Title: Microbial regulation of enteric eosinophils and its impact on tissue remodeling and Th2 immunity

Authors: Rodrigo Jiménez-Saiz[1§], Varun C. Anipindi[1], Heather Galipeau[2], Yosef Ellenbogen[1], Roopali Chaudhary[1], Joshua F. Koenig[1], Melissa E. Gordon[1], Tina D. Walker[1], Talveer S. Mandur[1], Soumeya Abed[1], Alison Humbles[3], Derek K. Chu[4], Kjetil Ask[1], Elena F. Verdú[2], Manel Jordana[1].

Affiliations:

[1]McMaster Immunology Research Centre (MIRC), Department of Pathology & Molecular Medicine, and [4]Department of Medicine, McMaster University, Hamilton, ON, L8N 3Z5, Canada.

[2]Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, ON, L8N 3Z5, Canada.

[3]Department of Respiratory, Inflammation and Autoimmunity, MedImmune, Gaithersburg, Maryland, USA.

Correspondence to: Manel Jordana, MD, PhD. Dept. of Pathology & Molecular Medicine, McMaster Immunology Research Centre, McMaster University, MDCL 4013, 1280 Main St W, Hamilton, ON, L8S 4K1, Canada. Email address: jordanam@mcmaster.ca

§Current address: Vaccines & Dendritic Cells Lab, Department of Biochemistry & Molecular Biology, Chemistry School, Complutense University, 28040, Madrid, Spain

ABSTRACT

Eosinophils have emerged as multifaceted cells that contribute to tissue homeostasis. However, the factors that control their frequency and function at mucosal sites remain unclear. Here, we investigated the role of the microbiota in regulating enteric eosinophils. We found that small intestinal (SI) eosinophilia was significantly greater in germ-free (GF) mice compared to specific pathogen free (SPF) controls. This phenomenon was associated with enteric overexpression of signals that mediate attraction, retention and survival of eosinophils, and was reversed by colonization. Additionally, we generated a novel strain of eosinophil-deficient GF mice. These mice displayed intestinal fibrosis and were less prone to allergic sensitization as compared to GF controls. Overall, our study demonstrates that commensal microbes regulate intestinal eosinophil frequency and function, which impacts tissue repair and allergic sensitization to food antigens. These data support a critical interplay between the commensal microbiota and intestinal eosinophils in shaping homeostatic, innate and adaptive immune processes in health and disease.

INTRODUCTION

Eosinophils have traditionally been described as effector inflammatory cells that are protective against parasitic infections but detrimental in allergic disease [1,2]. New evidence has considerably broadened this paradigm as eosinophils have now been shown to play complex roles in mucosal immunity and tissue remodelling [3-8]. For example, intestinal eosinophils and eosinophil peroxidase (EPO) are critical for the initiation of Th2 responses (IgE) to food allergens in the gastrointestinal (GI) tract [6,9]. Conversely, intestinal eosinophils secrete large quantities of IL-1 receptor antagonist, thus reducing inflammation in inflammatory bowel disease (IBD) [10,11]. Eosinophils have also been proposed to play a role in homeostatic tissue remodeling as their presence is increased at tissues with a high turnover such as the intestine and the uterus [4,12]. Eosinophils are equipped with damage-sensing receptors which stimulate them to produce factors involved in tissue repair (*eg.* TGF-α and -β, and fibroblast-, platelet- and vascular-endothelial growth factors, *etc.*) [13]. Hence, deciphering the factors that regulate eosinophils in the GI mucosa is relevant to understand the role of these cells in health and disease.

There is growing evidence on the role of the microbiota in regulating immune responses and maintaining intestinal homeostasis [14,15]. Studies in germ-free (GF) mice have demonstrated that the microbiota is crucial for the maturation of the adaptive immune system in the small intestine (SI) [16]. For example, GF mice have fewer CD4 T cells, intraepithelial lymphocytes, and lower IgA-producing plasma cells in the lamina propria (LP) of the SI as compared to specific pathogen free (SPF) mice [14,15,17,18]. Yet, knowledge on the effect of the microbiota on innate cells that are indigenous to the intestinal tract, such is eosinophils, is scarce [11,19].

