Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE MEDICO-CHIRURGICHE
GASTROENTEROLOGICHE E DEI TRAPIANTI

Ciclo XXV

Settore concorsuale di afferenza: 06/D4
Settore scientifico-disciplinare di afferenza: MED/12

TITOLO TESI

PATHOGENIC ROLE OF IL-33-MEDIATED EOSINOPHIL INFILTRATION AND FUNCTION IN EXPERIMENTAL INFLAMMATORY BOWEL DISEASE

Presentata da
Dott. Carlo DE SALVO

Coordinatore Dottorato
Chiarissimo Prof. Andrea STELLA

Relatore
Chiarissima Prof. Theresa T. PIZARRO

Esame finale anno 2013

TABLE OF CONTENTS

ABSTRACT

IL-33/ST2 axis is known to promote Th2 immune responses and has been linked to several autoimmune and inflammatory disorders, including inflammatory bowel disease (IBD), and recent evidences show that it can regulate eosinophils (EOS) infiltration and function. Based also on the well documented relationship between EOS and IBD, we assessed the role of IL-33-mediated eosinophilia and ileal inflammation in SAMP1/YitFc (SAMP) murine model of Th1/Th2 chronic enteritis, and we found that IL-33 is related to inflammation progression and EOS infiltration as well as IL-5 and eotaxins increase. Administering IL-33 to SAMP and AKR mice augmented eosinophilia, eotaxins mRNA expression and Th2 molecules production, whereas blockade of ST2 and/or typical EOS molecules, such as IL-5 and CCR3, resulted in a marked decrease of inflammation, EOS infiltration, IL-5 and eotaxins mRNA expression and Th2 cytokines production. Human data supported mice's showing an increased colocalization of IL-33 and EOS in the colon mucosa of UC patients, as well as an augmented IL-5 and eotaxins mRNA expression, when compared to non-UC. Lastly we analyzed SAMP raised in germ free (GF) condition to see the microbiota effect on IL-33 expression and Th2 responses leading to chronic intestinal inflammation. We found a remarkable decrease in ileal IL-33 and Th2 cytokines mRNA expression as well as EOS infiltration in GF versus normal SAMP with comparable inflammatory scores. Moreover, EOS depletion in normal SAMP didn't affect IL-33 mRNA expression. These data demonstrate a pathogenic role of IL-33-mediated eosinophilia in chronic intestinal inflammation, and that blockade of IL-33 and/or downstream EOS activation may represent a novel therapeutic modality to treat patients with IBD. Also they highlight the gut microbiota role in IL-33 production, and

the following EOS infiltration in the intestinal mucosa, confirming that the microbiota is essential in mounting potent Th2 response leading to chronic ileitis in SAMP.

INTRODUCTION

The IL-33

IL-33, also known as IL-1F11, is the newest member of the IL-1 family and represents a protein with dual function that can act as both a signaling cytokine as well as an intracellular nuclear factor (Baekkevold et al., 2003; Carriere et al., 2007). IL-33 was originally localized to high endothelial venules of human tonsils, Peyer's patches, and lymph nodes (Baekkevold et al., 2003). More recent reports suggest its wide distribution throughout various organ systems, with primary expression in non-hematopoietic cells, including fibroblasts, adipocytes, smooth muscle cells, endothelial cells, bronchial and intestinal epithelial cells (Moussion et al., 2008; Schmitz et al., 2005; Wood et al., 2009), but also in cells of hematopoietic origin, particularly in restricted populations of professional antigen presenting cells, such as macrophages and dendritic cells (Schmitz et al., 2005). IL-33 was initially associated with the development of Th2 immunity, based on the expression of its cell-bound receptor, ST2L (IL-1R4), on polarized Th2 lymphocytes (Schmitz et al., 2005) and more recently, on innate lymphoid cells (Neill et al., 2010; Price et al., 2010), as well as its ability to potently induce the production of Th2 cytokines (*e.g.*, IL-4, IL-5, and IL-13) in both *in vitro* and *in vivo* systems. In regard to disease pathogenesis, IL-33 has been shown to be involved in Th2-mediated disorders, such as airway inflammation (Kurowska-Stolarska et al., 2008; Kurowska-Stolarska et al., 2009; Liu et al., 2009) and allergic reactions (Halim et al., 2012; Komai-Koma et al., 2012; Matsuba-Kitamura et al., 2010); however, IL-33 has also been described to exacerbate arthritis, widely considered to be a Th1/Th17-mediated pathology (Palmer et al., 2009; Xu et al., 2008).

IL-33 and eosinophils

One of the original observations and prominent functions of IL-33 is its ability to activate and induce the infiltration of eosinophils (EOS) into mucosal organs exposed to the external environment, including the respiratory and gastrointestinal tracts (Schmitz et al., 2005). In fact, IL-33 has emerged as an essential mediator in the development of EOS-mediated allergic inflammation and asthma (Chow et al., 2010; Stolarski et al., 2010), and plays a pivotal role in EOS recruitment and helminth expulsion following intestinal parasitic hookworm infection (Hung et al., 2013). IL-33 has been shown to potently stimulate ST2-bearing EOS to express CD11b and ICAM-1, produce superoxide anions, IL-6, the chemokines IL-8/CXCL8 and MCP-1/CCL2, and promote EOS adhesiveness, degranulation, and survival (Cherry et al., 2008; Chow et al., 2010; Suzukawa et al., 2008a). Importantly, EOS have been identified as critical players in innate immunity, expressing toll-like receptors (TLRs), particularly TLR7 and TLR8 (Mansson and Cardell, 2009; Nagase et al., 2003), and respond to both pattern- and damage/danger-associated molecular pattern molecules (PAMPs and DAMPs, respectively). EOS, upon activation, can also produce pre- and *de novo*-formed factors, including granule proteins and cytokines/chemokines, that are important in innate responses during inflammation and infection (reviewed in Hogan et al., 2013).

EOS and Inflammatory Bowel Disease

Interestingly, a growing body of evidence also suggests the importance of EOS in the pathogenesis of inflammatory bowel disease (IBD), a disorder previously thought to occur as the result of dysregulated and overexuberant T cell responses. This paradigm, however, has been challenged in recent years by the concept that the primary cause of IBD resides in dysfunction of innate immunity (Arseneau et al., 2007; Asquith and Powrie, 2010) and its interaction with the gut microbiota (Nell et al., 2010), which together shapes subsequent downstream adaptive immune responses. In regard to EOS, while their numbers are reported to be elevated in both Crohn's disease (CD) and ulcerative colitis (UC), two of the idiopathic forms of IBD (Bischoff et al., 1996; Carvalho et al., 2003), the majority of studies report the preponderance of EOS and EOS activity in UC compared to CD patients (Ahrens et al., 2008; Lampinen et al., 2008; Lampinen et al., 2005; Wallon et al., 2011). To date, the mechanism(s) of action attributed to EOS in the pathogenesis of chronic intestinal inflammation have not been fully elucidated, although studies in animal models of IBD indicate that epithelial barrier disruption and damage to gut mucosal surfaces occur following EOS degranulation and release of EOS granule proteins, such as eosinophil cationic protein (ECP) and major basic protein (MBP), resulting in acute colitis (Forbes et al., 2004; Furuta et al., 2005; Shichijo et al., 2005; Vieira et al., 2009). Other studies have suggested that EOS can also act as immunoregulatory cells, stimulating T cell activation and proliferation, and subsequent release of Th1 and Th2 cytokines (Lampinen et al., 2005; Woerly et al., 1999), thereby perpetuating disease chronicity.

IL-33 and IBD

In 2010, four independent groups reported the increased expression and association of IL-33 with IBD, primarily in UC and to a lesser extent in CD (Beltran et al., 2010; Kobori et al., 2010; Pastorelli et al., 2010; Seidelin et al., 2010). Other groups have since confirmed these findings and together have established that in active UC, IL-33 is localized to, and potently upregulated in, intestinal epithelial cells and to a lesser extent, infiltrating lamina propria (LP) mononuclear cells belonging to the monocyte/macrophage and B-cell lineages (Beltran et al., 2010; Pastorelli et al., 2010). IL-33 has also been reported to be expressed in activated subepithelial myofibroblasts situated below ulcerative lesions in UC patients (Kobori et al., 2010; Sponheim et al., 2010), indicating a potential role for IL-33 in ulcer/wound healing, which may be different in UC compared to CD. In fact, although it is now widely accepted that IL-33 plays a central role in the pathogenesis of IBD, particularly UC, its precise function in this process has not been clearly defined. Mechanistic studies primarily utilizing acute, chemically-induced models of colitis have resulted in ambiguous findings, at best, supporting both a pathogenic (Imaeda et al., 2011; Oboki et al., 2010; Rani et al., 2011; Sedhom et al., 2012) as well as protective (Duan et al., 2012; Grobeta et al., 2012) function for IL-33 in intestinal inflammation. To date, the IL-33/ST2 axis has not been mechanistically evaluated in a chronic, immunologically-mediated model of IBD.

The SAMP1/YitFc mouse model of IBD

The SAMP1/YitFc (SAMP) mouse strain represents a chronic model of IBD and provides an excellent system in which to study the contributions made by specific cell types to the development of enteritis, prior to the onset of inflammation,

during the initiation and acute phase of inflammation, and when chronic disease is established (reviewed in Pizarro et al., 2011). Additionally, since SAMP mice were derived from brother-sister mating of WT AKR mice (parental strain) (Matsumoto et al., 1998), the phenotype occurs spontaneously as in the human condition, without chemical, genetic, or immunologic manipulation. Inflammation is fully developed by 10-12 wks of age (Rivera-Nieves et al., 2003), with Th1-type immune responses predominating early during the inductive phase (Bamias et al., 2002; Kosiewicz et al., 2001), and a mixed Th1/Th2 phenotype evolving as ileitis develops into chronic, established disease (Bamias et al., 2005). Of note, an inherent epithelial barrier defect characterizes SAMP mice that occurs prior to the onset of intestinal inflammation and persists when SAMP are raised under germ-free (GF) conditions (Olson et al., 2006). Relevant to the present study, elevated levels of serum and ileal IL-33 have been reported in SAMP compared to control AKR mice (Pastorelli et al., 2010), while adoptive transfer of SAMP $CD4^+$ T cells into naïve SCID recipients results in ileitis and colitis that is abrogated by neutralizing IL-5, the major maturation and differentiation factor for EOS in mice and humans (Takedatsu et al., 2004).

PURPOSE OF THE STUDY

In this study, we utilized the SAMP model of Th1/Th2-driven enteritis to mechanistically investigate whether IL-33 served any role in the development of chronic intestinal inflammation mediated by EOS. Our results show that (a) EOS increase with the progression and severity of chronic intestinal inflammation that is reversed upon EOS depletion, (b) exogenous IL-33 administration induces EOS homing to the gut and a potent intestinal mucosal Th2 immune response, while IL-33 blockade reduces these effects and diminishes the overall severity of disease, and (c) the commensal flora is essential for the induction of IL-33, subsequent EOS infiltration, and the production of Th2 cytokines that exacerbates ileitis characteristic of SAMP mice. Taken together, these experiments demonstrate an important role for IL-33 in the infiltration and activation of EOS and the development of chronic intestinal inflammation. As such, targeting the IL-33/ST2 axis for therapeutic purposes may prove to be beneficial for the treatment of IBD, particularly in patients with UC.

MATERIALS AND METHODS

Animals. SAMP1/YitFC (SAMP) and AKR/J (AKR) mice were maintained under specific pathogen free (SPF) conditions in the Animal Resource Core Facility of Case Western Reserve University. GF SAMP mice where purchased from Taconic Farms Inc (One Hudson City Centre, Hudson, NY). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University.

Patients. Mucosal biopsies were obtained during ileocolonoscopy from the ileum and colon of adult patients with IBD. Diagnosis of UC was established by clinical, macroscopical, and histological criteria. Endoscopies were performed at the Endoscopy Unit of the 1st Dpt of Internal Medicine, Propaedeutic, at Laikon Hospital, Athens, Greece, and at the Gastroenterology and Endoscopy Unit of IRCCS Policlinico San Donato, San Donato Milanese, Milano, Italy. In most cases, samples were taken from the same individual both from normal-looking mucosa and from areas with evident inflammation. The control group consisted of individuals undergoing screening colonoscopy, who were included in the study only when endoscopy did not reveal any mucosal abnormalities. Specimens were maintained at 4 °C for 1–2 days in RNAlater® RNA stabilization solution (Ambion Inc., CA) and then stored at −80 °C for total RNA extraction purpose. All diagnoses were confirmed by clinical, macroscopic, and histologic criteria. Full-thickness colon tissues from UC patients and controls were obtained as formalin-fixed, paraffin-embedded surgical specimens from the Pathology Department of the University Hospitals (UH) of

Cleveland for histology and IHC staining purpose. All studies were approved by Laikon Hospital and IRCCS Policlinico San Donato Ethics Committee, and by the Internal Review Board of the UH.

Mice Timecourse and Treatments. SAMP and AKR of 4-, 12-, and-20-wk-old were used for the timecourse study. EOS depletion was performed by intraperitoneal (i.p.) injection 2 times a week for 6 weeks of antibodies against mouse IL-5 (GS2-19-4; Lee Laboratories, Mayo Clinc, AZ) and mouse CCR3 (TRFK-5 mAb; Lee Laboratories, Mayo Clinic, AZ) administered either singularly and simultaneously (5mg/Kg) on 20-wk-old SAMP mice. Control mice were treated with an isotype mouse IgG antibody (IR-MS-GF-ED; Innovative Research). For IL-33 administration study 4-and 12-wk-old SAMP and AKR mice were i.p. injected with recombinant mouse IL-33 (ALX-522-101; Enzo Life Sciences) daily for one week (33μg/Kg). Control mice were injected with PBS. IL-33 neutralization was achieved by i.p. administration in 10-and 20-wk-old SAMP mice with an antibody against mST2L (mu anti-huAGP3pep-4D2 or rat anti muST2 M955, 1201 Amgen Court West AW2/D4262, Seattle, WA 98101-2936) 2 times a week for 6 weeks (5mg/Kg). Control mice were administered with an isotype antibody (4D2 mouse IgG1 mAb # P62382.3; 1201 Amgen Court West AW2/D4262, Seattle,WA 98101-2936).

Tissue Harvest and Histologic Assessment of SAMP Ileitis. Terminal ilea were removed from mice, rinsed with cold PBS, opened longitudinally, and submerged in Bouin's solution (LabChem). Fixed tissues were processed, paraffin-embedded, sectioned at 3-4μm, stained with Hematoxylin & eosin (H&E), and

resulting ileal sections were histologically evaluated by a single pathologist in a blinded fashion, using a validated semiquantitative scoring system.

Isolation and Culture of mesenteric lymph node (MLN) Cells. MLNs were collected and processed for cells isolation as follows: briefly organs were aseptically removed and pressed through a 70-μm cell strainer to obtain single cells suspensions. Lysis of erythrocytes was performed with ACK lysis buffer (Lonza). Resulting cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 with 10% FBS and 1% penicillin/streptomicyn, in the presence of immobilized anti-CD3 (5 μg/mL) and soluble anti-CD28 (1μg/mL) for 48-72 h. Supernatants were subsequently collected for further analysis.

