis as in acute colitis, confirming our previous findings with F4/80⁺ macrophages ¹¹. This pattern of infiltration was largely mirrored by the profile of cytokine and chemokine concentrations, including the inflammation promoting IL-1 β , macrophage maturation promoting GM-CSF and the neutrophil attracting CXCL2. Colonic IL-10 concentrations were only modestly decreased in reactivated relative to acute colitis, most likely reflecting the decrease in infiltration by monocyte / macrophages at this stage. The colonic concentration of the constitutive and inflammatory chemokine CCL2 was not altered at any

stage of colitis, however CCL2 displays attractant properties for a much broader range of cell types than CXCL2, which is predominately a neutrophil attractant. Our previous results indicate that T_{HELPER} infiltration into the colon in remission and reactivated colitis coincides with increased $\alpha_4\beta_7$ integrin expression¹¹, suggesting that CCL2 is not required for T cell or innate cell infiltration into the colon in TNBS colitis. Interestingly however, infiltration by Ly6G neutrophils increased to a greater extent in reactivated colitis than in acute colitis. This observation is perplexing given that neutrophil infiltration is typically associated with tissue damage and ulceration³⁸, however recent studies indicate that the role of neutrophils in colitis is complex and their infiltration may actually be beneficial^{39, 40}. Neutrophil infiltration in reactivated colitis occurred independently of CXCL2, which may provide a key insight as it has previously been demonstrated that CXCL2 recruited neutrophils are tissue damaging while CXCR4 expressing neutrophils are phenotypically different and promote angiogenesis⁴¹. CXCR4 is the receptor for CXCL12, a constitutive chemokine secreted by epithelial cells and fibroblasts that is increased in mucosal biopsies from people with active IBD⁴². However, it is currently unclear whether recruiting CXCR4 neutrophils promotes wound healing in TNBS colitis or in human IBD.

Trained memory in TNBS colitis

Interestingly we observed that the pattern of cytokine and chemokine secretion by stimulated innate immune cells in the bone marrow differed markedly between colitis stages. The increase in stimulated IL-10 secretion in the absence of altered IL-1 β , GM-CSF, CXCL2 or CCL2 in acute colitis suggests that these cells are driven toward a M2 macrophage lineage. The differences we observed in the LPS stimulated cytokine and chemokine secretion between health and remission and between acute and reactivated colitis were unexpected given the short life span of myeloid cells and the reduced innate

immune responses in reactivated colitis. However, we have previously shown that colonic explants from reactivated colitis are more sensitive to innate immune LPS stimulation than explants from acute colitis¹¹. Combined, these observations intriguingly suggest that acute colitis persistently alters innate immune responses long after resolution of inflammation, and shape innate immune responses to subsequent exposure to the same antigen. Growing evidence supports the concept of innate immune memory, termed 'trained memory'⁴³. While there is currently little evidence to support or oppose trained memory in human IBD or animal colitis models, our results suggest that the reactivated TNBS colitis model may provide insight into the regulation of this newly described phenomenon.

Conclusions

In summary, our results indicate that colonic para-cellular permeability, microbiota composition and adaptive and innate immune function are persistently altered by acute TNBS colitis during remission, and these changes substantially impact on responsiveness to reactivated colitis. We observed that colonic microbiota profiles differed between health and remission but were similar in acute and reactivated colitis despite the tolerance driven reduction in inflammatory damage in the latter. This indicates that immune responsiveness, including epithelial barrier and both innate and adaptive immune cells, drive inflammatory responses to colitogenic microbiota.

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Methods

Animals

All experiments were approved by the Animal Ethics Committees of The University of Adelaide and SA Pathology. Male C57BL/6 mice were sourced from Animal Resource Centre (Canning Vale, Australia) and were group housed in a conventional setting prior to experiments starting. Mice were individually housed following TNBS treatment and were humanely killed via CO₂ inhalation and cervical dislocation.

TNBS colitis model

0.1ml TNBS (130µl of 1M TNBS solution in 30% EtOH / ml, Sigma Aldrich, NSW, Australia) was administered via enema as previously described^{11, 13} and allowed to recover for 2 (Acute) or 28 (Remission) days. Some Remission mice were re-treated with the same TNBS protocol and allowed to recover for a further 2 days (Reactivated; 30 days). Healthy mice were age matched across the acute to reactivated time span. Colon length was measured on excised colons dissociated free from surrounding tissue from the tip of the mouse anus to the distal end of the cecum.

