



## Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis

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### ABSTRACT

Chronic psychosocial stress is a risk factor for many affective and somatic disorders, including inflammatory bowel diseases. In support chronic subordinate colony housing (CSC, 19 days), an established mouse model of chronic psychosocial stress, causes the development of spontaneous colitis. However, the mechanisms underlying the development of such stress-induced colitis are poorly understood. Assessing several functional levels of the colon during the initial stress phase, we show a pronounced adrenal hormone-mediated local immune suppression, paralleled by impaired intestinal barrier functions, resulting in enhanced bacterial load in stool and colonic tissue. Moreover, prolonged treatment with broad-spectrum antibiotics revealed the causal role of these early maladaptations in the development of stress-induced colitis.

Together, we demonstrate that translocation of commensal bacteria is crucial in the initiation of stress-induced colonic inflammation. However, aggravation by the immune-modulatory effects of fluctuating levels of adrenal hormones is required to develop this into a full-blown colitis.

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### 1. Introduction

Inflammatory disorders, such as Crohn's disease (CD) and ulcerative colitis (UC) represent a major health concern, particularly in Western societies, with a life time prevalence of approximately 0.1% (Singh et al., 2001). Despite this, the mechanisms underlying the development of these disorders, particularly those that initiate the disease, remain poorly understood. However, a number of risk factors are known, including chronic psychosocial stress exposure (Bernstein et al., 2010; Duffy et al., 1991; Salem and Shubair, 1967), and we recently demonstrated that 19 days of chronic subordinate colony housing (CSC) causes spontaneous (Reber et al., 2007) and aggravates chemically-induced (Reber et al., 2008) colitis in male mice. The CSC-induced spontaneous colitis is reflected by an increase in the histological damage score (Reber et al., 2007), in the cytokine secretion from isolated lamina propria mononuclear cells (LPMC) and mesenteric lymph node cells (mes-LNC), in the number of colonic macrophages, dendritic cells, and T helper cells, and colonic IFN- $\gamma$  and TNF mRNA expression (Supplementary Fig. 1 and (Reber et al., 2007, 2008)).

The hyperactive immune system following 19 days of CSC exposure is driven by the combination of low plasma glucocorticoid (GC) levels due to adrenal insufficiency, and GC resistance, which both occur during the later stages of chronic psychosocial stressor exposure (Reber et al., 2007). However, an initial stress-induced rise in GC during the first 10 h of CSC exposure (Reber et al., 2007) (see also Supplementary Fig. 2) seems critically involved in these pathological adaptations, as adrenalectomy (ADX) prior to CSC ameliorates cytokine secretion from isolated mesLNC and completely prevents the development of histological abnormalities (Reber et al., 2007, 2008). As ADX not only removes circulating GC but also mineralocorticoids and catecholamines, it can not be 100% excluded that adrenal hormones different from corticosterone at least partly are also involved in this initial pro-inflammatory effect of the adrenal glands.

Therefore, to confirm an initial local immunosuppression by high GC levels (Dhabhar and McEwen, 2001, 1997, 1999), we compared the immune status at the colonic tissue of CSC and single housed control (SHC) mice. High GC levels also result in increased colonic permeability (for review see Soderholm and Perdue, 2001), thus we characterized colonic-barrier functions during the initial 10 h of CSC. ADX prior to CSC should thereby dissect the causal involvement of adrenal hormones, most likely GC, in the development of barrier and immune defects during the initial phase of chronic stress. Prolonged stressor exposure increased the number

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of commensal gut bacteria in stool samples collected from the large intestine (Bailey et al., 2010) and a stress-induced increase in epithelial permeability of the gastrointestinal tract promotes bacterial translocation into various organs and body compartments (Ando et al., 2000; Bailey et al., 2006; Ding et al., 2004; Everson and Toth, 2000; Wang et al., 2004). Furthermore, suppression of the colonic immune system by excessive GC might result in unhindered proliferation of luminal, as well as translocated, bacteria of the commensal flora. Therefore, we assessed the bacterial load in different tissues during the initial phase of CSC. High levels of colonic antigens during the initial phase of CSC are likely to be a prerequisite for the subsequent development of spontaneous colitis. According to our hypothesis, during later stages of chronic psychosocial stress, when there is a lack of immunosuppressive GC signaling, mediated via both GC resistance (Reber et al., 2007; Schmidt et al., 2010) and hypocorticism (Reber et al., 2007; Reber and Neumann, 2008; Schmidt et al., 2010; Veenema et al., 2008), the over-reactive immune system targets excessive colonic antigens and consequently promotes the inflammatory processes. Accordingly, we investigated the causal involvement of intestinal bacteria in the development of CSC-induced colitis using prolonged treatment with broad-spectrum antibiotics prior to, and during 19 days of CSC exposure.

Together, the overall aim of the study was to identify relevant mechanisms that underlie stress-induced initiation of colitis by dissecting the early interplay between the endocrine system and the colonic immune and barrier functions.