Total RNA Extraction and Reverse Transcription. Mouse ilea and endoscopic biopsies were placed in RNA Later (Applied Biosystems), left at 4 °C overnight, then frozen and stored at -80 °C. Total RNA was isolated by using the Rneasy kit (Qiagen) and reverse-trinscribed by using the High Capacity RNA-to cDNA kit (Applied Biosystem).

qRT-PCR. Expression of mouse IL-33, IL-4, IL-5, IL-13, CCL11, CCL24 and human IL-5, CCL11, CCL24, CCL26 mRNA transcripts relative to mouse and human β-actin (houskeeping gene) was measured in mouse ileal tissue and in endoscopic biopsies using the following primers for mouse and Qiagen kits for human: mouse IL-33 sense (5'-TCCTTGCTTGGCAGTATCCA-3'), mouse IL-33 antisense (5'-

TGCTCAATGTGTCAACAGACCT-3'), mouse IL-4 sense (5'-GCTAGTTGTCATCCTGCTCTTC-3'), mouse IL-4 antisense (5'-GGCGTCCCTTCTCCTGTG-3'), mouse IL-5 sense (5'-GCTTCTGCACTTGAGTGTTCTG-3'), mouse IL-5 antisense (5'-CCTCATCGTCTCATTGCTTGTC-3'), mouse IL-13 sense (5'-TTGCTTGCCTTGGTGGTCTC-3'), mouse IL-13 antisense (5'-GGGAGTCTGGTCTTGTGTGATG-3') mouse CCL11 sense (5'-TGTCTCCCTCCACCATGCA-3'), mouse CCL11 antisense (5'-GATCTTCTTACTGGTCATGATAAAGCA-3'), mouse CCL24 sense (5'-TGCATCTCCCCATAGATTCTGT-3') mouse CCL24 antisense (5'-ACTCGGTTTTCTGGAATTTTCTTG-3'), mouse β-actin sense (5'-CAGGGTGTGATGGGAATG-3'), mouse β-actin antisense (5'-GTAGAAGGTGTGGTGCCAGAT-3'), human IL-5 (PPH00692A-200), human CCL11 (PPH0057B-200), human CCL 24 (PPH01162B-200), human CCL26 (PPH0163E-200), human β-actin (330001 PPH00073E-200). RT-PCRs were performed using an Applied Biosystems Step Plus machine (Applied Biosystems). Reaction mixture consisted of 15% volume first-strand synthesis in a total volume of 20 μL that included Power SYBR Green core reagents (Applied Byosistems) and 500 nM final concentration of primers. Thermal cycling conditions were 95 °C/10 min followed by 40 cycles of 95 °C/15 sec and 60 °C/1 min. Expression of all the genes of interest was normalized to β-actin.

Proteins assays. For the IL-5 and CCR3 neutralization and the IL-33 administration studies Mouse Cytokine Screen-IR 16-plex (Quansys Biosciences)

were used to measure IL-1α, IL-1β, IL-6, IL-12p70, IL-2, IFNγ, TNFα, IL-17, IL-4, IL-5, IL-10, MCP-1, MIP-1α, RANTES cytokines levels in mln's CD3/CD28 stimulated supernatants. Mouse IL-13 was measured by Ready-Set-Go ELISA assays (eBioscience cat # 88-7137-22). For the IL-33 neutralization study Ready-Set-Go ELISA kits (eBioscience) were used for measuring levels of the following mouse Th2 cytokines: IL-4 (88-7044-22), IL-5 (88-7054-22), IL-10 (88-7104-22), IL-13 (88-7137-22).

Immunohistochemistry. Immunohistochemistry (IHC) staining was performed using a polyclonal goat anti-mouse IL-33 IgG (AF3626; R&D Systems) at a 1:100 dilution or goat anti-human IL-33 IgG (AF3625; R&D Systems) at a 1:50 dilution, and a monoclonal (clone MT-14.7) rat anti-mouse MBP IgG (Lee Laboratories, Mayo Clinic, AZ) at a 1:500 dilution or a monoclonal (clone MM25-82.2) mouse anti-mouse and-human EPX IgG (Lee Laboratories, Mayo Clinic, AZ) at a 1:500 dilution. Tissue samples from mice were fixed by immersion in Bouin's solution, human samples in 10% neutral buffered formalin (Thermo Scientific), processed, embedded in paraffin, and 3-μm-thick sections were placed on glass slides. Sections were then deparaffinized and incubated in normal serum (Vector Labs) for nonspecific blocking. Samples were blocked for endogenous peroxidase activity by using 1.75% H_2O_2.. Antigen retrieval was performed on human samples heating them in microwave (2x5 min) while submerged in unmasking solution (Vector Labs). After incubation with primary antibodies at 4 °C, slides were rinsed in PBS and incubated with an appropriate biotinylated secondary antibody (Vector Labs). Slides were then rinsed in PBS and incubated with Vectastain ABC Kit (Vector Labs)

for 30 min. Immunoreactive cells were visualized by addition of a diaminobenzidine substrate (Vector Labs), were counterstained with hematoxylin and mounted using an 80% glycerol mount. All incubations were conducted at room temperature unless otherwise noted. Negative controls were prepared under identical condition as described above avoiding to put primary antibody.

EOS count. EOS, stained for MBP, were counted as number of EOS/high power field by a single pathologist in a blinded fashion.

Images acquisition. Images were obtained on an Axiophot microscope, captured on an Axiocam and assembled on an Axiovision Release 4.5 (Carl Zeiss, Inc., Thornwood, NY). The objective lenses used were a Plan-Apochromat 20X/0.60 ∞/0.17 for mice tissues and a EC-Plan-neofluor 10X/0.3 Ph1 ∞/-.

Statistical analysis. Statistical analysis was performed by using, paired Student's *t* test for paired data, and linear regression, as appropriate.

RESULTS

Marked increase in EOS infiltration is associated with disease progression and severity in the SAMP model of chronic intestinal inflammation

In order to determine whether EOS played an important role in the pathogenesis of chronic intestinal inflammation in an established Th1/Th2-driven model of IBD, we utilized SAMP mice and performed a detailed time course study comparing the presence of EOS, its correlation to disease severity, and expression of EOS-associated mediators in 4-, 12- and 20-wk-old SAMP with their age- and gender-matched parental (AKR) controls. These distinct ages represent critical time points in the development of SAMP ileitis, based on phenotypic and functional differences as follows: 3-4 wks (prior to histologic evidence of ileal inflammation, but epithelial permeability defect present), 12 wks (early, active phase of inflammation with prominent polymorphonuclear leukocytes (PMN) infiltration, expansion of macrophages and lymphocytes, and dominant Th1 cytokine profile), and 20 wks (established, chronic inflammation with prevalence of lymphocytic and plasma cell infiltration, and mixed Th1/Th2 cytokine profile). After 20 wks of age, no significant differences are observed in the severity of intestinal inflammation assessed by several parameters, including histology, percent of immune cell subsets in MLNs and LP, MLN cytokine profiles, and epithelial permeability (Pizarro et al., 2011). In this study, EOS were identified by MBP-positivity since MBP represents one of the most abundant proteins produced by EOS and exists as two different homologues (*i.e.*, MBP1 and MBP2), with the former produced at significantly lower levels in EOS, but also in basophils, while the latter is specific for EOS (Furuta et al., 2005; Woodruff et al., 2011).

Our results showed that prior to the onset of histologically-evident inflammation, 4-wk-old SAMP displayed sparse, scattered MBP^+ cells, identified as EOS, within the ileal LP, similar to that observed in age-matched AKR controls, but with evident accumulation already present within the base of the crypts (Fig. 1, left panels). By 12 wks, massive EOS infiltration was seen throughout the LP of SAMP ilea, with marked accumulation close to the base of the crypts, and in the deep submucosa immediately adjacent to the muscularis propria. Histologically, frank ileal inflammation was present at 12 wks, with expansion of the LP and loss of conventional villous architecture (Fig. 1, lower middle panel). This phenotype persisted in 20-wk-old SAMP (Fig. 1, lower right panel), while age-matched AKR control groups showed scant numbers of EOS scattered throughout the LP with no histologic evidence of inflammation (Fig. 1, upper middle & right panels).

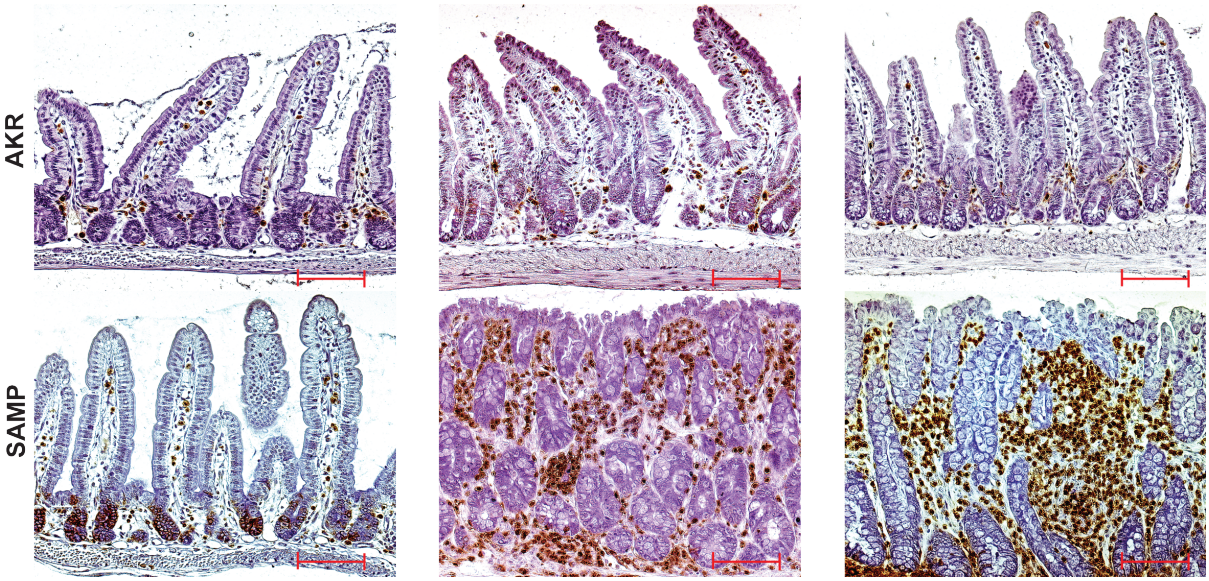

Fig 1. Increased infiltration of EOS is associated with the progression of disease severity in SAMP mice. Representative photomicrographs of full-thickness ilea from 4-, 12-, and 20-wk-old SAMP and AKR mice (N=4), evaluated for EOS by IHC for major basic protein (MBP), show sparse, scattered staining within the lamina propria of 4-wk-old SAMP, prior to histologic evidence of ileitis (lower, left panel), while 12- and 20-wk-old SAMP with established disease show a marked and progressive increase in EOS infiltration as disease severity escalates (lower, middle and right panels). Little to any staining is observed in age-matched AKR control mice (upper panels). All panels X20 + 1.25 original mag; scale bar=100μm.

Quantitative enumeration of EOS revealed no difference between 4-wk-old SAMP and AKR mice (86.50 ± 34.53 versus 80.50 ± 6.17 EOS/HPF) (Fig. 2, left panel), which both strains showed little to no ileal inflammation (1.33 ± 0.54, SAMP and 0.63 ± 0.26, AKR) (Fig. 2, middle panel). Conversely, the number of EOS in SAMP ilea was vastly increased by 12 wks of age compared to AKRs (500.00 ± 71.53 versus 69.00 ± 19.21 EOS/HPF), with an associated elevation in inflammation (9.13 ± 1.05, versus 1.75 ± 0.41). These trends were sustained in SAMP compared to AKR ilea through 20 wks (441.00 ± 71.50 versus 53.00 ± 9.28 EOS/HPF and 8.33 ± 0.53, versus 1.50 ± 0.27), with no significant differences in eosinophilia and ileal inflammation versus 12-wk-old SAMP (Fig. 2, left & middle panels). Overall, EOS number positively correlated with the total inflammatory scores of experimental mice (r = .8100) (Fig. 2, right panel), and confirmed the association of EOS infiltration and the severity of ileitis in SAMP mice.

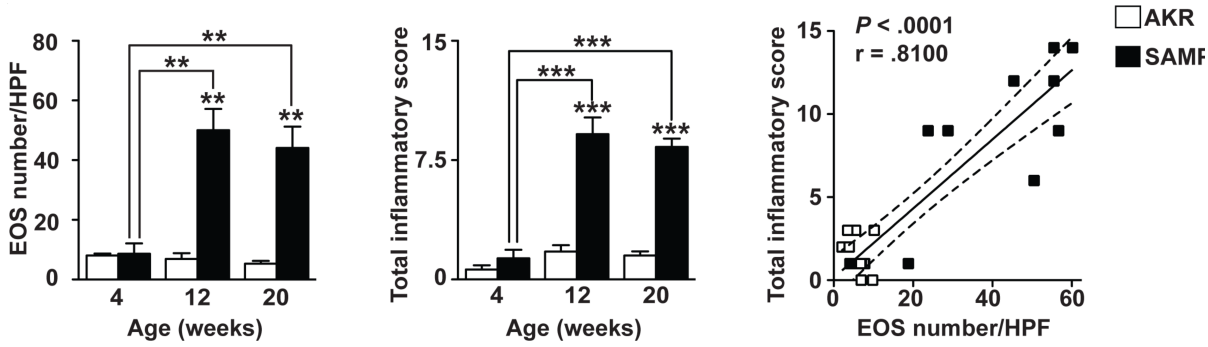

Fig. 2. Increased infiltration of EOS is associated with the progression of disease severity in SAMP mice. EOS count, total inflammatory score (TIS), and correlation between EOS count and TIS (N≥5); **p<0.01, ***p<0.001 vs. age-matched AKR controls and as indicated.

To further characterize the importance of EOS in SAMP mice, we next measured prominent mediators important in EOS development and recruitment, including IL-5, CCL11, and CCL24. IL-5 represents the primary factor for EOS

maturation and differentiation (Takedatsu et al., 2004), but is also important in EOS activation and recruitment (Lampinen et al., 2001; Mishra et al., 1999), while CCL11 and CCL24, also known as eotaxin-1 and -2, respectively, are EOS-specific chemokines that bind to the chemokine receptor, CCR3, expressed on the surface of EOS and are critical for EOS recruitment (Hogan et al., 2008; Masterson et al., 2011). Our results showed no differences in either IL-5, CCL11, or CCL24 mRNA expression comparing non-inflamed, 4-wk-old SAMP to age-matched AKRs (Fig. 3). However, by 12 wks, IL-5 and CCL11 mRNA levels showed a 11.6- and 2.7-fold increase, respectively, compared to age-matched AKRs. The most dramatic difference was observed at 20 wks, wherein IL-5, CCL11, and CCL24 mRNA transcript levels were substantially increased by 13.3-, 3.9-, and 11.4-fold, respectively, compared to age-matched AKRs; in fact, mRNA expression of these particular EOS-associated mediators were potently elevated in 20-wk-old SAMP compared to earlier time points evaluating younger SAMP mice (Fig. 3).

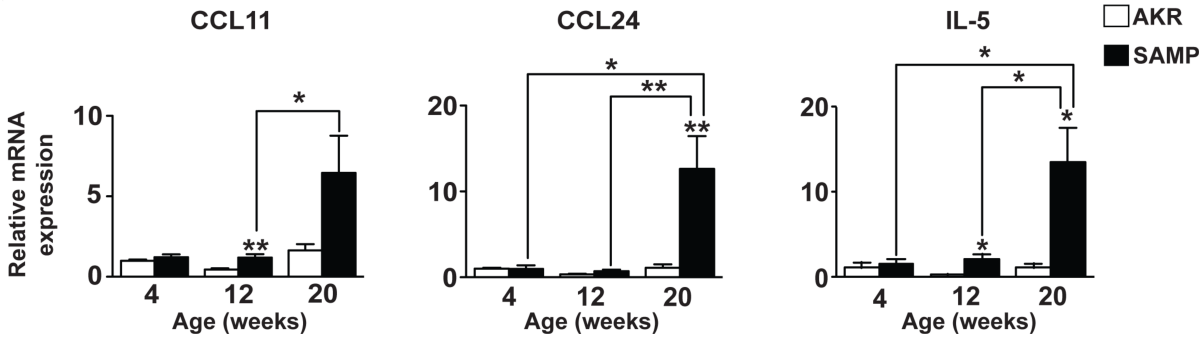

Fig. 3. Increased infiltration of EOS-associated chemokines/cytokines is associated with the progression of disease severity in SAMP mice. Relative mRNA levels of the EOS-associated cytokines/chemokines, IL-5 and eotaxin-1 (CCL11) and -2 (CCL24), in full-thickness ileal tissues from SAMP vs. age-matched AKR control mice (N≥5); *p<0.05, **p<0.01 vs. age-matched AKR controls and as indicated.

