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Acute colitis chronically alters immune infiltration mechanisms and sensory neuro-immune interactions

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ABSTRACT

Objective: Little is understood regarding how disease progression alters immune and sensory nerve function in colitis. We investigated how acute colitis chronically alters immune recruitment and the impact this has on re-activated colitis. To understand the impact of disease progress on sensory systems we investigated the mechanisms underlying altered colonic neuro-immune interactions after acute colitis.
Design: Inflammation was compared in mouse models of health, acute tri-nitrobenzene sulphonic acid (TNBS) colitis, Remission and Reactivated colitis. Cytokine concentrations were compared by ELISA *in-situ* and in explanted colon tissue. Colonic infiltration by CD11b/F4-80 macrophage, CD4 T_{HELPER} (T_H) and CD8 $T_{CYTOTOXIC}$ (T_C) and $\alpha_4\beta_7$ expression on mesenteric lymph node (MLN) T_H and T_C was determined by flow cytometry. Cytokine and effector receptor mRNA expression was determined on colo-rectal afferent neurons and the mechanisms underlying cytokinergic effects on high-threshold colo-rectal afferent function were investigated using electrophysiology.
Results: Colonic damage, MPO activity, macrophage infiltration, IL-1β and IL-6 concentrations were lower in Reactivated compared to Acute colitis. T_H infiltration and $\alpha_4\beta_7$ expression on T_H MLN was increased in Remission but not Acute colitis. IFN-γ concentrations, T_H infiltration and $\alpha_4\beta_7$ expression on T_H and T_C MLN increased in Reactivated compared to Acute colitis. Reactivated explants secreted more IL-1β and IL-6 than Acute explants. IL-6 and TNF-α inhibited colo-rectal afferent mechanosensitivity in Remission mice via a BK_{Ca} dependent mechanism.
Conclusions: Acute colitis persistently alters immune responses and afferent nerve signalling pathways to successive episodes of colitis. These findings highlight the complexity of viscero-sensory neuro-immune interactions in painful remitting and relapsing diseases.

1. Introduction

Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS) are chronic diseases of the lower gastrointestinal (GI) tract. IBD is characterised by remitting and relapsing inflammatory lesions in the intestinal wall (Roberts-Thomson et al., 2011). IBS, on the other hand, is diagnosed according to ROME criteria based on symptoms of lower abdominal pain with altered bowel habit that occur in the absence of readily identifiable pathology (Longstreth et al., 2006). Immune function is clearly abnormal in active IBD, but there is increasing evidence that the immune system is also altered in IBS consistent with a chronic low grade activation, and these alterations often correlate with symptoms of pain (Hughes et al., 2013b). More recently it has also been proposed that IBD patients in remission experience symptoms typically associated with IBS, including abdominal pain (Bryant et al., 2011; Grover et al., 2009; Spiller, 2009), suggesting that the mechanisms underlying these symptoms share commonalities (Keohane et al., 2010; Quigley, 2016). Effective treatments for chronic gastrointestinal pain remain elusive despite the prevalence and debilitating nature of these diseases, highlighting the lack of understanding of

Abbreviations: BK_{Ca}, large conductance voltage dependent and Ca^{2+} modulated K^+ ion channel; DRG, dorsal root ganglia; IBD, Inflammatory Bowel Disease; IBS, Irritable Bowel Syndrome; GI, gastrointestinal; IL, interleukin; MLN, mesenteric lymph node; LPMC, lamina propria mononuclear cell; T_C, $T_{CYTOTOXIC}$; T_H, T_{HELPER}; TNBS, Tri-nitrobenzene sulphonic acid; TRPA1, Transient Receptor Potential Ankyrin 1; PI3K, phosphoionositide-3-kinase.

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the mediators and molecular pathways involved in pain sensing in diseases of the lower GI tract.

Animal models of colitis have provided important insights into understanding how altered immune function initiates and maintains inflammation in the colo-rectum (Neurath, 2012). There are currently more than 50 animal models of colitis, however the majority of them progress directly from acute through to fulminant colitis and therefore are not useful for investigating remission or re-activation of colitis, modelling relapse. This contrasts starkly with studies of human IBD which typically sample from relapsing and not naïve subjects. Consequently, little is understood regarding the persistent effects that acute colitis has on the gastrointestinal immune system despite the importance of acquired memory as a fundamental principal of immunity. Rectal instillation of Trinitrobenzene sulphonic acid (TNBS) causes a transmural colitis that heals spontaneously such that by 28 days after the initial instillation the gross morphology of the colon wall does not differ from health, modelling remission and also permitting re-activation of colitis, modelling relapse (Campaniello et al., 2016; Hughes et al., 2009a; Hughes et al., 2009b; Hughes et al., 2014b; Krauter et al., 2007). However, it is unknown whether acute TNBS colitis persistently alters immune function through remission and whether this alters immune responses to re-activated colitis.