Here we examined the impact of the microbiota on eosinophil frequency and function in the SI. We found that eosinophil frequency was enriched along the SI of GF mice relative to SPF controls. This was associated with the overexpression of signals involved in the attraction, retention and survival of eosinophils. This relative hypereosinophilia was also observed in other mucosal sites, but not in sterile tissues, and was corrected by repletion of microbiota through co-habitation with altered Schaedler flora (ASF)- or SPF-mice. In addition, we generated a novel strain of eosinophil-deficient (ΔdblGATA1) mice on a GF background. In this system, the absence of eosinophils was associated with increased collagen accumulation in the submucosa as well as reduced allergic sensitization. This study illustrates a novel role for the microbiota in regulating mucosal eosinophils and tissue homoeostasis.

MATERIAL AND METHODS

Mice and colonization procedures. Age-, vendor-, and strain-matched controls were used in all the experiments. C57BL/6 and BALB/c mice were obtained from Charles River. ΔdblGATA1 (GATA) mice were bred in house. A novel strain of GF GATA mice was generated by two-cell embryo transfer, as previously described [20]. Mice were bred and maintained in flexible film isolators in McMaster's Axenic Gnotobiotic Unit. GF status was monitored weekly by DNA immunofluorescence (SYTOX Green), as well as anaerobic and aerobic culture of cecal stool

samples. Mice had unlimited access to autoclaved food and water. ASF-colonized mice were originally generated by co-housing female colonizers harbouring ASF, with GF mice. ASF-colonized mice were then bred for 3 generations in individually ventilated racks within the Axenic Gnotobiotic Unit [21]. Pathogen contamination and microbiota diversification were evaluated in mouse fecal contents every 2 weeks in sentinels by PCR for *Helicobacter bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *Helicobacter spp.*, *H. typhlonius*, and *Pneumocystis murina*. Mouse serum was also tested for murine viral pathogens by multiplexed fluorometric immunoassay/enzyme-linked immunosorbent assay (ELISA)/indirect fluorescent antibody tests [21]. SPF colonization was performed by co-habitation of GF mice with SPF mice for a minimum of 1 month. In some experiments, mice were fed an elemental diet based on amino acids (TD1084 and TD 130916; Harlan Laboratories Inc.) for 3 generations prior to use. All procedures were approved by the McMaster University Animal Research Ethics Board.

Intestinal cell isolation. As previously described [22,23], after flushing intestinal contents with cold PBS, fat was removed, and intestines were opened longitudinally and cut into approximately 3-5 mm pieces. Mucus was eliminated by washing with phosphate-buffered saline (PBS) containing 10 mM HEPES and 4 µM dithiothreitol (DTT) (Sigma) for 15 min at 37ºC while on a shaker. Epithelial cells were removed by 3 rounds of 10 min-washes at 37ºC, under shaking, in PBS containing 10% fetal bovine serum (FBS), 10 mM HEPES and 5 mM ethylenediaminetetraacetic acid (EDTA). The tissues were then digested in 0.125 U/mL Collagenase A (Roche) with 130 U/mL DNase I (Roche) in 10% FBS containing RPMI for 50–60 min in a shaker at 37ºC. Lastly, the digested tissues were pressed through a 40 µm nylon strainer (Falcon) and immune cells were purified via 40/70% Percoll (GE Healthcare) gradient and centrifugation.

Tissue processing and cell isolation. Bone marrow [6], spleens [24], uterus [25], vaginal tract [25], and blood [6] were collected and processed as previously described.

EPO assay. Intestinal tissue was made up to 100 mg/mL, w/v suspension, in PBS containing complete protease inhibitors (Roche) and rotor-stator homogenized (Polytron; Kinematica, Lucerne, Switzerland). The intestinal homogenate was centrifuged (1952 g x 10 min, 4ºC) and red blood cells were lysed in the pellet via ACK lysis buffer. The pellet was resuspended in 2 mL of 0.2 % NaCl for 30 sec followed by 2 mL of 1.6% NaCl before centrifugation and resuspension at 100 mg/mL in Hank's balanced salt solution (HBSS) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma: H5882). Pellets were homogenized and freeze/thawed 3 times using liquid nitrogen. Finally, samples were centrifugated, the supernatants were transferred to clean tubes and EPO activity was measured as previously described [26].