Taken together, these data showed that, overall, cytokines/chemokines associated with eosinophilia are elevated during the development of SAMP ileitis, and although they may be critical in the initial activation and infiltration of EOS, they also appear to be important in the exacerbation of chronic intestinal inflammation and the maintenance of established disease.

EOS depletion potently decreases ileal inflammation in SAMP mice with established disease

We next tested whether EOS depletion could significantly affect ileitis in chronically-inflamed SAMP mice. Treatment with anti-IL-5 was previously shown to successfully decrease ileitis and the presence of granulocytes, including EOS, in SAMP mice (Takedatsu et al., 2004), while effective *in vivo* depletion of EOS has been reported following administration of antibodies specific for CCR3 (Grimaldi et al., 1999). As such, 20-wk-old SAMP with established disease were treated with a combination of anti-CCR3 and IL-5 antibodies for a period of six wks to achieve complete EOS depletion, after which ileal tissues were evaluated for histologic evidence of inflammation, presence of EOS, as well as eotaxin mRNA expression.

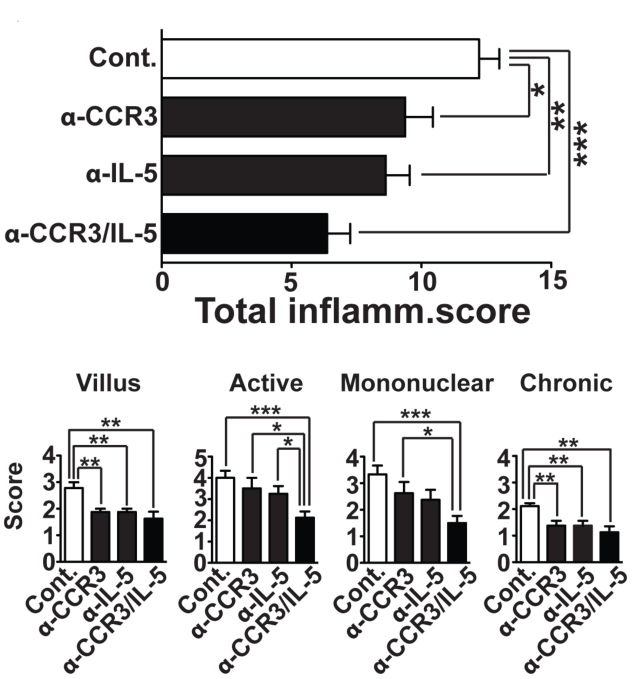

Figure 4. EOS depletion markedly reduces the severity of ileitis in ilea of SAMP mice with established disease. Histologic evaluation of ilea from EOS-depleted 20-wk-old SAMP mice compared to IgG-treated controls (Cont) (N≥6); *p<0.05, **p<0.01, ***p<0.001 vs. Cont. and as indicated.

Our results showed that treatment with either anti-CCR3 or anti-IL-5 had a moderate, albeit significant, effect at decreasing total inflammation (9.38 ± 1.07 and 8.63 ± 0.92, respectively), while combination anti-CCR3/anti-IL-5 was more effective than either antibody administered alone at potently downregulating ileitis compared to IgG-treated controls (6.38 ± 0.89 versus 12.22 ± 0.78) (Fig. 4). All three treatment modalities primarily decreased villous and chronic inflammatory indices, while active and mononuclear inflammatory features did not significantly change upon single antibody treatment, but were reduced upon combination anti-CCR3/anti-IL-5 administration (Fig. 4, lower panels).

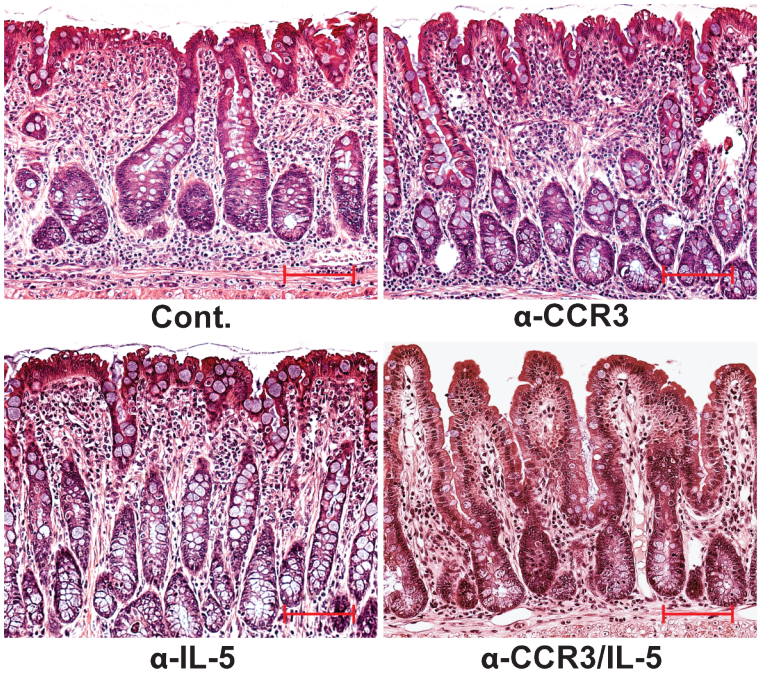

Figure 5. EOS depletion markedly reduces the severity of ileitis in ilea of SAMP mice with established disease. Representative photomicrographs of H&E- stained ileal tissues show a striking improvement in overall gut morphology, with restoration of villous architecture and decreased inflammatory cell infiltrates in SAMP mice treated with either anti-CCR3 or anti-IL-5 neutralizing Abs alone or in combination compared to IgG-treated controls (Cont). All panels X20 + 1.25 original mag; scale bar=100μm.

Histologically, control IgG-treated SAMP mice displayed severe chronic active ileitis characterized by marked villous blunting, increase of monocytic inflammatory cells, especially plasma cells in the LP, as well as active cryptitis and ulceration (Fig. 5). Upon either anti-CCR3 or anti-IL-5 treatment, both the activity and severity of chronic ileitis showed slight

dampening, featured by partial restoration of villi and reduction in LP inflammatory infiltrates, while anti-CCR3 and anti-IL-5 together led to improved restoration of epithelial villous structure and an impressive decrease of overall ileal inflammation compared to IgG-treated control mice.

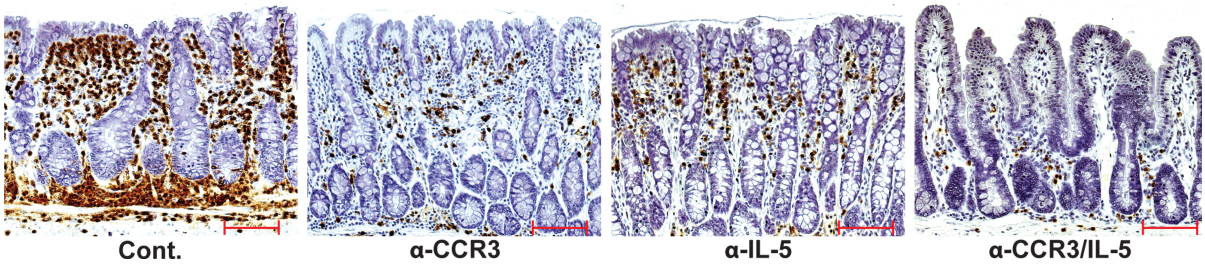

Figure 6. EOS depletion potently decreases EOS infiltration in ilea of SAMP mice with established disease. Representative photomicrographs of MBP-stained ileal tissues show a marked reduction in EOS, in SAMP mice treated with either anti-CCR3 or anti-IL-5 neutralizing Abs alone or in combination compared to IgG-treated controls (Cont). All panels X20 + 1.25 original mag; scale bar=100μm.

In addition, evaluation of EOS by IHC of MBP^+ cells revealed a dramatic decrease in ileal EOS infiltration (Fig. 6) and the total number of EOS (Fig. 7) after either anti-CCR3 or anti-IL-5 antibody administration (35.53 ± 7.95 and 23.55 ± 5.28 EOS/HPF), which was even more impressive with combination anti-CCR3/anti-IL-5 treatment compared to IgG-treated SAMP (17.78 ± 2.39 versus 57.00 ± 3.48 EOS/HPF)

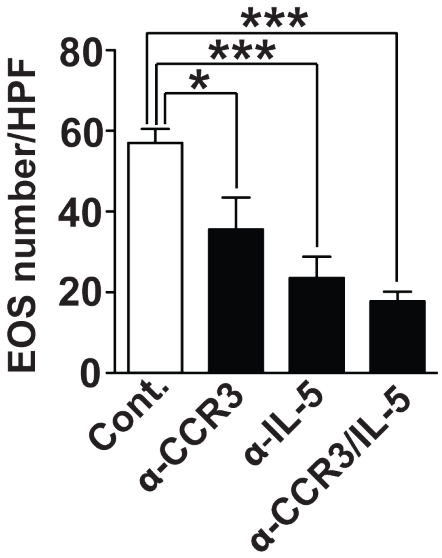

Figure 7. EOS depletion potently decreases EOS infiltration in ilea of SAMP mice with established disease. EOS count in EOS-depleted vs. IgG control-treated SAMP mice (N≥6); *p<0.05, ***p<0.001 vs. Cont. and as indicated.

Moreover, anti-CCR3 and anti-IL-5 administered either alone or together strongly

downregulated mRNA expression of ileal CCL11 by 3.4-, 1.9-, and 2.1-fold, respectively, while only combination anti-CCR3/anti-IL-5 treatment was effective at decreasing CCL24 mRNA transcripts (by 2.9-fold) compared to IgG-treated controls (Fig. 8).

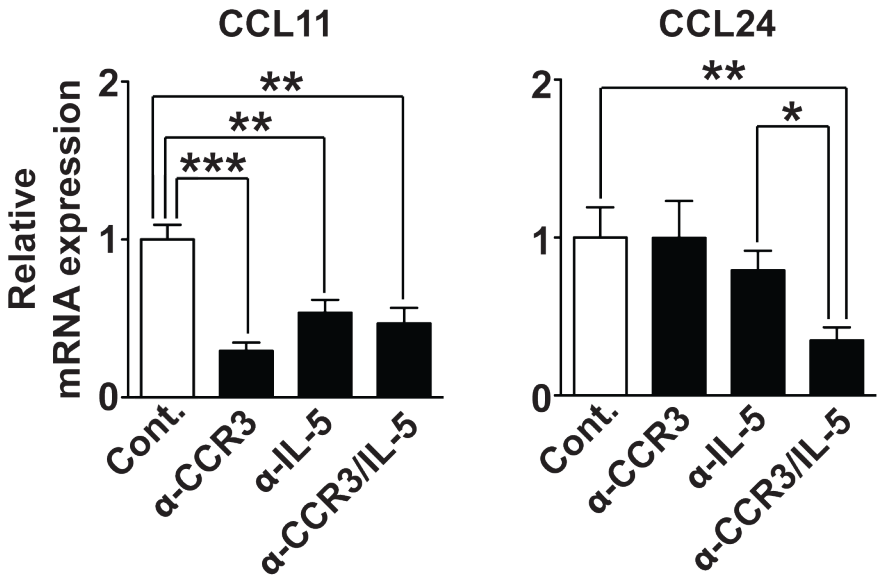

Figure 8. EOS depletion potently decreases eotaxin expression in ilea of SAMP mice with established disease. eotaxin mRNA expression in EOS-depleted vs. IgG control-treated SAMP mice (N≥6); *p<0.05, **p<0.01, ***p<0.001 vs. Cont. and as indicated.

To further investigate the downstream effects of EOS depletion in chronic intestinal inflammation, cytokine profiles were evaluated in gut-associated lymphoid tissue (GALT), specifically the draining MLN, of SAMP mice following anti-CCR3/anti-IL-5 antibody treatment (Fig. 9). Previous studies have established that unfractionated, anti-CD3/anti-CD28-activated MLN cells from inflamed SAMP mice with advanced disease produced elevated levels of both Th1 and Th2 cytokines, promoting a mixed Th1/Th2 gut mucosal immune response compared control AKRs (Bamias et al., 2005; Kosiewicz et al., 2001; Olson et al., 2006). Using a similar protocol, TCR-activated MLN cells from EOS depleted 20-wk-old SAMP were

assayed for the production of the classic proinflammatory cytokines, IL-1α, IL-1β, IL-6, and IL-12, prototypic Th1/Th2/Th17 cytokines, and the chemokines, MCP-1, MIP-1α, and RANTES.

In general, classic, acute proinflammatory cytokines were trending towards a decreased production in which single administration of either anti-CCR3 (for IL-6) or anti-IL-5 (for IL-1 and IL-12) was more effective than both antibodies given together (Fig. 9, top row). Interestingly, anti-IL-5 appeared to have a greater effect in reducing the Th1 cytokines, IFNγ and TNF, but not IL-2, while anti-CCR3 was more efficient a decreasing the Th2 cytokines, IL-4 and IL-5, but had no effect on IL-13 (Fig. 9, middle rows). Production of both IL-17 and RANTES decreased upon either anti-IL-5 or anti-CCR3 treatment, while MCP-1 and MIP-1α were differentially reduced after either anti-CCR3 or anti-IL-5 administration, respectively (Fig. 9, bottom row). Of note, for the majority of immune mediators measured, combination anti-CCR3/anti-

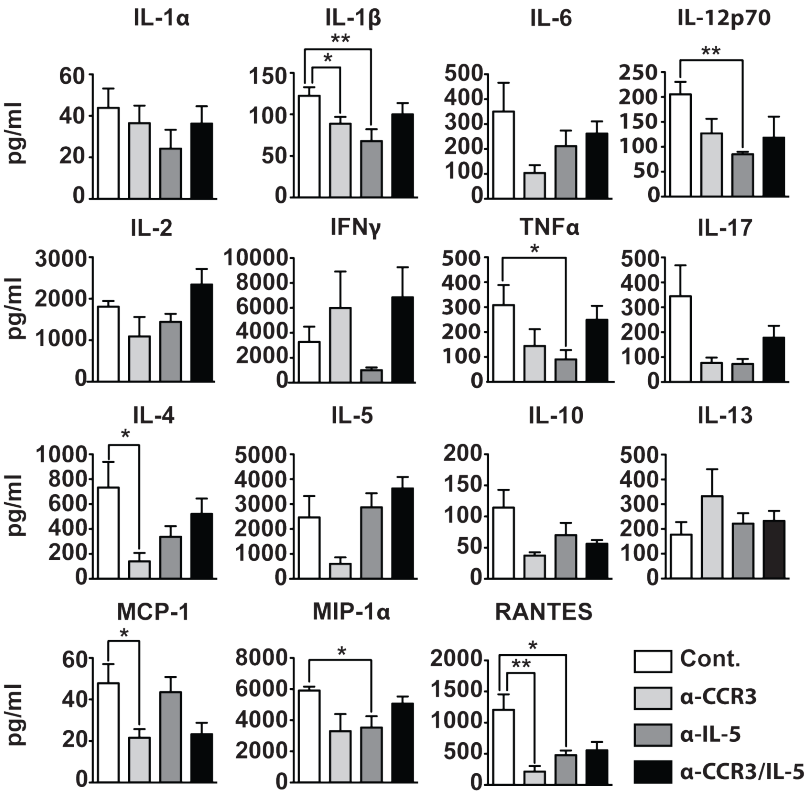

Figure 9. Cytokine profiles in SAMP mice following EOS depletion. Protein levels of classic proinflammatory (top row), Th1/Th17 and Th2, cytokines (2nd and 3rd rows), as well as chemokines (bottom row) in supernatants of *ex vivo* anti-CD3/anti-CD28-activated MLN cells from EOS-depleted vs. IgG Cont.-treated SAMP mice (N≥4); *p<0.05, **p<0.01.