Painful stimuli in the colon and rectum are sensed by high-threshold extrinsic sensory nerve fibres which have their cell bodies in the Dorsal Root Ganglion (DRG) and signal to higher CNS regions via the spinal dorsal horn (Brierley and Linden, 2014). High-threshold colo-rectal afferents are not only sensitised to mechanical stimuli during TNBS colitis but also remain sensitised through the remissive period when inflammation has healed, modelling aspects of the chronic visceral pain experienced in IBS and IBD (Campaniello et al., 2016; Deiteren et al., 2015; Hughes et al., 2009b). Immune mediators alter nociceptive colo-rectal afferent function (Barbara et al., 2007; Brierley et al., 2005; Brierley and Linden, 2014; Hughes et al., 2009c, 2014b, 2013b), and we have previously demonstrated that the cytokines IL-1β, IL-6 and TNF-α sensitise high-threshold colo-rectal afferents to mechanical stimuli in health (Hughes et al., 2013a). However, the overwhelming majority of studies have concentrated on the functional effects of immune mediators on GI nerves in the healthy state and it remains to be determined whether these effects are relevant in disease. Here we demonstrate that immune responses differ markedly between acute, remissive and relapsing colitis, and the effects that the cytokines Interleukin (IL)-1β, IL-6 and Tumor Necrosis Factor (TNF)-α have on colo-rectal afferent function in remissive TNBS colitis profoundly contrast from those we previously observed in health.

2. Methods

All experiments were approved by the Animal Ethics Committees of The University of Adelaide and SA Pathology. Experiments were restricted to male mice to negate the potential confounding effects of estrus cycle.

2.1. TNBS colitis model

TNBS colitis (0.1 ml of 130 μl of 1M TNBS solution/ml in 30% EtOH) (Sigma, NSW, Australia) or 30% EtOH alone (Vehicle) was induced in isoflurane anaesthetized (2–4% in oxygen) 20–30 g C57/Bl6 male mice as previously described (Hughes et al., 2009b). Briefly, 0.1 ml of 130 μl of 1M TNBS solution/ml in 30% EtOH via a polyethylene catheter inserted 3 cm from the anus. Mice were provided with post-operative care 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. In all experiments outlined below Healthy, Acute, Remission and Reactivated mice were humanely killed via CO_2 inhalation. Colon length was measured from the anus to the distal tip of the caecum (N = 10/group).

2.2. Gross damage scores

The colon was taken, residual fat and blood vessels removed and flushed with PBS to remove faeces. A 1 cm section of colon removed starting 1 cm proximal from the anus was fixed in 4% paraformaldehyde (Sigma) in 0.1 mol/L phosphate-buffered saline (PBS) overnight at 4 °C. 10 haematoxylin and eosin stained paraffin embedded colon sections (6 μm) per mouse (N = 4/group) were processed for image capture (Nanozoomer (Hamamatsu, Japan)), and analysis (NDPI View, Hamamatsu) and scored by 2 blinded investigators for gross histology as previously described (Hughes et al., 2009b). Microscopic damage was blindly scored according to the following: mucosal architecture (0–3, normal to extensive damage), cellular infiltrate (0–3, no infiltrate to transmural infiltration), muscle thickening (0–3, normal to extensive thickening), crypt abscesses (0 absent, 1 present), goblet cell depletion (0 absent, 1 present) as previously described. A maximum possible total score is 11.

2.3. MPO

A 1 cm section of colon was processed for myeloperoxidase (MPO) and activity determined as previously described (Campaniello et al., 2016). A 1 cm piece of colon (n = 5/group) was homogenized in hexadecyl-trimethylammonium bromide (HTAB) (Sigma) at 0.5% in 50 mM/L phosphate buffer, subjected to 3 cycles of freeze/thaw in liquid nitrogen and centrifuged at 14,000 rpm for 15 min at 4 °C. Supernatants were collected and stored at −80 °C until analysis. For analysis, supernatants were reacted with O-dianisidine hydrochloride (Sigma) with H_2O_2 (Sigma) as a substrate. One unit of MPO activity is defined as the amount that degraded 1 μmol of H_2O_2/minute at 25 °C. Changes in MPO absorbance were measured every 30 s on a Versa-Max microplate reader (Molecular Devices, California, USA), averaged over 2.5 min and expressed as units/mg tissue.

2.4. Cytokine measurements

2.4.1. mRNA expression

mRNA was isolated from a 1 cm section of colon was stored in RNAlater (Qiagen, CA, USA) until extraction with RNeasy extraction kits according to manufacturers instructions (Qiagen) as previously described (Hughes et al., 2013a). Equal concentrations of mRNA was pooled from 5 mice per group and expression of IL-1β, IL-6, IFN-γ and TNF-α determined relative to the housekeeper GAPDH by quantitative RT-PCR using QuantiTect SYBR kits (Qiagen). using a Chromo4 real time instrument (MJ Research, USA) attached to a PTC-200 Peltier thermal cycler (MJ Research) and Opticon Monitor software (MJ Research). Qiagen QuantiTect SYBR Green RT-PCR one-step kits were used according to the manufacturer's specifications with the following conditions: Reverse Transcription: 50 °C (30 min); Initial PCR activation: 95 °C (15 min); Annealing, denaturing and extension cycles: 94 °C (15 s), 55 °C (30 s) and 72 °C (30 s) repeated for 50 cycles. The comparative cycle threshold method was used to quantify the abundance of targets relative to the housekeeper GAPDH. Experiments were performed in triplicate and averaged as previously described (Campaniello et al., 2016; Hughes et al., 2014a, 2013a, 2014b). Primer (Geneworks, SA, Australia) details are outlined in Table 1.