## 2. Materials and methods

### 2.1. Animals

As previously described (Reber et al., 2007, 2008; Reber and Neumann, 2008; Schmidt et al., 2010; Singewald et al., 2009; Veenema et al., 2008) male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19–22 g (experimental mice) or 30–35 g (used as residents during the CSC housing) were used in the current study.

## 3. Experimental procedure

Immune and barrier functions and the number of colony forming units (CFU) in stool, colonic and mesenteric lymph node tissue, and plasma corticosterone were assessed following 10 h of CSC. In another set of mice ADX was performed 2 weeks prior to the start of a 10-h CSC exposure. Finally, experimental mice were treated with four different antibiotics (see below) during the 4 weeks before as well as during subsequent 19 days CSC exposure. Another set of mice was killed following 19 days of CSC for detailed immunological characterization of CSC-induced spontaneous colitis (see Supplementary Fig. 1).

### 3.1. Stress procedure and surgical procedure

The chronic subordinate colony housing (CSC) paradigm (Reber et al., 2007, 2008; Reber and Neumann, 2008; Schmidt et al., 2010; Singewald et al., 2009; Veenema et al., 2008) and adrenalectomy (ADX) (Reber et al., 2007, 2008) was conducted as described previously.

### 3.2. Antibiotic treatment

Prolonged antibiotic treatment was performed as described previously (Rakoff-Nahoum et al., 2004). Mice received a cocktail of ampicillin (1 g/l), vancomycin (500 g/l), neomycin sulfat (1 g/l), and metronidazole (1 g/l) ordered from Sigma Aldrich (Deisenho-

fen, Germany) or purchased from the pharmacy affiliated to the University Hospital of Regensburg, via the drinking water during 4 weeks prior and subsequent CSC (19 days) exposure.

### 3.3. Blood sampling and ELISA for corticosterone (results see Supplementary Fig. 2)

All mice of one cage were rapidly killed by decapitation under CO<sub>2</sub> anaesthesia within 3 min after entering the animal room. Approximately 200 µl trunk blood was collected in EDTA-coated tubes on ice (Sarstedt Nümbrecht, Germany) and centrifuged at 4 °C (5000 rpm, 10 min). Plasma samples were stored at –20 °C until assayed using a commercially available ELISA for corticosterone (DRG, Marburg, Germany).

### 3.4. Immunohistochemistry (IHC)

Frozen and acetone-post-fixated cross sections were incubated with 100 µl of a 5 µg/ml F4/80 (NatuTec, Frankfurt am Main, Germany), or of a 10 µg/ml CD11c (BD Biosciences, Heidelberg, Germany), CD3 (BD Biosciences, Heidelberg, Germany), CD4 (BD Biosciences, Heidelberg, Germany), or of a 150 µg/ml Ki67 (DakoCytomation, Hamburg, Germany) solution (in 1% goat serum) for 1 h at RT, before respective biotinylated secondary antibodies (1:500 in 5% goat serum; Jackson ImmunoResearch, Suffolk, UK) were added for 1 h at RT. After visualization using Vectastain ABC-Kit followed by Vector NovaRed Substrate Kit (Vector Laboratories, Loerrach, Germany), positive cells were counted and averaged from 3 to 5 cross-sectioned slices per mouse. The mean + SEM per group was calculated and presented in the graphs as percentage of respective SHC mice set to 100%. Isotype controls were used in each approach to verify specificity.

### 3.5. Alcian Blue staining

Formalin-fixated colon tissue was embedded in paraffin and cut longitudinally. Two to three 3 µm sections were taken at 100 µm distance and used for acidic Alcian Blue staining as described previously (Lev and Spicer, 1964). Alcian Blue positive pixels were quantified in 2–3 slices per mouse (4–5 fields of view (magnification 1:20) per slice) using Leika FW4000 detection software. Respective mean + SEM per group were calculated and presented in the graphs as percentage of respective SHC mice set to 100%.

### 3.6. TUNEL labelling

Formalin-fixated colon tissue was embedded in paraffin and cut longitudinally. Three 3 µm sections taken at 100 µm distance were used for TUNEL labelling (Merck, Darmstadt, Germany) performed according to manufacturer's recommendation. Labelled apoptotic epithelial nuclei were counted in 4–5 fields of view (magnification 1:20) per slice (fluorescein filter, 465–495 nm) and averaged per mouse. The mean + SEM per group was calculated and presented in the graphs as percentage of respective SHC mice set to 100%. Positive controls [DNase I (Roche, Mannheim, Germany)] and negative controls [no TdT enzyme] were used in each approach to verify specificity.

### 3.7. Isolation/incubation of mesenteric lymph node cells (mesLNC)

Isolation/incubation of mesLNC was performed as previously described (Blaas et al., 2009; Reber et al., 2007, 2008; Veenema et al., 2008). After isolation mesenteric lymph node cells (mesLNC; pooled from each group; *n* = 6–8 mice per group) were incubated over 24 h either in *anti*-CD3-coated wells in the presence of IL-2 or in non-coated wells in the presence of CpG (50 µg/100 µl) (Blaas

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