IL-5 treatment was less effective at decreasing cytokine production compared to each antibody administered alone.

Taken together, these data show that EOS depletion by either anti-CCR3 or anti-IL-5, and more potently with a combination of both antibodies together, effectively decreased ileal inflammation in SAMP mice with established disease. Importantly, while EOS depletion was successfully achieved with all three treatments, each of the aforementioned antibodies administered separately or in combination had a differential effect on several immune mediators, including eotaxins, chemokines, and Th1 versus Th2 cytokines.

Exogenous administration of IL-33 induces robust EOS infiltration into the ilea and a potent gut mucosal Th2 response

Based on the importance of EOS in the pathogenesis of SAMP ileitis and our previous report demonstrating the increase of IL-33 in this IBD model (Pastorelli et al., 2010), we investigated whether IL-33 had a direct effect on EOS infiltration and function in the gut mucosa that could potentially lead to chronic intestinal inflammation. We confirmed that IL-33 mRNA expression was upregulated in the ilea of 12- and 20-wk-old SAMP compared to age-matched AKRs, and potently increased with disease progression (4.1- and 6.8-fold, respectively, versus 4-wk-old SAMP) (Fig. 10).

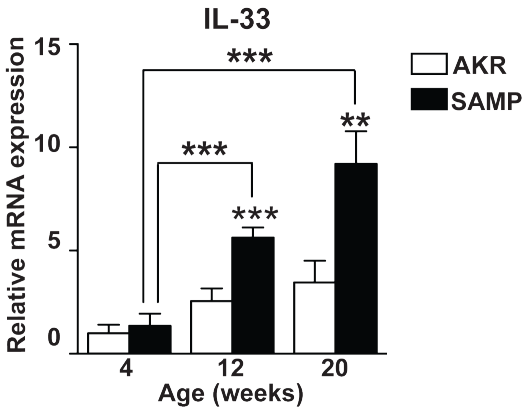

Figure 10. Increased expression of IL-33 is associated with the progression of disease severity in SAMP mice. Time course of IL-33 mRNA levels in full-thickness ilea from native SAMP and age-matched AKR controls (N≥9); *p<0.05, **p<0.01, ***p<0.001 vs. age-matched AKR.

Importantly, ileal IL-33 mRNA levels positively correlated with ileal inflammation (Pastorelli et al., 2010) and the number of infiltrating EOS (r = .7593) (Fig 11).

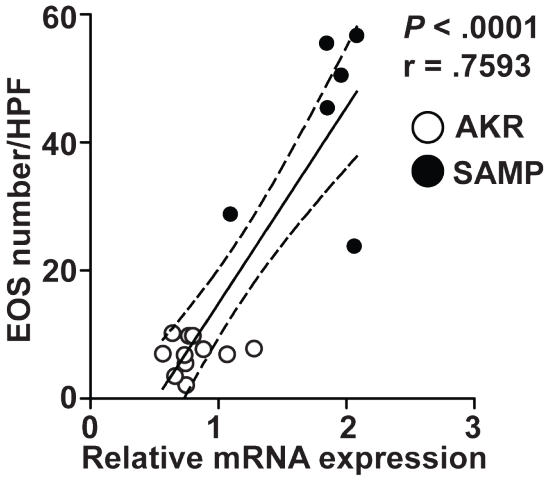

Figure 11. Increased expression of IL-33 is associated with the progression of disease severity in SAMP mice. IL-33 mRNA levels in full-thickness ilea from native SAMP and age-matched AKR controls and correlation with EOS number (N=17).

To determine whether IL-33 had the ability to induce EOS infiltration into the gut in the absence of background inflammation, recombinant IL-33 was administered to non-inflamed AKR control mice at 4 and 12 wks of age.

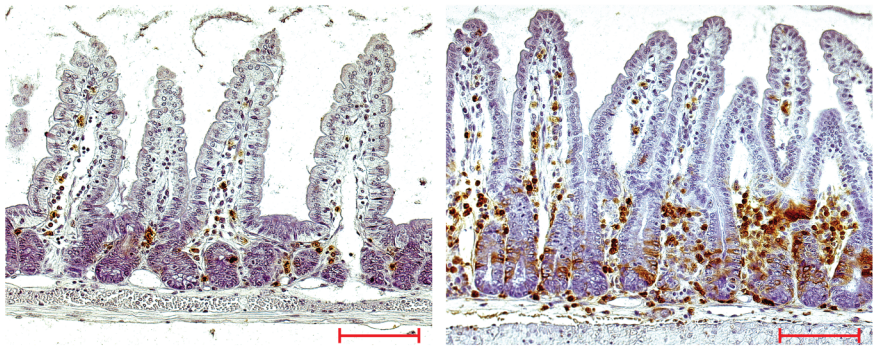

Figure 12. Exogenous administration of rIL-33 induces EOS infiltration into the ilea of normal, non-inflamed AKR mice. Representative photomicrographs of full-thickness ilea from 4-wk-old AKR mice showed abundant infiltration of EOS by IHC for MBP following administration of rIL-33 (right panel) compared to vehicle-treated Cont. (N=4, left panel); all panels X20+1.25 original mag, scale bar=100μm.

Daily administration of IL-33 for one week induced robust infiltration of EOS into the ilea for which total EOS number was greater in both 4- and 12-wk-old AKR compared to vehicle controls (25.18 ± 1.70 versus 7.23 ± 0.83 and 22.89 ± 4.45 versus 5.43 ± 0.44 EOS/HPF, respectively) (Fig. 12 & 13).

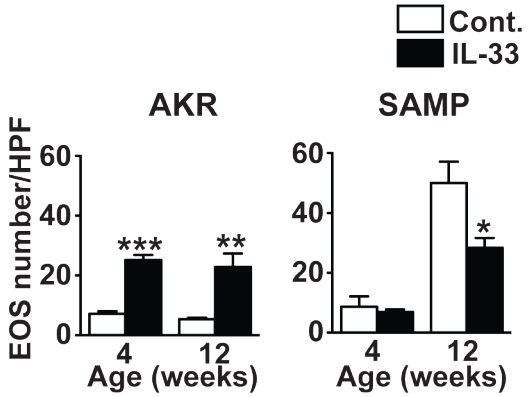

Figure 13. Exogenous administration of rIL-33 induces EOS infiltration into the ilea of normal, non-inflamed AKR mice. EOS number (N=4) in ilea of IL-33- and vehicle Cont.-treated mice. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle Cont.-treated mice.

Interestingly, exogenous administration of rIL-33 to SAMP mice did not increase the number of infiltrating EOS into the ilea of 4-wk-old mice (6.93 ± 0.85 versus 8.65 ± 3.45 EOS/HPF), and actually decreased EOS numbers in inflamed, 12-wk-old SAMP compared to vehicle controls (28.35 ± 3.32 versus 50.00 ± 7.15 EOS/HPF) (Fig. 13), suggesting that, in the setting of mucosal inflammation and when EOS number is already high, further increasing IL-33 may provoke a negative feedback mechanism to downregulate EOS infiltration into the

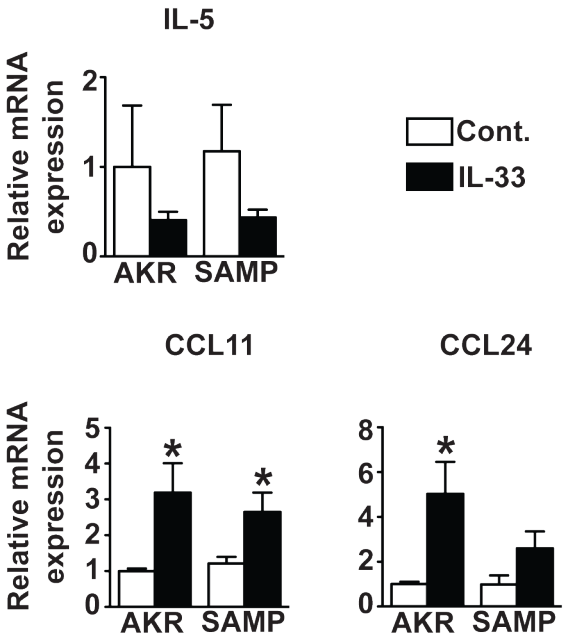

Figure 14. Exogenous administration of rIL-33 induces a relevant increase in eotaxins mRNA expression into the ilea of IL-33 treated mice. Relative mRNA expression of IL-5 and eotaxins in ilea of IL-33- and vehicle Cont.-treated mice (N=5-6). *p<0.05, vs. AKR vehicle Cont.-treated mice.

gut mucosa. This phenomenon was also observed for IL-5 mRNA transcript levels, wherein IL-33 administration to both mouse strains caused a decreased trend for local ileal IL-5 expression, while eotaxin mRNA levels were increased by 3.1- and 2.2-fold for CCL11 and 5- and 2.6-fold for CCL24 (both AKR and SAMP, respectively) compared to vehicle controls (Fig. 14).

Exogenous IL-33 administration also had the ability to induce a potent Th2 mucosal immune response in GALT by producing copious amounts of IL-4 (3.29 ± 0.68 versus 1.39 ± 0.77 ng/ml), IL-5 (11.14 ± 1.04 versus 2.30 ± 1.08 ng/ml), IL-10 (2.03 ± 0.80 versus 0.15± 0.10 ng/ml), and IL-13 (0.74 ± 0.067 versus 0.397 ± 0.060 ng/ml) in *ex vivo* TCR-activated unfractionated MLN cells derived from 12-wk-old SAMP after IL-33 treatment compared to vehicle-treated controls (Fig. 15).

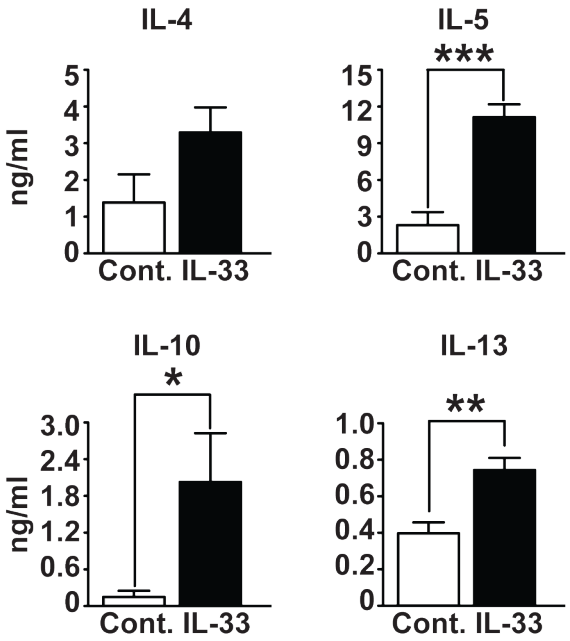

Figure 15. Exogenous administration of rIL-33 induces a potent Th2 response from draining MLN of normal, non-inflamed AKR mice. Th2 cytokine levels in supernatants of *ex vivo* anti-CD3/anti-CD28-activated MLN cells from IL-33- vs. vehicle Cont.-treated SAMP mice (N=4-6). *p<0.05, **p<0.01, ***p<0.001 vs. vehicle Cont.-treated mice.

These data confirm the ability of IL-33 to strongly induce the infiltration of EOS into the gut mucosa, and report the novel finding that IL-33 potently elicits Th2 cytokine production from GALT in a model of chronic intestinal inflammation. However, in the setting gut inflammation and when IL-33 production is high, exogenous administration of more IL-33 induces a potential negative feedback mechanism that appears to downregulate both EOS infiltration and IL-5 mRNA expression.

Neutralization of IL-33 by blockade of ST2/IL-33R decreases EOS infiltration, Th2 cytokine expression, and overall severity of ileitis in SAMP mice

To mechanistically address whether neutralization of IL-33 had the ability to ameliorate Th1/Th2-driven ileitis in SAMP mice, we designed two different experimental strategies to test the efficacy of: 1) preventing the progression of ileal inflammation, and 2) treating established disease. IL-33 activity was blocked by administration of an antibody against the IL-33 receptor, ST2, twice weekly for a period of six wks to 10- (preventive protocol) and 20-wk-old (therapeutic protocol) SAMP mice.

Our results showed that both neutralizing IL-33 early in the disease process, and treating established ileitis, was effective in downregulating ileal inflammation (Fig. 16 and 17).

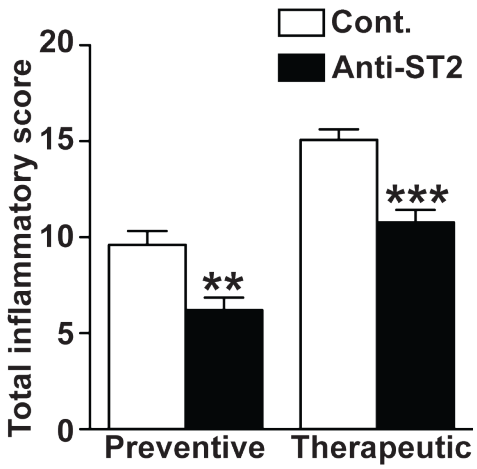

Figure 16. IL-33 neutralization in SAMP mice results in decreased ileitis severity Blockade of IL-33 in SAMP mice was achieved by administration of an anti-ST2 antibody given either prior to the onset of ileitis (preventive protocol) or during established disease (Histologic evaluation therapeutic protocol), both for a 6-wk period. Histologic evaluation demonstrate a marked reduction in total inflammation using both preventive and therapeutic strategies with anti-ST2- compared to IgG-treated Cont. (N=13-14); $p<0.05$, ***$P<0.001$ vs. IgG-treated Cont.

Using the preventive protocol, anti-ST2 was effective in reducing the severity of ileitis by 35% compared to IgG-treated controls (6.21 ± 0.65 versus 9.62 ± 0.71). Similarly, although baseline inflammation was greater in older mice, therapeutically treating established disease with anti-ST2 was equally effective, decreasing ileitis severity by 29% (10.77 ± 0.65 versus 15.07 ± 0.55) (Fig. 16). Histologically, anti-ST2 treatment decreased inflammatory cell infiltration, improved villous structure and

overall gut morphology, as well as reduced the number of infiltrating EOS compared to IgG-treated controls (Fig. 17).