Quantitative real-time PCR for gene validation. Intestinal tissue was homogenized, and RNA was extracted using the RNeasy Kit (Qiagen). RNA was quantified and normalized, and RNA integrity was assessed by Agilent Bioanalyzer (Agilent Scientific). cDNA was generated using the SuperScript III Reverse Transcriptase kit (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Relative transcript expression assay was conducted, as described

previously [27], using the Fluidigm Biomark system (Fluidigm, San Francisco, CA). BestKeeper (version 1) [28] was used to identify the stably expressed housekeeping gene to be used as an internal reference. Among the four housekeeping genes [β-actin (Actb), β-2-microglobulin (β2m), Gapdh and hypoxanthine phosphoribosyltransferase1 (Hprt1)], Hprt1 emerged as the most stably expressed and, thus, was selected for normalizing genes of interest. To visualize broad differences in the chemokine and cytokine signals, raw ΔΔCT values were converted to a log scale. The fold change in gene expression between GF and SPF mice was represented on a heatmap with R software using the heatmap package. Data analysis of cycle threshold values was conducted using the Relative Expression Software Tool-384 (REST-384) version 1.

Flow cytometry. Antibodies were obtained from eBioscience, BD Biosciences, or BioLegend. In all assays, cells were incubated with anti-FcγRII/III before incubation with fluorochrome-conjugated antibodies. Dead cells were excluded by propidium iodide uptake (Sigma) or fixable viability dye eFluor780 (eBioscience) and gated on singlets. On average, a minimum of 300,000 alive and singlet cells were analyzed. Fluorescence minus one (FMO) and isotype controls were used for gating. Data were acquired on an LSR II or Fortessa (BD) and analyzed using FlowJo (Treestar). Flow-sorting experiments were performed on a FACS ARIA III (BD).

Food allergy model. Peanut butter (3.75 mg; ~1 mg of protein; Kraft, Northfield, IL) with 10 μg of cholera toxin (List Biologicals, Campbell, CA) in 0.5 mL of PBS was administered intragastrically (Delvo SA, Biel, Switzerland) weekly for 4 weeks. Serum was collected by retro-orbital bleeding and analyzed for peanut-specific Igs via sandwich ELISA [29-31].

Histology and imaging systems. Intestinal segments were collected and fixed in 10% formalin for 24 h, and then washed with 70% ethanol and paraffin embedded. Sections were stained with the Protocol Hema 3 stain set (Fisher Scientific, Hampton, NH) and Masson's Trichome method [32,33]. For image analysis of histological sections, Masson's trichrome stained tissue slides were scanned (VS120-ASW v2.9 slide scanner, with UPlanSApo 20x objective, Olympus) and analyzed using HALO® Image Analysis Platform (v2.2.1870.34, Indica Labs Inc, Corrales, New Mexico) using Area Quantification module (v1.0).

Statistics. Data were analyzed and graphed with GraphPad Prism 7 software (GraphPad Software). Continuous data are expressed as means ± SEMs and were analyzed by using 1-way ANOVA with Bonferroni *post hoc* tests and unpaired Student's *t* test. Differences were considered statistically significant at a *P* value of less than 0.05 or as indicated.

RESULTS

The microbiota regulates the frequency of intestinal eosinophils

In order to quantify eosinophil frequency, LP cells from the SI were isolated and evaluated by flow cytometry [6]. In agreement with previous reports [6,34], intestinal eosinophils were identified as $CD45^+$ SSC^{high} Siglec-F^+ cells and expressed high levels of CD11b (**Fig. 1A**). This putative population of eosinophils was flow-sorted and stained using Hema 3 to validate their identity. The microscopic analysis showed hallmark morphological features (*i.e.* lobular

polymorphic nucleus and eosinophilic granular cytoplasm) of eosinophils [1,2] in >95% of the cells (**Fig. 1B**).