Alcian blue staining

5µm paraffin embedded sections were cut from Carnoys buffer fixed distal colon sections and stained with Alcian Blue (Sigma Aldrich) with Nuclear Fast Red (Sigma Aldrich) counterstain. Alcian blue staining was analysed by examining areas of epithelia that were adjacent to submucosal folds and scoring 1 for mucous touching or 0 where there was clear space (i.e. no mucous). Scores were averaged across 2 representative areas of

approx. 20 crypts each per section, with 3 colon sections scored per section and averaged per mouse. Values were expressed as % and averaged across each mouse.

Ussing chamber studies

Distal colon segments were mounted into 0.1 cm² aperture sliders bathed in 5ml baths with oxygenated glucose-fortified Ringers solution for Ussing chamber analysis (Physiologic instruments, CA, USA) as previously described¹³ (See supplementary methods). Briefly, voltage clamped tissue were equilibrated for 20 min before baseline baseline I_{sc} (μA/cm²), conductance (G, S/cm²) and trans-epithelial electrical resistance (R_{TE}; Ω.cm²) was averaged over 10 min, after which 20 μmol/L Amiloride (Sigma Aldrich) was added apically for 10 min. prior to 10 μmol/L Forskolin (0.1% DMSO, Sigma Aldrich) application to the basolateral surface for 20 minutes, followed by 100 μmol/L Carbachol (Sigma Aldrich) application to the basolateral surface for 10 minutes. I_{sc} was continuously measured and the response determined as the change in I_{sc} following agonist administration (ΔμA/cm²).

Microbiota 16S rRNA gene amplicon sequencing and bioinformatics analysis

Colonic luminal faecal pellets and 1cm sections of distal colon were stored at -80°C after removal. Bacterial DNA was extracted using the Powerlyzer Powersoil DNA Isolation kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions and then used to generate amplicons of the V4 hypervariable region for 16S rRNA sequencing. Barcoded amplicons of colonic and faecal DNA extracts were generated based on the Illumina Miseq 16S Metagenomic Sequencing Library Preparation protocol with several modifications as described (See supplementary methods)⁴⁴. Paired-end (2 x 300 bp) sequencing was performed on an Illumina Miseq platform. Bioinformatics analysis of the sequences were

performed using the Quantitative Insights Into Microbial Ecology (QIIME, v19.1) ⁴⁵, based on a previously described bioinformatics pipeline (See supplementary methods) ⁴⁶. The SILVA reference dataset (release 128) clustered at 97% identity was used for chimera filtering and open reference OTU picking. All colonic and faecal samples were analysed at a sequencing depth of 4,682 reads, which provided an average Good's coverage score of 96.5% ($\pm 1.2\%$) and 96.6% ($\pm 0.5\%$), respectively.

Immune cell isolation

Tissue was dissociated essentially as previously described ^{11, 13} (See supplementary methods). Briefly, colonic LPMC were isolated enzymatically (1mg/ml Collagenase D, 3mg/ml Dispase, 0.5mg/ml DNase1, (Sigma Aldrich) strained (40 μ M, Corning, NY, USA), centrifuged (500g, 10 min.) and the pellet resuspended over a 40/80 Percoll (Sigma Aldrich) layer. The interphase layer was collected, centrifuged (500g, 10 min.) and the pellet collected. MLN and femur BM were mechanically dissociated (Supplementary methods), passed through a 40 μ M strainer and centrifuged in PBS (500g, 10 min). Cell numbers and viability were determined by trypan blue exclusion and immediately processed for flow cytometry or cultured for stimulations. Only preparations with $\geq 80\%$ viability were processed.

Flow cytometry

0.5 x 10⁶ Fc blocked (BD Biosciences, NSW, Australia, dilutions bracketed) were stained with the following antibodies (BD Bioscience, NSW, Australia): a) CD45 BV605, CD326 BV510, CD3 BUV 395, CD4 BUV737 (1:10), CD8 APC-H7, CD25 BV421, followed by fixation / permeabilization (BD Bioscience) and a further stain with FOXP3 PE-CF594 (1:5) b) CD45 BV605, CD326 BV510, CD3 BUV 395, CD11b APCCy7, CD11c PE-CF594, Ly6C

BB515 (1:5), Ly6G PECy7 (1:5). 20,000 events / tube were analysed on a LSRFortessa X-20 (BD Biosciences) and proportions of live singlets were determined using FlowJo (Tree Star, OR, USA) as previously described ^{11, 13, 47}.