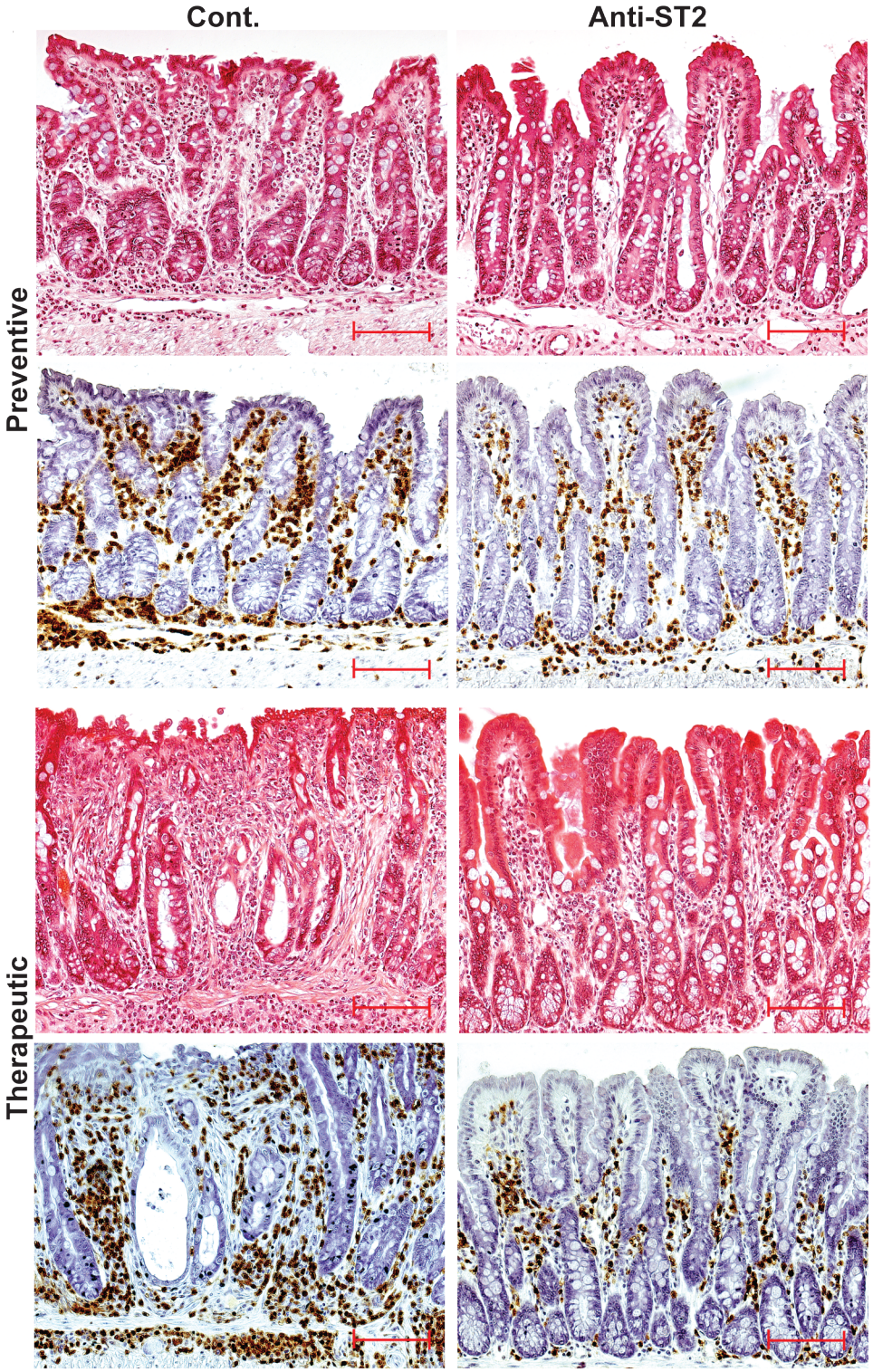

Figure 17. IL-33 neutralization in SAMP mice results in decreased ileitis severity and reduction of EOS infiltration. Representative photomicrographs of H&E- and MBP-stained full-thickness ileal tissues demonstrate a marked reduction in total inflammation, remarkable improvement in villous structure and overall gut morphology, and a prominent decrease in infiltrating EOS using both preventive and therapeutic strategies with anti-ST2- compared to IgG-treated Cont. All panels X20 + 1.25 original mag; scale bar=100μm.

A decreased trend of 3.3- and 4.6-fold was also observed for ileal IL-5 and CCL24 mRNA transcripts, respectively, from SAMP treated with anti-ST2 compared to IgG controls, particularly using the preventive protocol, while minimal changes were observed for CCL11 (Fig. 18).

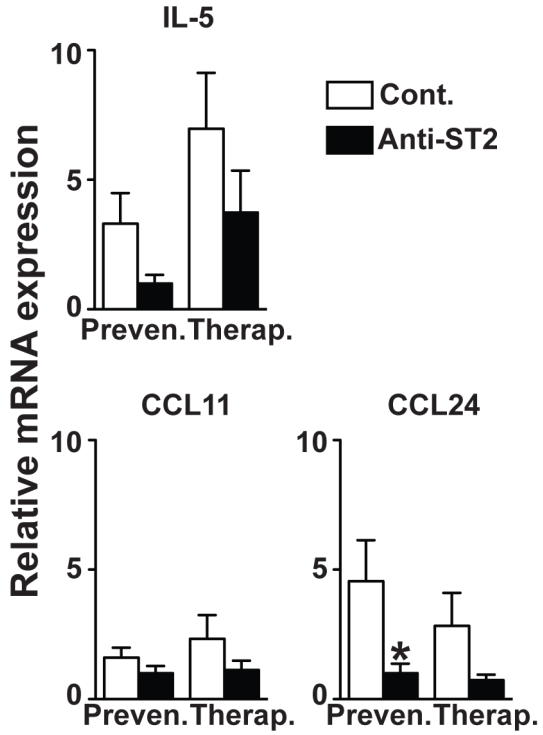

Figure 18. IL-33 neutralization in SAMP mice results in modulation of eotaxin expression. Relative mRNA expression of ileal IL-5 and eotaxins (N≥4); *p<0.05 vs. IgG-treated Cont.

Similarly, anti-ST2 administration using either a preventive or therapeutic approach dampened the production of the Th2 cytokines, IL-4 (318.67± 63.35 versus 146.17 ± 4.96 pg/ml; therapeutic protocol), IL-5 (365.25 ± 28.11 versus 191.30 ± 28.07 pg/ml; preventive protocol), and IL10 (1766.88 ± 245.20 versus 1010.50 ± 214.84 pg/ml; preventive protocol), but not IL-13, from activated MLN cells from treated SAMP mice (Fig. 19).

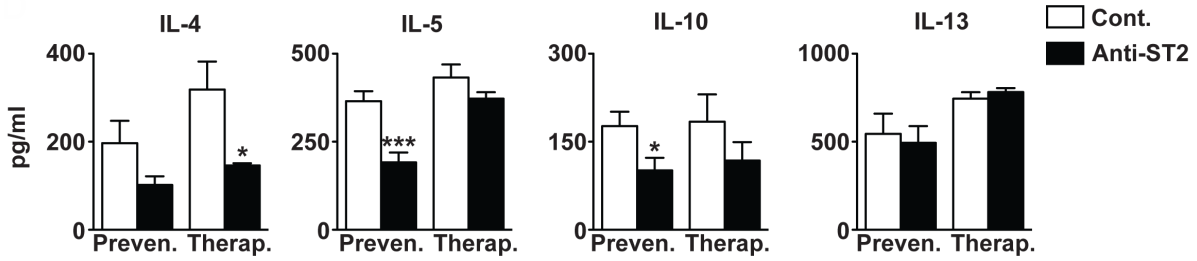

Figure 19. IL-33 neutralization in SAMP mice results in modulation of Th2 cytokine expression. Th2 cytokine levels in supernatants of *ex vivo* anti-CD3/anti-CD28-activated MLN cells (N≥4); *p<0.05, ***P<0.001 vs. IgG-treated Cont.

Together, these results indicate that neutralization of IL-33 in a chronic, Th1/Th2-mediated model of IBD is effective in decreasing overall gut inflammation, the infiltration of EOS, and GALT-derived Th2 immune responses.

EOS are abundantly expressed and co-localize with IL-33 in the inflamed colonic mucosa of UC patients

Previous studies have reported the widespread presence of EOS in the gut mucosa of patients with IBD, and that EOS infiltration and activity is particularly high in UC (Ahrens et al., 2008; Lampinen et al., 2008; Lampinen et al., 2005; Wallon et al., 2011). Likewise, several groups have recently established that IL-33 is upregulated in IBD, and also predominates in patients with UC (Beltran et al., 2010; Kobori et al., 2010; Pastorelli et al., 2010; Seidelin et al., 2010). We therefore performed confirmatory experiments to determine whether results obtained in the chronic, SAMP Th1/Th2-mediated IBD model reflects those in the human condition, comparing UC patients to normal controls. IHC studies confirmed abundant expression of IL-33 in UC patients, which intensified as disease became more severe and ulcerated (Fig. 20).

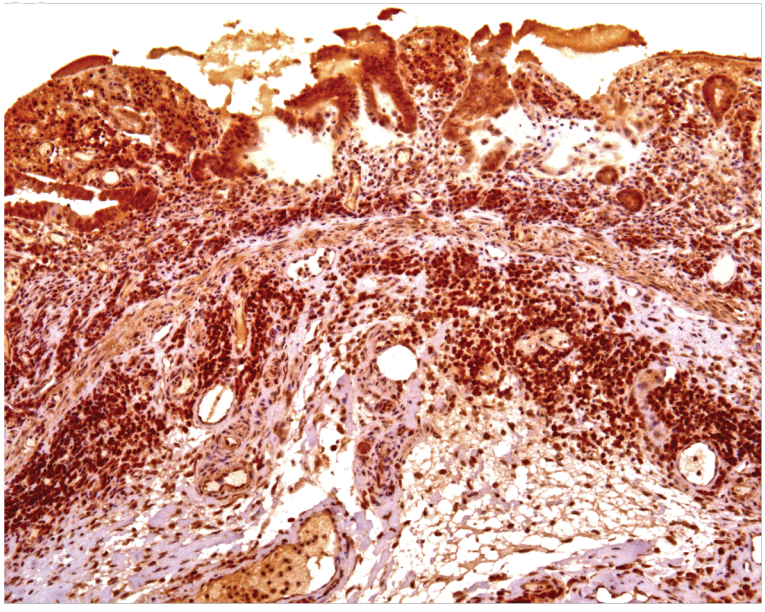

Figure 20. IL-33 is elevated in the colonic mucosa of UC patients. Representative photomicrographs of resected, full-thickness colonic tissue stained for IL-33 showed abundant IL-33 localized within the mucosa and submucosa of ulcerated, inflamed areas of UC patients. All panels X10+1.25 original mag; scale bar=100μm.

Diffuse IL-33 staining was observed throughout the LP, in intestinal epithelial cells and scattered mononuclear cells, within the inflamed gut mucosa. In addition, comparable to the distribution of EOS, IL-33 was found in submucosal mononuclear cells accumulating in areas marginating the muscularis mucosa. EOS localization was performed by IHC for eosinophil protein X (or EPX), specific for the detection of human EOS (Protheroe et al., 2009; Willetts et al., 2011). Similar to the staining pattern in SAMP mice, EPX^{+} EOS were found to primarily collect at the mucosal/submucosal interface adjacent to the muscularis propria, and co-localized, but did not overlap, with cells that were positive for IL-33 (Fig. 21).

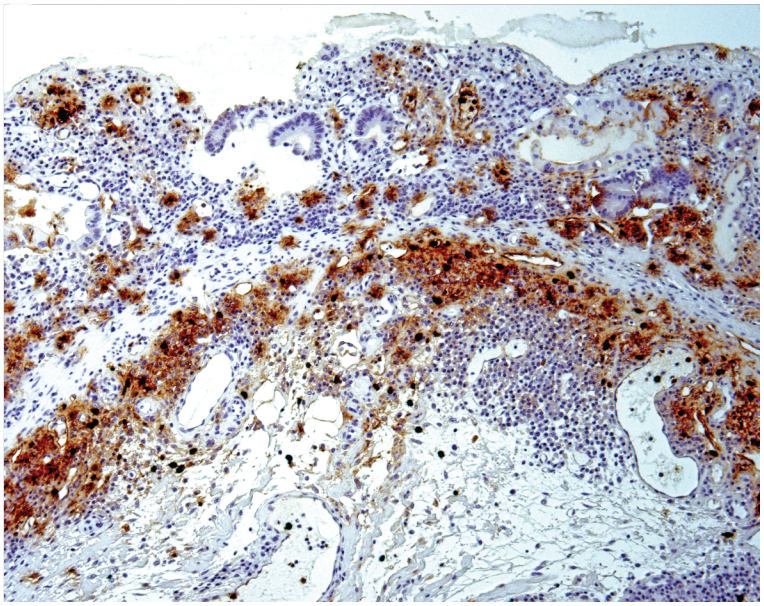

Figure 21. EOS infiltration is elevated in the colonic mucosa of UC patients. Representative photomicrographs of resected, full-thickness colonic tissue stained for EPX-specific EOS showed a marked accumulation of EOS distributed at the mucosal/submucosal interface in close proximity of IL-33 staining. All panels X10+1.25 original mag; scale bar=100μm.

Conversely, in non-inflamed controls, IL-33 staining was largely limited to epithelial cells with scattered staining of LP mononuclear cells, while EOS were sparsely present in areas close to the colonic crypts (Fig. 22 & 23).

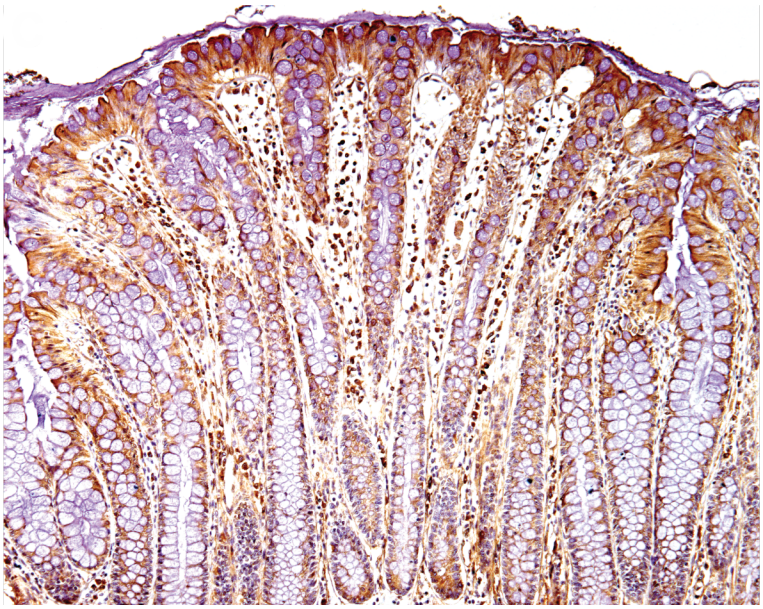

Figure 22. IL-33 is low in the colonic mucosa of control patients. Representative photomicrographs of resected, full-thickness colonic tissue stained for IL-33. Healthy, non-inflamed areas of control patients showed less IL-33, primarily limited to surface epithelial cells. All panels X10+1.25 original mag; scale bar=100μm.

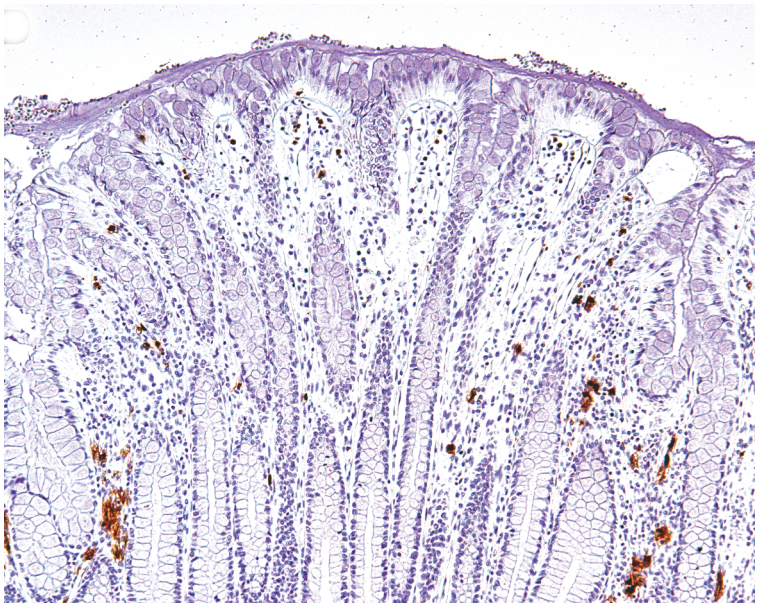

Figure 23. EOS infiltration is low in the colonic mucosa of control patients. Representative photomicrographs of resected, full-thickness colonic tissue stained for EPX-specific EOS. Healthy, non-inflamed areas of control patients showed sparsely scattered EOS staining,. All panels X10+1.25 original mag; scale bar=100μm.