Next, we compared eosinophil frequency in the SI of SPF and GF mice. To account for possible differences in strains biased towards Th1 and Th2 immunity [35], both C57BL/6 and BALB/c strains were assessed. The frequency of intestinal eosinophils in GF mice was ~2-fold higher than in SPF controls, regardless of the strain (**Fig. 1C**). We then assessed the distribution of eosinophils along the SI tract of GF and SPF mice. Regardless of colonization status, eosinophils were enriched predominantly in the proximal end of the SI (duodenum) and reduced in the distal end (ileum) (**Fig. 1D**). Nevertheless, within all sections of the SI, GF mice harbored a greater proportion of eosinophils than SPF control mice. These data demonstrate that the microbiota significantly influences the basal tissue eosinophilia of the SI LP.

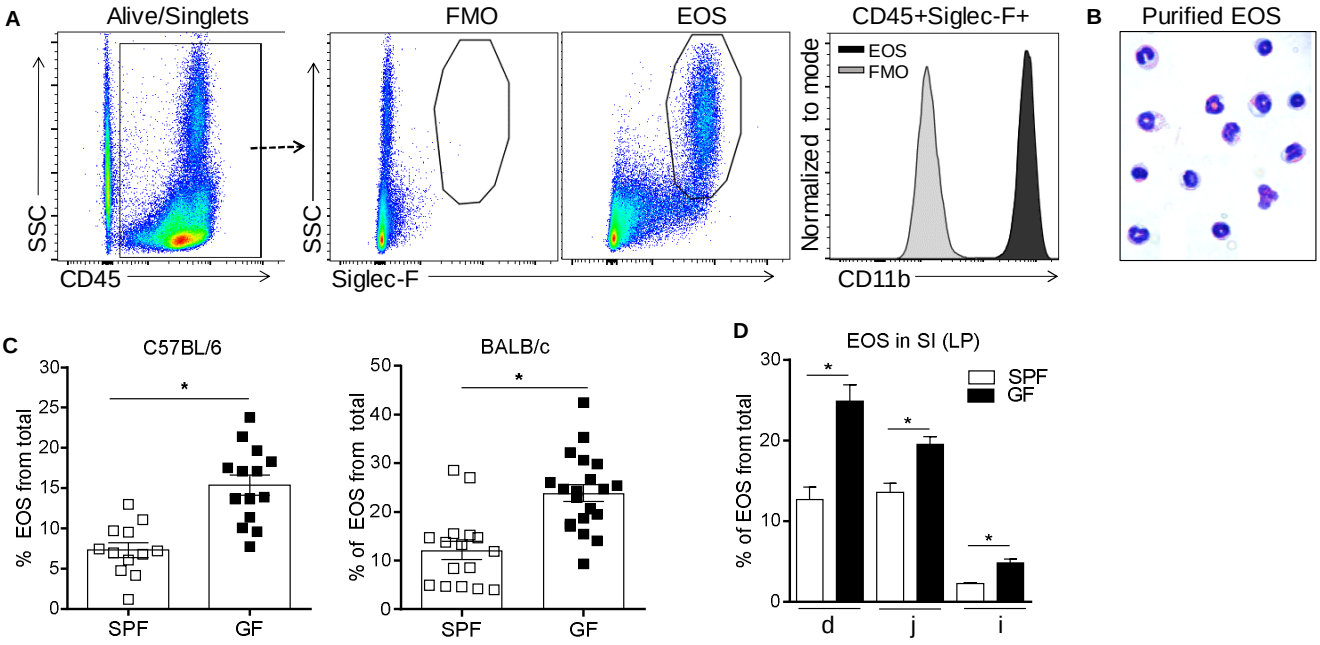

Figure 1. Flow cytometric identification of small intestinal eosinophils (EOS) as alive singlet CD45+Siglec-F+ cells (A), morphologic validation (B) and assessment of their frequency in the small intestine (SI) of SPF and GF C57BL/6 and BALB/c mice (C, D). The frequency of EOS from total cells in the lamina propria (LP) of different sections of the SI including duodenum (d), jejunum (j) and ileum (i) of C57BL/6 mice (D). Pooled data from 3-4 experiments (n=12-20) (C) or representative data from 3 experiments (D) represented as mean ± SEM, *P<0.05

The microbiota sets the basal eosinophilic tone in naturally colonized mucosal surfaces

To test whether the differences in eosinophils between GF and SPF mice were directly related to the microbiota, we colonized GF mice with either a complex (SPF) or simple (ASF) microflora [21]. To this end, we co-housed separate groups of GF mice with either ASF or SPF mice. The data show that the hypereosinophilia seen in the SI of GF mice was partially

attenuated by colonization with a minimal assortment of only 8 well-defined bacterial species [21] using ASF mice. Hypereosinophilia was fully attenuated with complex colonization using SPF flora (**Fig. 2A**). These data show a graded regulatory relationship between the complexity of the microbiota and tissue eosinophil levels.