MLN and bone marrow stimulations

MLN and BM were plated at 1×10^5 cells / 200uL in complete media in 96 well U-bottom plates. MLN were cultured for 96 hours in the presence of CD3/CD28 beads (ThermoFisher Scientific, MA, USA) (1:4 cell:bead). BM were cultured for 16 hours in the presence of 1 μ g/ml Lipopolysaccharide (LPS) (Sigma Aldrich). Cultures were centrifuged, supernatant collected and stored at -80°C until ELISA analysis.

Colon protein extraction

Distal colon proteins were extracted in the presence of protease inhibitor cocktail (Sigma Aldrich) essentially as previously described ^{11, 13} (See supplementary methods). Total protein concentration was calculated by BCA assay (Abcam, UK) (See supplementary methods).

ELISA

Colon tissue and cultured supernatant concentrations of TGF- β_1 , IFN- γ , IL-10, IL-1 β , GM-CSF, CXCL2 (R&D Systems, MN, USA) and CCL2 were measured by ELISA (all from BD Bioscience unless specifically stated) essentially according to manufacturer's protocol as previously demonstrated ¹¹. CXCL2 was purchased pre-labelled, and all other ELISA required plate coating (See supplementary methods). Samples were acidified prior to TGF- β_1 analysis. All colon concentrations were determined neat, however some culture samples were diluted as follows to remain within the upper and lower limits of the standard

curve: CD3/CD28: IFN- γ 1:50, LPS: CXCL2 1:10, CD3/CD28: IFN- γ 1:50. Concentrations were corrected for dilution factor and normalized to total protein concentration. The limit of detections were TGF- β_1 <250pg/ml, IFN- γ <3.1 pg/ml, IL-10 <31.3 pg/ml, IL-1 β <31.3 pg/ml, GMCSF <15.6 pg/ml, CXCL2 <24.7 pg/ml, CCL2 <15.6 pg/ml.

Statistical Analysis

In all cases results are expressed as Mean \pm SEM. N=number of animals. One-way ANOVA with Bonferonni's post-hoc test (Epithelial barrier and immune) or Kruskal-Wallis with Dunn's post-hoc test (Microbiota) determined the significance of findings, selecting for significance between Healthy vs. Acute, Healthy vs. Remission, Acute vs. Reactivated and Remission vs. Reactivated groups.

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Figure legends:

Fig 1: Acute TNBS colitis chronically alters epithelial responsiveness to subsequent colitis.

A) Colon length decreases in Acute colitis, but subsequently normalizes in Remission.

Colon length does not decrease to the same extent in Reactivated colitis relative to Acute colitis (N=12 / group). **B)** Mucosal thickness decreases in Acute colitis, normalizes in

Remission and decreases to the same extent in Reactivated colitis as observed in Acute colitis (N=6 / group). Representative images of colonic sections stained with Alcian Blue, scale bar = 50µm. **Ci)** Baseline epithelial conductance and **Cii)** trans-epithelial electrical

resistance (R_{TE}) are altered in Acute colitis, normalise in remission and are not affected by Reactivated colitis. **Ciii)** Baseline epithelial short circuit current (I_{SC}) decreases in Acute

colitis, but in Remission increases to levels higher than in Health. I_{SC} decreased in Reactivated colitis to a similar extent as in Acute colitis (N=8 / group). **Di)** Forskolin and

Dii) Carbachol secretagogue I_{SC} responses were blunted in Acute, Remission and Reactivated colitis relative to Health (N=6 / group). **Diii)** Baseline normalised traces of all secretagogue data. All data Mean \pm SEM. *n.s.* not significant. * $p < 0.05$, ** $p < 0.01$, ***

$p < 0.001$.

Figure 2. Colonic adherent microbiota composition converges is similar in Acute and Reactivated colitis despite differing between Health and Remission. Non-metric multidimensional scaling (NMDS) plot of the colonic adherent microbiota in Healthy (N=12), Acute (N=12), Remission (N=13) and Reactivated (N=13) mice.