Interestingly, IL-5 mRNA levels also recapitulated results obtained from IL-33 administration studies in SAMP mice (Fig. 10-15), wherein UC non-involved areas expressed a 5.7-fold increase in IL-5 mRNA levels compared to non-inflamed controls, but were downregulated in involved areas of severe disease (Fig. 24), supporting the concept that in the setting of heightened inflammation, high levels of IL33 may incite a negative feedback mechanism to downregulate IL-5. Conversely,

mRNA expression for the eotaxins, CCL11, CCL24, and CCL26 were all found to be increased by 4.3-, 1.9-, and 2.6-fold, respectively, in severely involved colonic biopsies from patients with UC compared to non-inflamed controls (Fig. 24).

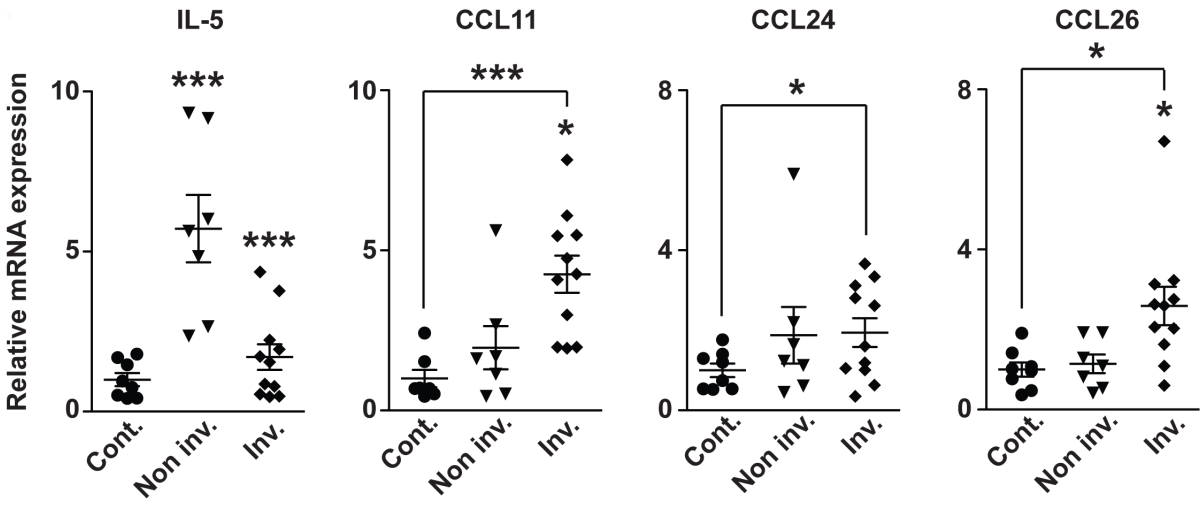

Figure 24. EOS-associated cytokines/chemokines are elevated in the colonic mucosa of UC patients. Relative mRNA expression of IL-5 and eotaxin-1 (CCL11), -2 (CCL24), and-3 (CCL26) in involved and non-involved colonic mucosal biopsies from UC patients compared to healthy Cont. (N≥7); p*<0.05, p***<0.001 vs. Cont.

As such, the overall results obtained from UC patients regarding co-localization of IL-33/EOS and EOS mediator expression confirm the observed trends found in the SAMP model of Th1/Th2-driven IBD. In both UC patients and SAMP mice, IL-33 and EOS co-localize, but do not overlap, within the inflamed gut mucosa and uniquely accumulate in areas adjacent to the muscularis propria. In addition, eotaxin expression is increased as disease becomes more severe.

Gut microflora is essential for the induction of IL-33, EOS infiltration, and Th2 immune responses during SAMP ileitis

Previous studies from our laboratory indicated that, despite being raised under germ-free (GF) conditions, SAMP mice develop chronic ileitis with a delayed

onset (later than 13 wks of age), lower penetrance (70%) and decreased severity (1/3 of mice had severe ileitis) compared to age- and gender-matched specific pathogen free (SPF)-reared SAMP (Bamias et al., 2007). Interestingly, although TCR-activated cells from the draining MLN of GF-SAMP displayed a robust, intact Th1 immune response, they were unable to mount a vigorous Th2 effector response in the absence of bacterial flora, as shown by a dramatic decrease in IL-5, IL-13, and in the ratio of GATA3:T-bet compared to SPF-SAMP; this effect could be reversed upon exposure of fecal antigens, which resulted in expansion of a distinct population of IL-4 secreting mucosal lymphocytes (Bamias et al., 2007). As such, since IL-33 is best known to promote Th2 immunity (Schmitz et al., 2005), we tested the hypothesis that the intestinal microflora may be responsible for driving the early induction of IL-33, leading to the infiltration of EOS and downstream Th2 immune responses that ultimately exacerbates ileitis in SAMP mice.

Our results showed that 20- to 26-wk-old GF-SAMP displayed a 5.2-fold decrease in IL-33 mRNA levels compared to age-matched SPF-SAMP with comparable inflammatory scores (6.25 ± 1.60 versus 8.33 ± 0.53, n.s.) (Fig. 25).

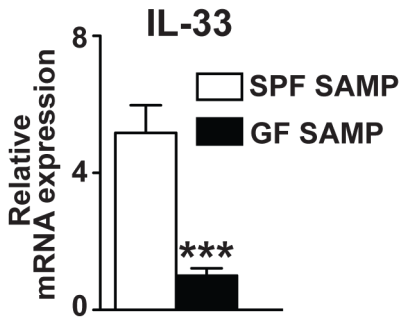

Figure 25. IL-33 mRNA level is reduced in the ileal mucosa of GF compared to age matched SPF SAMP mice. Relative ileal IL-33 mRNA expression in 20- to 26-wk-old SPF compared to age matched GF SAMP mice (N=7-8); p***<0.001 vs SPF SAMP mice.

In fact, in the absence of the gut commensal flora, a dramatic decrease was observed in the number of infiltrating EOS in GF- compared to SPF- raised SAMP (22.67 ± 4.65 versus 44.10 ± 7.15 EOS/HPF) (Fig. 26 & 27).

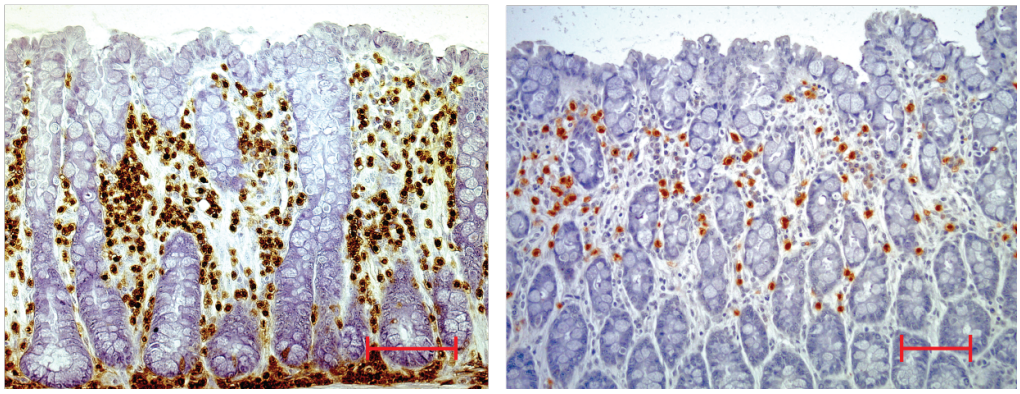

Figure 26. EOS infiltration, is reduced in the ileal mucosa of GF compared to age matched SPF SAMP mice. Representative photomicrographs of MBP-stained full thickness ileal tissues from SPF and GF SAMP mice show a remarkable higher amount of EOS infiltrating the mucosa of equally inflamed areas (respectively from left to right) all panels X20+1.25 original mag; scale bar=100μm.

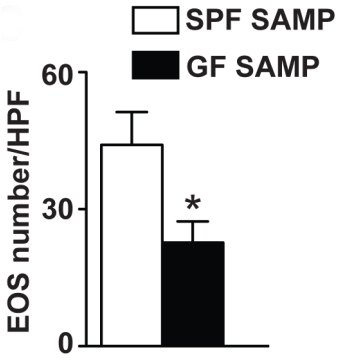

Figure 27. EOS infiltration, is reduced in the ileal mucosa of GF compared to age matched SPF SAMP mice. EOS count (N= 4-11); p*<0.05, vs SPF SAMP mice.

In addition, local, gut mucosal levels of Th2 cytokines were reduced in GF versus SPF-SAMP (Fig. 28) by 12.7-fold, 2-fold, and 3.5-fold for IL-4, IL-5, and IL-13, respectively, supporting our original observation that SAMP Th2 effector responses are dependent on the presence of the gut microflora (Bamias et al., 2007). Importantly, we also confirmed that IL-33 induction occurs independently of EOS infiltration as EOS depletion by combination anti-CCR3/anti-IL-5 treatment did not affect IL-33 mRNA levels compared to IgG-treated controls (Fig. 29), suggesting that upregulation of IL-33 likely occurs before EOS activation and homing to the gut and is dependent on the presence of the intestinal microflora.

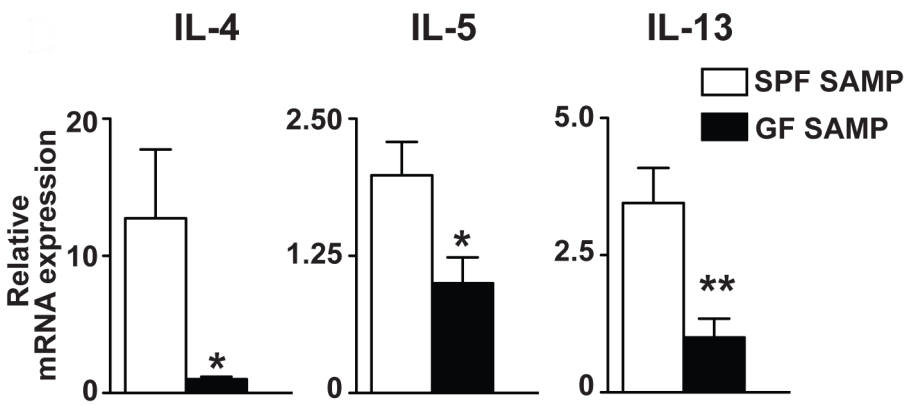

Figure 28. Th2 cytokines mRNA levels is reduced in the ileal mucosa of GF compared to age matched SPF SAMP mice. Relative ileal mRNA expression in 20- to 26-wk-old GF of Th2 cytokines (N=7-8); p*<0.05, p**<0.01, vs SPF SAMP mice.

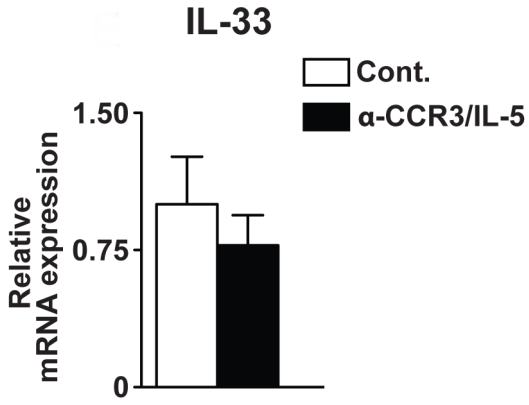

Figure 29. IL-33 is independent from EOS infiltration. Relative mRNA expression of IL-33 in 20-wk-old SAMP mice treated with a combination of anti-CCR3 and anti-IL5 neutralizing Abs compared to IgG-treated Cont. (N=7-8).

Together, these data indicate that the gut microbiota induces the production of IL-33 that can promote EOS infiltration into the intestinal mucosa. We also confirm that the commensal flora is responsible for the ability of SAMP mice to mount a vigorous Th2 immune response that leads to the exacerbation of chronic ileitis in this model of spontaneous IBD.

DISCUSSION

In this work we found an association between eosinophilia and IL-33 in an IBD model of spontaneous ileitis, the SAMP Yit/Fc mouse. The connection between eosinophilia and IBD is already well documented. Especially UC, being a Th2-driven disease (Seidelin et al., 2010), is the IBD that shows more eosinophil-associated features in humans. Our group, in the other hand, found increased levels of IL-33 in UC patients compared to nonaffected controls (Pastorelli et al., 2010). Moreover, we found that gut microbiota is essential for IL-33 production and downstream EOS infiltration in the intestinal mucosa as well as to generate Th2 response leading to chronic ileitis in SAMP mice.

EOS and IBD

EOS are leukocytes whose main function is supposed to be killing invading parasites. However, in absence of parasites, activated EOS can cause tissue damage and inflammation as it happens in a number of well known inflammatory diseases associated with eosinophilia such as asthma, allergic rhinitis, atopic skin disease, idiopathic hypereosinophilic syndrome and IBD (Makiyama et al., 1995; Silberstein, 1995). The recurrent and paroxysmal fashion of the inflammation found in UC associated with a characteristic feature of inflammatory diseases reminiscent of allergy, such as activated lymphocytes, macrophages, neutrophils and EOS mucosal infiltration, put UC close to inflammatory disease with an allergic component such as bronchial asthma (MacDermott et al., 1998; Makiyama et al., 1995). In normal, non inflamed tissue there is a baseline level of EOS resident in the lamina propria which trafficking and activation may be regulated by eotaxin and IL-5 (Matthews et al., 1998). During intestinal inflammation EOS number is highly

increased as shown by morphological and immunohistochemical studies that revealed activation of intestinal EOS in IBD (Bischoff et al., 1996; Carvalho et al., 2003; Dubucquoi et al., 1995) and increased intestinal release of ECP, EPO and EPX/EDN in UC (Carlson et al., 1999). Our data in fact confirm these evidences showing a massive eosinophilic infiltration in inflamed ilea of mid-age to older SAMP compared to AKR control mice as well as in human inflamed colon of UC patients compared to non UC control tissues. Eotaxins and IL-5 increase accordingly in human, in SAMP are more elevated at older age, that is at a more established stage of the disease, compared to AKR mice. This could just mean that EOS activate and migrate at the site of inflammation independently from the influence of IL-5 and eotaxins at an early stage of the disease, or, maybe, that IL-33 has a role in activating and recruiting EOS at the site of inflammation, and indeed our IL-33 timecourse data corroborate the latter hypothesis showing an increase that goes along with EOS infiltration and the overall inflammation. In a more established and chronic stage of the disease then, EOS should be able to produce molecules, like IL-5 and eotaxins, necessary to recruit and activate other EOS in a positive feedback mechanism that perpetuates the inflammation. IL-5 in particular increases the pool of eotaxin-responsive cells and primes EOS to respond to CCR3 ligands (Zimmermann et al., 2003), in the other hand Th2 cytokines such as IL-4, IL-10, IL-13 stimulate the expression and production of CCR3 specific ligands such as eotaxins (Manousou et al., 2010; Zimmermann et al., 2003), however Th2 molecules are generally the most involved cytokines in EOS trafficking (Horie et al., 1997; Manousou et al., 2010; Moser et al., 1992). This supports again our findings of an increased number of EOS that infiltrates the mucosa and submucosa of colon tissues of UC patients compared to non-UC ones. Our data moreover clearly show that when blocking either IL-5 or

CCR3 there is an evident decrease in EOS infiltration and in the overall inflammation in ilea of mice in which the disease is already established. Theoretically combination of anti-IL-5 and a CCR3 antagonist is supposed to be more effective in reducing eosinophilia than blocking IL-5 or CCR3 alone as this would inhibit both bone marrow EOS maturation, mainly induced by IL-5, and their tissue accumulation, predominantly a CCR3 effect (Hogan et al., 2008). As a matter of fact the inflammation and EOS infiltration as well as eotaxin-2 levels had a marked beneficial effect from the additional effect of the combined blockade treatment.