Since the microbiota regulated the frequency of enteric eosinophils, we next examined whether germ-free induced hypereosinophilia was present in other naturally colonized mucosal sites (large intestine and vaginal tract), sterile ─or poorly colonized─ mucosal sites (uterus) [36] and non-mucosal sites (spleen). The frequency of eosinophils was also significantly higher in the large intestine and vaginal tract of GF mice compared to SPF (**Fig. 2B, C**) but not in sterile tissues such as the spleen (**Fig. 2D**) and uterus (**Fig. 2E**). These data further suggest that the frequency of tissue eosinophils is dependent on the natural colonization status.

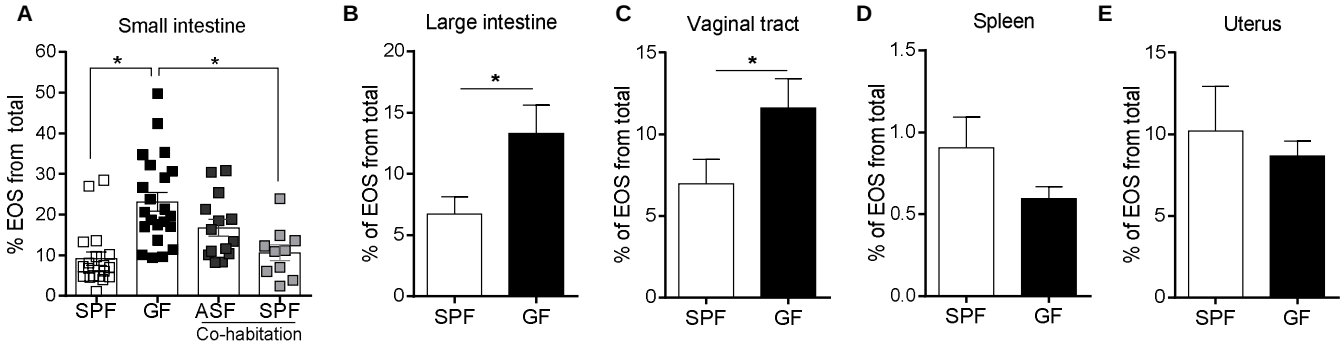

Figure 2. Separate groups of GF mice were colonized by co-habitation with ASF or SPF mice and the presence of small intestinal eosinophils (EOS) was assessed (A). Assessment of EOS frequency in the large intestine, vaginal tract, spleen and uterus of SPF and GF mice by flow cytometry (B-E). Pooled data from 2-5 experiments (n=8-22) represented as mean ± SEM, *P<0.05.

The maturity of the immune system is not associated with intestinal hypereosinophilia

It is well established that the adaptive immune system of GF mice is immature, particularly as it refers to T cells [37]. Consistent with previous observations [38,39], we found that the frequency of $CD4^+$ T cells was significantly lower in the SI of GF mice compared to SPF controls (**Fig. 3A**). In contrast, there were no statistically significant differences in the frequency of B cells, dendritic cells (DCs), macrophages or mast cells (**Fig. 3A**). To investigate whether the hypereosinophilia observed in GF conditions was due to the absence of colonization or inherent to an immature immune system, we generated microbiota-competent mice with an immature immune system [40]. To this end, BALB/c mice, fed with an elemental (amino acid) diet and housed in a SPF environment, were bred for 3 generations. Fewer Peyer's patches and a lower frequency of $CD4^+$ T cells were recapitulated in the LP, confirming the immaturity of the adaptive immune system of these mice (**Fig. 3B**). We did not find changes in the frequency of B cells, macrophages or eosinophils. These findings indicate that the relative hypereosinophilia in

GF mice is likely due to a lack of microbial-derived signals rather than immune immaturity *per se*.