Figure 3. Selected bacterial taxa within colonic adherent microbiota are differentially affected by Acute colitis. The relative abundances of **A) *Bacteroides***, **B)**

Parabacteroides, **C)** *Escherichia-Shigella* and **D)** *Enterococcus* are increased in Acute colitis, normalize in remission and are similar between Acute and Reactivated colitis. **E)** Relative abundances of *Lactobacillus* were not significantly altered in Acute colitis, but were significantly increased in Reactivated colitis relative to Remission. **F)** *Erysipelatoclostridium* and **G)** RC9 Gut group were not affected in Acute colitis and decreased in Remission relative to Health. They were both increased in Reactivated colitis relative to Remission. **H)** *Tyzzarella* decreased in Acute colitis, normalized in remission and was similar in Acute and Reactivated colitis. **I)** *Helicobacter* was not affected by Acute colitis, but increased in Remission relative to Health and decreased in Reactivated colitis relative to Remission. N=12 / group for Healthy and Acute, N=13 for Remission and Reactivated. *n.s.* not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4: Acute TNBS colitis chronically increases T_{REG} infiltration into the colon. Ai)

Colonic T_{REG} proportions do not differ between Acute colitis and health but increase in Remission relative to Health and Reactivated colitis relative to Acute colitis. **Aii)** Mesenteric lymph node T_{REG} proportions are not affected by colitis. Representative plot of gating strategy for CD25 / FOXP3 staining in MLN. N=6 / group. **B)** Colonic TGF- β_1 concentration decreases in Acute colitis, normalizes in Remission and is not altered in Reactivated colitis. N=6 / group. All data Mean \pm SEM. *n.s.* not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5: Stimulated adaptive immune responses switch from pro-inflammatory in

Acute colitis to immune suppressive in Reactivated colitis. A) CD3/CD28 stimulated mesenteric lymph node secretion of IFN- γ increases in Acute colitis, normalizes in Remission and is reduced in Reactivated colitis relative to Acute colitis. N=6 / group. **B)**

CD3/CD28 stimulated mesenteric lymph node secretion of IL-10 increases in Acute colitis, normalizes in Remission and is increased in Reactivated colitis relative to Acute colitis. N=6 / group. All data Mean \pm SEM. *n.s.* not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6: Acute colitis chronically alters innate immune infiltration into the colon. A)

Colonic concentrations of **Ai)** IL-1 β , **Aii)** GMCSF and **Aiv)** CXCL2 increase in Acute colitis, normalize in Remission and are lower in Reactivated colitis compared to Acute colitis.

Colonic concentrations of **Aiii)** IL-10 and **Av)** CCL2 are not altered in Acute colitis or Remission. IL-10 concentrations are lower in Reactivated colitis than Remission but not Acute colitis, and CCL2 concentrations remained unaffected in Reactivated colitis. N=6 /

group. **B)** Colonic proportions of **Bi)** Ly6C+ / Ly6G- monocyte / macrophages, **Bii)** Ly6C- / Ly6G+ neutrophils and **Biii)** CD11c+ dendritic cells increase in Acute colitis compared to health and normalize in Remission. Colonic Ly6C+ / Ly6G- proportions and CD11c+ proportions are lower in Reactivated colitis compared to Acute colitis, but Ly6C- / Ly6G+ proportions are increased. Representative flow plots of Ly6C / Ly6G staining and CD11b+ CD11c+ in colonic tissue. All data Mean \pm SEM. *n.s.* not significant. N=6 / group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7: Acute colitis chronically alters stimulated innate immune responses. LPS

stimulated bone marrow cell secretion of **A)** IL-1 β , **B)** GMCSF, **D)** CXCL2 and **E)** CCL2 is not altered in Acute colitis, while secretion of **C)** IL-10 increases relative to health. In Remission, secretion of IL-1 β and CCL2 is increased, GMCSF and CXCL2 is decreased and IL-10 is unchanged relative to health. In Reactivated colitis, secretion of IL-1 β and GMCSF is increased, IL-10 and CCL2 is unchanged and CXCL2 is decreased relative to

Acute colitis. N=6 / group. All data Mean \pm SEM. *n.s.* not significant. * $p<0.05$, ** $p<0.01$,
*** $p<0.001$.

A) Colonic adherent microbiota			
Group comparison	t	P(perm)	Unique perms
Healthy vs Acute	2.290	0.0002	9902
Healthy vs Remission	2.099	0.0081	9922
Remission vs Reactivated	2.731	0.0003	9935
Acute vs Reactivated	1.111	0.2487	9932
B) Luminal faecal microbiota			
Group comparison	t	P(perm)	Unique perms
Healthy vs Acute	2.275	0.0001	9781
Healthy vs Remission	1.466	0.0481	9888
Remission vs Reactivated	1.504	0.018	9892
Acute vs Reactivated	1.264	0.1404	9774

Table 1. Microbiota composition did not differ between Acute and Reactivated colitis. PERMANOVA pairwise comparison indicated that A) colonic adherent and B) luminal faecal microbiota composition differed between Healthy mice and Acute colitis and between Healthy and Remission mice but not between Acute and Reactivated colitis.