In this scenario blocking IL-5 or CCR3 may not necessarily result in a decrease of IL-5 itself, in a mouse with an established stage of disease, in fact, besides EOS, other cellular types are thought to contribute to IL-5 production, during immunologically mediated intestinal inflammation, such as mast cells and lymphocytes (Lorentz et al., 1999). So, probably, blocking IL-5 in an advanced stage of chronic inflammation is not enough to completely stop its production by all the different cell types involved, and actually there could be some kind of positive feedback mechanisms that induce a replacement of IL-5, and consequently of CCR3 too, in an attempt to continue promoting the expansion of EOS progenitor cells in the bone marrow, their activation and migration in the site of inflammation. To mention also that IL-5 indirectly contributes to tissue remodeling by increasing indirectly the levels of pro-fibrotic effector molecules, such as TGFβ and IL-13, secreted by EOS (Blyth et al., 2000; Cho et al., 2004; Flood-Page et al., 2003; Huaux et al., 2003; Trifilieff et al., 2001). That could explain the inconsistently high levels of IL-5 found in the mlns supernatants of SAMP treated with anti-IL-5 and anti-CCR3. On the other hand we found that the cytokines most affected by this blocking treatment were, maybe more predictably, chemokines such as MCP-1, MIP-1α and RANTES. Along with those

also IL-1β, IL-12p70, TNFα and IL-4 showed a decrease when treated with anti-IL-5 or anti-CCR3 confirming that different cytokines from different compartments (IL-1 family, Th1, Th2), which normally are involved in the inflammatory process, are affected by the block of two key cytokines of EOS priming, migration and activation such as IL-5 and CCR3.

IL-33 as a possible trigger for eosinophilia in UC

IL-33 role in targeting Th2 responses is quite well documented in 2005 Schmitz work (Schmitz et al., 2005) and recently Seidelin et al. highlighted its function also in Th2-polarized intestinal inflammation (Seidelin et al., 2011). Furthermore IL-33 induces eosinophilia and EOS activation in expressing IL-8 and superoxide production (Cherry et al., 2008; Pecaric-Petkovic et al., 2009; Suzukawa et al., 2008b). Our data support and put together these findings, we found indeed a positive correspondence between augmented EOS infiltration in the gut mucosa and IL-33 mRNA relative expression in mice treated with IL-33 along with an increase in Th2 cytokines in the mln. The only cytokine that appears not so significantly increased in the mln (even having the same trend as the others), is the IL-4. This can find an explanation in a relatively recent work that hypothesize the presence of two different pathways for Th2 responses, the IL-4-dependent and the IL-33-dependent pathways, based on data showing that IL-33 drives a population of murine and human $CD4^+$ T cells which produce mainly IL-5 and IL-13 but not IL-4 (Kurowska-Stolarska et al., 2008). Eotaxins mRNA relative expression in the ileum was elevated as well, in IL-33 administered mice, giving to the whole picture also the chemotactic component in the site of the inflammation. In fact the IL-33/ST2 signaling pathway enhances the expression of CCR3 which plays an important role in recruiting EOS from bone marrow to the peripheral blood and then to the sites of inflammation

(Humbles et al., 2002) through the interaction with the eotaxins exactly. Cherry et al. found that IL-33 appeared to have stronger effect than IL-5 in activating EOS, to produce and release proinflammatory mediators, and to contribute to EOS survival (Cherry et al., 2008). So we, maybe hazardously, could hypothesize, then, that the lower IL-5 mRNA expression we found in response to IL-33 administration, can be due to an agonist mechanism in activating and stimulating EOS proliferation of IL-33 towards IL-5, directly in the site of inflammation, that can lead to a negative feedback mechanism and consequent inhibition of IL-5 transcription. Our data, from the IL-33 blocking through the anti-ST2 treatments, further confirmed that the IL-33/ST2 axis has a determinant role in eosinophilia in IBD showing decrease in the overall inflammation and EOS infiltration, along with a slight decrease of IL-5 and eotaxins, in the ileal mucosa either in early and in established disease. The notion of IL-33 as a Th2 polarizing cytokine is strengthened also by the fact that ST2 is expressed on Th2 and not on Th1 lymphocytes (Schmitz et al., 2005) (Chan et al., 2001), and, consistently with this, our data on mln's Th2 cytokines showed a quite distinctive common trend in decrease, except for IL-13 that didn't show any evident change, when we blocked the ST2 receptor. That being so, our human data supported all the evidences we had from mice showing an interesting correspondence between IL-33 accumulation and EOS infiltration in the colon mucosa of UC patients greater than in healthy tissues. As expected, IL-5 and eotaxins mRNA expression showed the same trend confirming also in humans the ostensible link between eosinophilia and IBD (particularly UC), that is the IL-33.

The importance of gut microbiota

Lastly, to confirm the strong connection between IL-33, EOS and Th2 immune response, we tested the hypothesis that the gut microflora is responsible for driving the early induction of IL-33 that leads to EOS infiltration and downstream the Th2 immune response that exacerbates Ileitis in SAMP, based on our previous finding that in GF SAMP the onset of ileitis is delayed and less severe compared to age-matched SPF SAMP mice (Bamias et al., 2007) along with a lack of Th2 effector response. Our results confirmed the hypothesis showing that either IL-33 and Th2 cytokines expression and EOS infiltration were significantly lower in 20- to 26-wk-old GF compared to age-matched SPF SAMP mice. Also depleting EOS didn't affect in any way IL-33 induction and this final finding complete the picture in which IL-33 upregulation occurs before EOS activation and homing to the gut and depends on the intestinal microflora. So we can conclude that the gut microbiota promotes IL-33 production and so EOS infiltration as well as induces a strong Th2 response that leads to the exacerbation of chronic ileitis in this model of spontaneous IBD.

CONCLUSIONS

In this work we highlighted and strengthen the relationship between eosinophilia and IBD (particularly UC) in a murine model that partially resemble a Th2-type of IBD, the SAMP mouse, focusing on some of the most important molecules that drive EOS activation and migration, such as IL-5, eotaxins and Th2 cytokines. We found them directly related between each other and furthermore we found IL-33 as a possible key molecule in driving EOS infiltration and subsequent inflammation in the gut, interacting with IL-5 and eotaxins through a Th2 driven mechanism and dependent on the intestinal microbiota. Taken together these findings demonstrate a pathogenic role of IL-33-mediated EOS infiltration and function in chronic intestinal inflammation and that blockade of IL-33 could represent a novel therapeutic modality to treat patients with IBD, especially UC. Also they highlight the pivotal role of the gut microbiota in inducing IL-33 production, the downstream EOS infiltration and the potent Th2 response that leads to chronic ileitis in SAMP.

AKNOWLEDGEMENTS

Co-Mentor

Maurizio Vecchi

Departments of [1]Pathology and [2]Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106

Xiao-Ming Wang[1]

Benedetta Mattioli[1]

Luca Pastorelli[1,3]

Rekha R. Garg[1]

Saleem Chowdhry[1]

Daniele Corridoni[2]

Wei Xin[1]

Fabio Cominelli[2]

Theresa T. Pizarro[1,2]

IRCCS Policlinico San Donato, Gastroenterology and Gastrointestinal Endoscopy Unit, San Donato Milanese, Italy; University of Milan School of Medicine, Milan 20097, Italy

Luca Pastorelli

Maurizio Vecchi

Department of Inflammation Research, Amgen, Seattle, WA 98119

Dirk E. Smith

1st Department of Internal Medicine, Propaedeutic, Ethnikon & Kapodistriakon University of Athens; Laikon Hospital, Athens 11527, Greece

Giorgos Bamias

Division of Pulmonary Medicine, Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Mayo Clinic Arizona, Scottsdale, AZ 85259

James J. Lee

REFERENCES

Ahrens, R., A. Waddell, L. Seidu, C. Blanchard, R. Carey, E. Forbes, M. Lampinen, T. Wilson, E. Cohen, K. Stringer, E. Ballard, A. Munitz, H. Xu, N. Lee, J.J. Lee, M.E. Rothenberg, L. Denson, and S.P. Hogan. 2008. Intestinal macrophage/epithelial cell-derived CCL11/eotaxin-1 mediates eosinophil recruitment and function in pediatric ulcerative colitis. *J Immunol* 181:7390-7399.

Arseneau, K.O., H. Tamagawa, T.T. Pizarro, and F. Cominelli. 2007. Innate and adaptive immune responses related to IBD pathogenesis. *Curr Gastroenterol Rep* 9:508-512.

Asquith, M., and F. Powrie. 2010. An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer. *J Exp Med* 207:1573-1577.

Baekkevold, E.S., M. Roussigne, T. Yamanaka, F.E. Johansen, F.L. Jahnsen, F. Amalric, P. Brandtzaeg, M. Erard, G. Haraldsen, and J.P. Girard. 2003. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol* 163:69-79.

Bamias, G., M. Marini, C.A. Moskaluk, M. Odashima, W.G. Ross, J. Rivera-Nieves, and F. Cominelli. 2002. Down-regulation of intestinal lymphocyte activation and Th1 cytokine production by antibiotic therapy in a murine model of Crohn's disease. *J Immunol* 169:5308-5314.

Bamias, G., C. Martin, M. Mishina, W.G. Ross, J. Rivera-Nieves, M. Marini, and F. Cominelli. 2005. Proinflammatory effects of TH2 cytokines in a murine model of chronic small intestinal inflammation. *Gastroenterology* 128:654-666.

Bamias, G., A. Okazawa, J. Rivera-Nieves, K.O. Arseneau, S.A. De La Rue, T.T. Pizarro, and F. Cominelli. 2007. Commensal bacteria exacerbate intestinal inflammation but are not essential for the development of murine ileitis. *J Immunol* 178:1809-1818.

Beltran, C.J., L.E. Nunez, D. Diaz-Jimenez, N. Farfan, E. Candia, C. Heine, F. Lopez, M.J. Gonzalez, R. Quera, and M.A. Hermoso. 2010. Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 16:1097-1107.

Bischoff, S.C., J. Wedemeyer, A. Herrmann, P.N. Meier, C. Trautwein, Y. Cetin, H. Maschek, M. Stolte, M. Gebel, and M.P. Manns. 1996. Quantitative assessment of intestinal eosinophils and mast cells in inflammatory bowel disease. *Histopathology* 28:1-13.

Blyth, D.I., T.F. Wharton, M.S. Pedrick, T.J. Savage, and S. Sanjar. 2000. Airway subepithelial fibrosis in a murine model of atopic asthma: suppression by dexamethasone or anti-interleukin-5 antibody. *Am J Respir Cell Mol Biol* 23:241-246.

Carlson, M., Y. Raab, C. Peterson, R. Hallgren, and P. Venge. 1999. Increased intraluminal release of eosinophil granule proteins EPO, ECP, EPX, and cytokines in ulcerative colitis and proctitis in segmental perfusion. *Am J Gastroenterol* 94:1876-1883.

Carriere, V., L. Roussel, N. Ortega, D.A. Lacorre, L. Americh, L. Aguilar, G. Bouche, and J.P. Girard. 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A* 104:282-287.

Carvalho, A.T., C.C. Elia, H.S. de Souza, P.R. Elias, E.L. Pontes, H.P. Lukashok, F.C. de Freitas, and J.R. Lapa e Silva. 2003. Immunohistochemical study of intestinal eosinophils in inflammatory bowel disease. *J Clin Gastroenterol* 36:120-125.

Chan, W.L., N. Pejnovic, C.A. Lee, and N.A. Al-Ali. 2001. Human IL-18 receptor and ST2L are stable and selective markers for the respective type 1 and type 2 circulating lymphocytes. *J Immunol* 167:1238-1244.

Cherry, W.B., J. Yoon, K.R. Bartemes, K. Iijima, and H. Kita. 2008. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *J Allergy Clin Immunol* 121:1484-1490.

Cho, J.Y., M. Miller, K.J. Baek, J.W. Han, J. Nayar, S.Y. Lee, K. McElwain, S. McElwain, S. Friedman, and D.H. Broide. 2004. Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest* 113:551-560.

Chow, J.Y., C.K. Wong, P.F. Cheung, and C.W. Lam. 2010. Intracellular signaling mechanisms regulating the activation of human eosinophils by the novel Th2 cytokine IL-33: implications for allergic inflammation. *Cell Mol Immunol* 7:26-34.

Duan, L., J. Chen, H. Zhang, H. Yang, P. Zhu, A. Xiong, Q. Xia, F. Zheng, Z. Tan, F. Gong, and M. Fang. 2012. Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3(+) regulatory T-cell responses in mice. *Mol Med* 18:753-761.

Dubucquoi, S., A. Janin, O. Klein, P. Desreumaux, P. Quandalle, A. Cortot, M. Capron, and J.F. Colombel. 1995. Activated eosinophils and interleukin 5 expression in early recurrence of Crohn's disease. *Gut* 37:242-246.

Flood-Page, P., A. Menzies-Gow, S. Phipps, S. Ying, A. Wangoo, M.S. Ludwig, N. Barnes, D. Robinson, and A.B. Kay. 2003. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 112:1029-1036.

Forbes, E., T. Murase, M. Yang, K.I. Matthaei, J.J. Lee, N.A. Lee, P.S. Foster, and S.P. Hogan. 2004. Immunopathogenesis of experimental ulcerative colitis is mediated by eosinophil peroxidase. *J Immunol* 172:5664-5675.

Furuta, G.T., E.E. Nieuwenhuis, J. Karhausen, G. Gleich, R.S. Blumberg, J.J. Lee, and S.J. Ackerman. 2005. Eosinophils alter colonic epithelial barrier function: role for major basic protein. *Am J Physiol Gastrointest Liver Physiol* 289:G890-897.

Grimaldi, J.C., N.X. Yu, G. Grunig, B.W. Seymour, F. Cottrez, D.S. Robinson, N. Hosken, W.G. Ferlin, X. Wu, H. Soto, A. O'Garra, M.C. Howard, and R.L. Coffman. 1999. Depletion of eosinophils in mice through the use of antibodies specific for C-C chemokine receptor 3 (CCR3). *J Leukoc Biol* 65:846-853.

Grobeta, P., K. Doser, W. Falk, F. Obermeier, and C. Hofmann. 2012. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflamm Bowel Dis* 18:1900-1909.

Halim, T.Y., R.H. Krauss, A.C. Sun, and F. Takei. 2012. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 36:451-463.

Hogan, S.P., H.F. Rosenberg, R. Moqbel, S. Phipps, P.S. Foster, P. Lacy, A.B. Kay, and M.E. Rothenberg. 2008. Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy* 38:709-750.

Hogan, S.P., A. Waddell, and P.C. Fulkerson. 2013. Eosinophils in infection and intestinal immunity. *Curr Opin Gastroenterol* 29:7-14.

Horie, S., Y. Okubo, M. Hossain, E. Sato, H. Nomura, S. Koyama, J. Suzuki, M. Isobe, and M. Sekiguchi. 1997. Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. *Intern Med* 36:179-185.

Huaux, F., T. Liu, B. McGarry, M. Ullenbruch, Z. Xing, and S.H. Phan. 2003. Eosinophils and T lymphocytes possess distinct roles in bleomycin-induced lung injury and fibrosis. *J Immunol* 171:5470-5481.