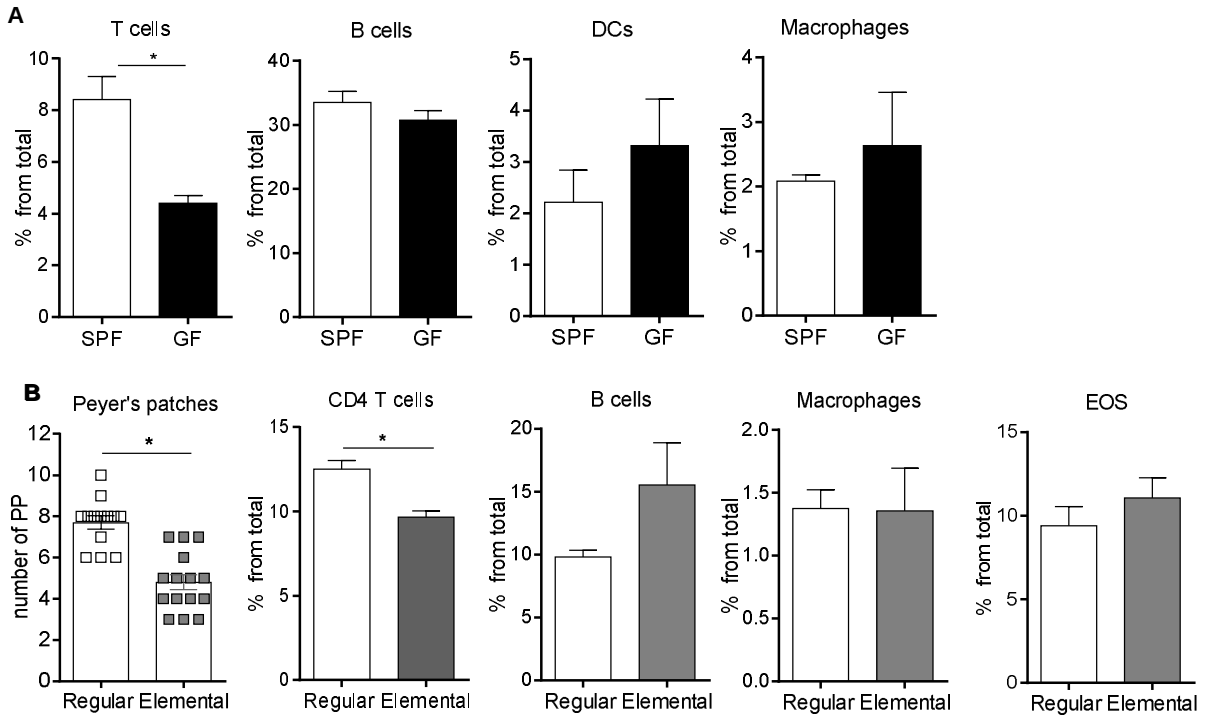

Figure 3. Flow cytometric characterization of adaptive and innate immune compartments (A, B) in the small intestine of GF and SPF mice fed a regular (A) or elemental (B) diet (B). Pooled data from 2-4 experiments (n=6-22) represented as mean ± SEM, *P<0.05.

The microbiota regulates eosinophil attraction and retention signals in mucosal tissues

Tissue accumulation of eosinophils associated with chronic intestinal inflammation is typically attributed to increased eosinopoiesis [41]. Here, we investigated whether the same mechanism applies to constitutive hypereosinophilia in healthy GF mice. We found that there were no differences in BM eosinophils between GF and SPF mice, nor were differences in EOS circulating in the peripheral blood (data not shown). This suggested that eosinophil accumulation in mucosal sites of otherwise healthy GF mice might be mediated by increased expression of attraction and/or retention signals in the SI, large intestine and vaginal tract.

To identify signals associated with eosinophil migration and retention at the tissue level, we analyzed chemokine and cytokine RNA expression in the proximal (duodenum) and distal (ileum) small intestinal segments of GF and SPF mice (**Fig. 4A**). The GF duodenum (GF D) exhibited a significant increase in eosinophil-associated chemotactic genes such as CCL5 (RANTES), CCR5 and TSLP as compared to SPF controls **(Fig. 4B**). Furthermore, within the GF ileum (GF I), we observed a significant increase in RANTES, CCR5, CCL24, CCL22 and GM-CSF transcripts, which are associated with eosinophil recruitment and survival (**Fig. 4C**) [42-

[45]. These data suggest that the microbiota downregulates signals involved in the attraction, retention and survival of eosinophils in the SI, the absence of which results in the relative hypereosinophilia found in GF conditions.

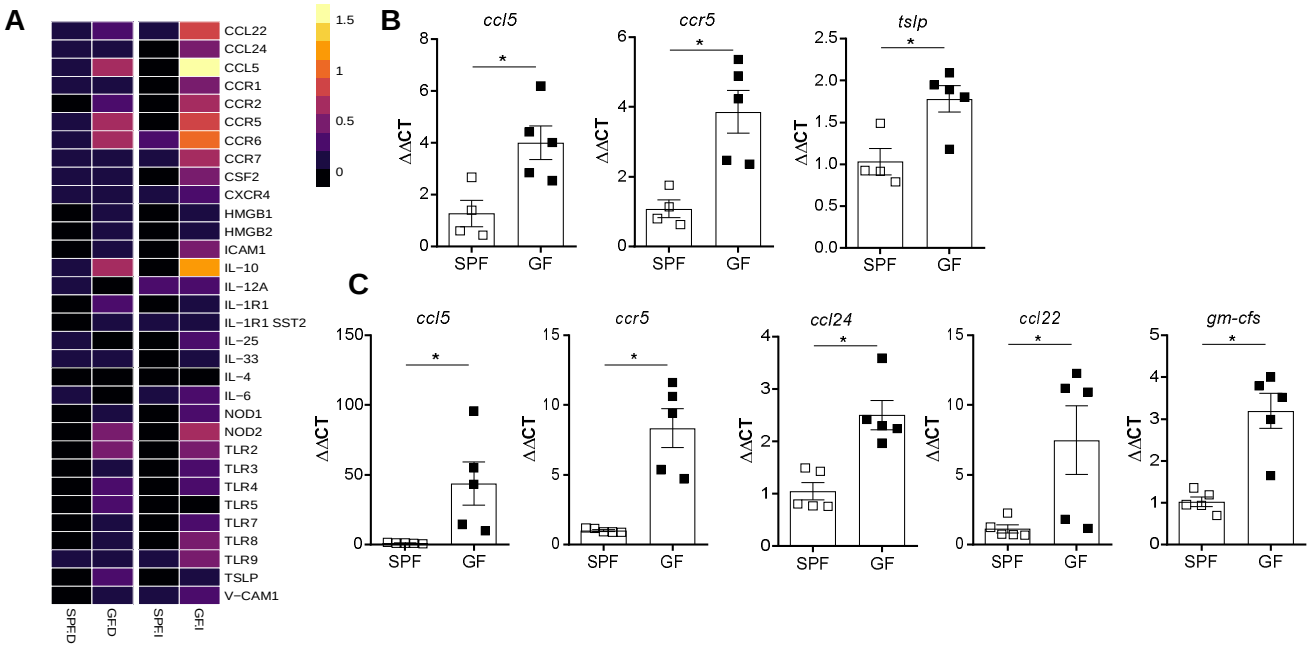

Figure 4. Heatmap of chemokine and cytokine RNA expression in the proximal (duodenum; D) and distal (ileum; I) small intestinal segments from GF and SPF mice as determined by quantitative, real time PCR (A). Genes differentially expressed in the proximal (B) and distal (C) small intestine of SPF and GF mice. Data from 4-5 mice (B,C) represented as mean ± SEM, *P<0.05.

The microbiota modulates eosinophils-mediated intestinal remodeling and allergic sensitization

In order to evaluate GF eosinophil function, we first determined the degranulation status of eosinophils by quantifying EPO activity [26,46] in SI homogenates of GF and SPF mice. Despite the extensive hypereosinophilia (**Fig. 1C**), significantly lower EPO activity was detected in GF mice (**Fig. 5A**) compared to SPF controls, thus suggesting ongoing eosinophil degranulation in GF mice.