Humbles, A.A., B. Lu, D.S. Friend, S. Okinaga, J. Lora, A. Al-Garawi, T.R. Martin, N.P. Gerard, and C. Gerard. 2002. The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc Natl Acad Sci U S A* 99:1479-1484.

Hung, L.Y., I.P. Lewkowich, L.A. Dawson, J. Downey, Y. Yang, D.E. Smith, and D.R. Herbert. 2013. IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity against hookworms. *Proc Natl Acad Sci U S A* 110:282-287.

Imaeda, H., A. Andoh, T. Aomatsu, K. Uchiyama, S. Bamba, T. Tsujikawa, Y. Naito, and Y. Fujiyama. 2011. Interleukin-33 suppresses Notch ligand expression and prevents goblet cell depletion in dextran sulfate sodium-induced colitis. *Int J Mol Med* 28:573-578.

Kobori, A., Y. Yagi, H. Imaeda, H. Ban, S. Bamba, T. Tsujikawa, Y. Saito, Y. Fujiyama, and A. Andoh. 2010. Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. *J Gastroenterol* 45:999-1007.

Komai-Koma, M., F. Brombacher, P.N. Pushparaj, B. Arendse, C. McSharry, J. Alexander, R. Chaudhuri, N.C. Thomson, A.N. McKenzie, I. McInnes, F.Y. Liew, and D. Xu. 2012. Interleukin-33 amplifies IgE synthesis and triggers mast cell degranulation via interleukin-4 in naive mice. *Allergy* 67:1118-1126.

Kosiewicz, M.M., C.C. Nast, A. Krishnan, J. Rivera-Nieves, C.A. Moskaluk, S. Matsumoto, K. Kozaiwa, and F. Cominelli. 2001. Th1-type responses mediate spontaneous ileitis in a novel murine model of Crohn's disease. *J Clin Invest* 107:695-702.

Kurowska-Stolarska, M., P. Kewin, G. Murphy, R.C. Russo, B. Stolarski, C.C. Garcia, M. Komai-Koma, N. Pitman, Y. Li, W. Niedbala, A.N. McKenzie, M.M. Teixeira, F.Y. Liew, and D. Xu. 2008. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol* 181:4780-4790.

Kurowska-Stolarska, M., B. Stolarski, P. Kewin, G. Murphy, C.J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I.B. McInnes, D. Xu, and F.Y. Liew. 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol* 183:6469-6477.

Lampinen, M., M. Backman, O. Winqvist, F. Rorsman, A. Ronnblom, P. Sangfelt, and M. Carlson. 2008. Different regulation of eosinophil activity in Crohn's disease compared with ulcerative colitis. *J Leukoc Biol* 84:1392-1399.

Lampinen, M., M. Carlson, P. Sangfelt, Y. Taha, M. Thorn, L. Loof, Y. Raab, and P. Venge. 2001. IL-5 and TNF-alpha participate in recruitment of eosinophils to intestinal mucosa in ulcerative colitis. *Dig Dis Sci* 46:2004-2009.

Lampinen, M., A. Ronnblom, K. Amin, G. Kristjansson, F. Rorsman, P. Sangfelt, B. Safsten, M. Wagner, A. Wanders, O. Winqvist, and M. Carlson. 2005. Eosinophil granulocytes are activated during the remission phase of ulcerative colitis. *Gut* 54:1714-1720.

Liu, X., M. Li, Y. Wu, Y. Zhou, L. Zeng, and T. Huang. 2009. Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma. *Biochem Biophys Res Commun* 386:181-185.

Lorentz, A., S. Schwengberg, C. Mierke, M.P. Manns, and S.C. Bischoff. 1999. Human intestinal mast cells produce IL-5 in vitro upon IgE receptor cross-linking and in vivo in the course of intestinal inflammatory disease. *Eur J Immunol* 29:1496-1503.

MacDermott, R.P., I.R. Sanderson, and H.C. Reinecker. 1998. The central role of chemokines (chemotactic cytokines) in the immunopathogenesis of ulcerative colitis and Crohn's disease. *Inflamm Bowel Dis* 4:54-67.

Makiyama, K., S. Kanzaki, K. Yamasaki, W. Zea-Iriarte, and Y. Tsuji. 1995. Activation of eosinophils in the pathophysiology of ulcerative colitis. *J Gastroenterol* 30 Suppl 8:64-69.

Manousou, P., G. Kolios, V. Valatas, I. Drygiannakis, L. Bourikas, K. Pyrovolaki, I. Koutroubakis, H.A. Papadaki, and E. Kouroumalis. 2010. Increased expression of chemokine receptor CCR3 and its ligands in ulcerative colitis: the role of colonic epithelial cells in in vitro studies. *Clin Exp Immunol* 162:337-347.

Mansson, A., and L.O. Cardell. 2009. Role of atopic status in Toll-like receptor (TLR)7- and TLR9-mediated activation of human eosinophils. *J Leukoc Biol* 85:719-727.

Masterson, J.C., E.N. McNamee, P. Jedlicka, S. Fillon, J. Ruybal, L. Hosford, J. Rivera-Nieves, J.J. Lee, and G.T. Furuta. 2011. CCR3 Blockade Attenuates Eosinophilic Ileitis and Associated Remodeling. *Am J Pathol* 179:2302-2314.

Matsuba-Kitamura, S., T. Yoshimoto, K. Yasuda, S. Futatsugi-Yumikura, Y. Taki, T. Muto, T. Ikeda, O. Mimura, and K. Nakanishi. 2010. Contribution of IL-33 to induction and augmentation of experimental allergic conjunctivitis. *Int Immunol* 22:479-489.

Matsumoto, S., Y. Okabe, H. Setoyama, K. Takayama, J. Ohtsuka, H. Funahashi, A. Imaoka, Y. Okada, and Y. Umesaki. 1998. Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. *Gut* 43:71-78.

Matthews, A.N., D.S. Friend, N. Zimmermann, M.N. Sarafi, A.D. Luster, E. Pearlman, S.E. Wert, and M.E. Rothenberg. 1998. Eotaxin is required for the baseline level of tissue eosinophils. *Proc Natl Acad Sci U S A* 95:6273-6278.

Mishra, A., S.P. Hogan, J.J. Lee, P.S. Foster, and M.E. Rothenberg. 1999. Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. *J Clin Invest* 103:1719-1727.

Moser, R., J. Fehr, and P.L. Bruijnzeel. 1992. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. *J Immunol* 149:1432-1438.

Moussion, C., N. Ortega, and J.P. Girard. 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* 3:e3331.

Nagase, H., S. Okugawa, Y. Ota, M. Yamaguchi, H. Tomizawa, K. Matsushima, K. Ohta, K. Yamamoto, and K. Hirai. 2003. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. *J Immunol* 171:3977-3982.

Neill, D.R., S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, H.E. Jolin, and A.N. McKenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.

Nell, S., S. Suerbaum, and C. Josenhans. 2010. The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. *Nat Rev Microbiol* 8:564-577.

Oboki, K., T. Ohno, N. Kajiwara, K. Arae, H. Morita, A. Ishii, A. Nambu, T. Abe, H. Kiyonari, K. Matsumoto, K. Sudo, K. Okumura, H. Saito, and S. Nakae. 2010. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A* 107:18581-18586.

Olson, T.S., B.K. Reuter, K.G. Scott, M.A. Morris, X.M. Wang, L.N. Hancock, T.L. Burcin, S.M. Cohn, P.B. Ernst, F. Cominelli, J.B. Meddings, K. Ley, and T.T. Pizarro. 2006. The primary defect in experimental ileitis originates from a nonhematopoietic source. *J Exp Med* 203:541-552.

Palmer, G., D. Talabot-Ayer, C. Lamacchia, D. Toy, C.A. Seemayer, S. Viatte, A. Finckh, D.E. Smith, and C. Gabay. 2009. Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis. *Arthritis Rheum* 60:738-749.

Pastorelli, L., R.R. Garg, S.B. Hoang, L. Spina, B. Mattioli, M. Scarpa, C. Fiocchi, M. Vecchi, and T.T. Pizarro. 2010. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proc Natl Acad Sci U S A* 107:8017-8022.

Pecaric-Petkovic, T., S.A. Didichenko, S. Kaempfer, N. Spiegl, and C.A. Dahinden. 2009. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 113:1526-1534.

Pizarro, T.T., L. Pastorelli, G. Bamias, R.R. Garg, B.K. Reuter, J.R. Mercado, M. Chieppa, K.O. Arseneau, K. Ley, and F. Cominelli. 2011. SAMP1/YitFc mouse strain: a spontaneous model of Crohn's disease-like ileitis. *Inflamm Bowel Dis* 17:2566-2584.

Price, A.E., H.E. Liang, B.M. Sullivan, R.L. Reinhardt, C.J. Eisley, D.J. Erle, and R.M. Locksley. 2010. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* 107:11489-11494.

Protheroe, C., S.A. Woodruff, G. de Petris, V. Mukkada, S.I. Ochkur, S. Janarthanan, J.C. Lewis, S. Pasha, T. Lunsford, L. Harris, V.K. Sharma, M.P. McGarry, N.A. Lee, G.T. Furuta, and J.J. Lee. 2009. A novel histologic scoring system to evaluate mucosal biopsies from patients with eosinophilic esophagitis. *Clin Gastroenterol Hepatol* 7:749-755 e711.

Rani, R., A.G. Smulian, D.R. Greaves, S.P. Hogan, and D.R. Herbert. 2011. TGF-beta limits IL-33 production and promotes the resolution of colitis through regulation of macrophage function. *Eur J Immunol* 41:2000-2009.

Rivera-Nieves, J., G. Bamias, A. Vidrich, M. Marini, T.T. Pizarro, M.J. McDuffie, C.A. Moskaluk, S.M. Cohn, and F. Cominelli. 2003. Emergence of perianal fistulizing disease in the SAMP1/YitFc mouse, a spontaneous model of chronic ileitis. *Gastroenterology* 124:972-982.

Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D.M. Gorman, J.F. Bazan, and R.A. Kastelein. 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23:479-490.

Sedhom, M.A., M. Pichery, J.R. Murdoch, B. Foligne, N. Ortega, S. Normand, K. Mertz, D. Sanmugalingam, L. Brault, T. Grandjean, E. Lefrancais, P.G. Fallon, V. Quesniaux, L. Peyrin-Biroulet, G. Cathomas, T. Junt, M. Chamaillard, J.P. Girard, and B. Ryffel. 2012. Neutralisation of the interleukin-33/ST2 pathway

ameliorates experimental colitis through enhancement of mucosal healing in mice. *Gut*

Seidelin, J.B., J.T. Bjerrum, M. Coskun, B. Widjaya, B. Vainer, and O.H. Nielsen. 2010. IL-33 is upregulated in colonocytes of ulcerative colitis. *Immunol Lett* 128:80-85.

Seidelin, J.B., G. Rogler, and O.H. Nielsen. 2011. A role for interleukin-33 in T(H)2-polarized intestinal inflammation? *Mucosal Immunol* 4:496-502.

Shichijo, K., K. Makiyama, C.Y. Wen, M. Matsuu, T. Nakayama, M. Nakashima, M. Ihara, and I. Sekine. 2005. Antibody to eosinophil cationic protein suppresses dextran sulfate sodium-induced colitis in rats. *World J Gastroenterol* 11:4505-4510.

Silberstein, D.S. 1995. Eosinophil function in health and disease. *Crit Rev Oncol Hematol* 19:47-77.

Sponheim, J., J. Pollheimer, T. Olsen, J. Balogh, C. Hammarstrom, T. Loos, M. Kasprzycka, D.R. Sorensen, H.R. Nilsen, A.M. Kuchler, M.H. Vatn, and G. Haraldsen. 2010. Inflammatory bowel disease-associated interleukin-33 is preferentially expressed in ulceration-associated myofibroblasts. *Am J Pathol* 177:2804-2815.

Stolarski, B., M. Kurowska-Stolarska, P. Kewin, D. Xu, and F.Y. Liew. 2010. IL-33 exacerbates eosinophil-mediated airway inflammation. *J Immunol* 185:3472-3480.

Suzukawa, M., M. Iikura, R. Koketsu, H. Nagase, C. Tamura, A. Komiya, S. Nakae, K. Matsushima, K. Ohta, K. Yamamoto, and M. Yamaguchi. 2008a. An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J Immunol* 181:5981-5989.

Suzukawa, M., R. Koketsu, M. Iikura, S. Nakae, K. Matsumoto, H. Nagase, H. Saito, K. Matsushima, K. Ohta, K. Yamamoto, and M. Yamaguchi. 2008b. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Lab Invest* 88:1245-1253.

Takedatsu, H., K. Mitsuyama, S. Matsumoto, K. Handa, A. Suzuki, H. Funabashi, Y. Okabe, T. Hara, A. Toyonaga, and M. Sata. 2004. Interleukin-5 participates in the pathogenesis of ileitis in SAMP1/Yit mice. *Eur J Immunol* 34:1561-1569.

Trifilieff, A., Y. Fujitani, A.J. Coyle, M. Kopf, and C. Bertrand. 2001. IL-5 deficiency abolishes aspects of airway remodelling in a murine model of lung inflammation. *Clin Exp Allergy* 31:934-942.

Vieira, A.T., C.T. Fagundes, A.L. Alessandri, M.G. Castor, R. Guabiraba, V.O. Borges, K.D. Silveira, E.L. Vieira, J.L. Goncalves, T.A. Silva, M. Deruaz, A.E. Proudfoot, L.P. Sousa, and M.M. Teixeira. 2009. Treatment with a novel chemokine-binding protein or eosinophil lineage-ablation protects mice from experimental colitis. *Am J Pathol* 175:2382-2391.

Wallon, C., M. Persborn, M. Jonsson, A. Wang, V. Phan, M. Lampinen, M. Vicario, J. Santos, P.M. Sherman, M. Carlson, A.C. Ericson, D.M. McKay, and J.D. Soderholm. 2011. Eosinophils express muscarinic receptors and corticotropin-releasing factor to disrupt the mucosal barrier in ulcerative colitis. *Gastroenterology* 140:1597-1607.

Willetts, L., K. Parker, L.J. Wesselius, C.A. Protheroe, E. Jaben, P. Graziano, R. Moqbel, K.O. Leslie, N.A. Lee, and J.J. Lee. 2011. Immunodetection of occult eosinophils in lung tissue biopsies may help predict survival in acute lung injury. *Respir Res* 12:116.

Woerly, G., N. Roger, S. Loiseau, D. Dombrowicz, A. Capron, and M. Capron. 1999. Expression of CD28 and CD86 by human eosinophils and role in the secretion of

type 1 cytokines (interleukin 2 and interferon gamma): inhibition by immunoglobulin a complexes. *J Exp Med* 190:487-495.

Wood, I.S., B. Wang, and P. Trayhurn. 2009. IL-33, a recently identified interleukin-1 gene family member, is expressed in human adipocytes. *Biochem Biophys Res Commun* 384:105-109.

Woodruff, S.A., J.C. Masterson, S. Fillon, Z.D. Robinson, and G.T. Furuta. 2011. Role of eosinophils in inflammatory bowel and gastrointestinal diseases. *J Pediatr Gastroenterol Nutr* 52:650-661.

Xu, D., H.R. Jiang, P. Kewin, Y. Li, R. Mu, A.R. Fraser, N. Pitman, M. Kurowska-Stolarska, A.N. McKenzie, I.B. McInnes, and F.Y. Liew. 2008. IL-33 exacerbates antigen-induced arthritis by activating mast cells. *Proc Natl Acad Sci U S A* 105:10913-10918.

Zimmermann, N., G.K. Hershey, P.S. Foster, and M.E. Rothenberg. 2003. Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J Allergy Clin Immunol* 111:227-242; quiz 243.