To investigate the functional significance of EPO degranulation in GF mice, we generated a novel eosinophil-deficient mouse strain (ΔdblGATA; GF-GATA) on a GF background. Given that eosinophils are known to contribute to tissue remodeling, intestinal tissues were assessed with Masson's Trichome staining [32,33] to evaluate collagen deposition (**Fig. 5B**). To remove potential bias of field selection, these differences were quantified using the HALO Image Analysis software. This analysis demonstrated higher collagen density in the submucosal layers of eosinophil-deficient mice, particularly in GF conditions, as compared to

SPF controls **(Fig. 5C).** These findings suggest that the absence of eosinophils in GF GATA mice is associated with increased fibrotic tissue remodelling.

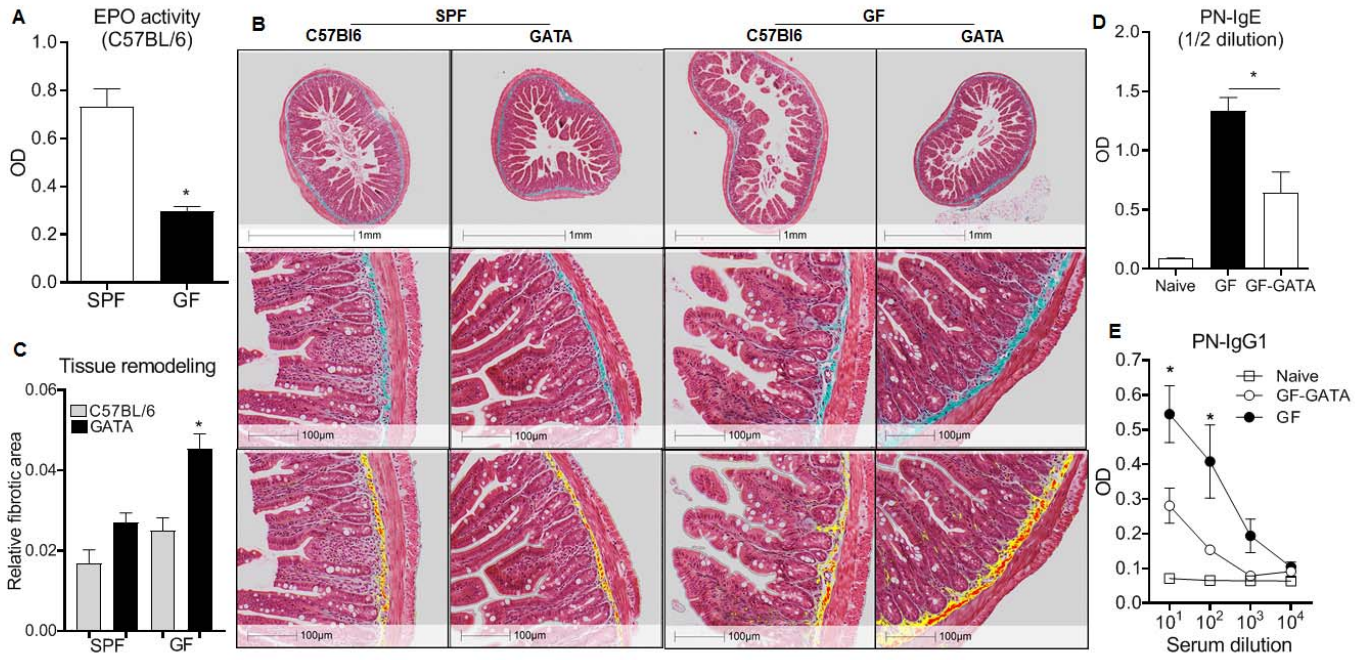

Figure 5. Assessment of eosinophil peroxidase activity (EPO) in intestinal homogenates of SPF and GF mice (A). Representative histological examples of intestinal fibrosis (B, upper panels) and associated Halo quantification of fibrotic surface on digitalized (20X) sections where yellow and red represents Masson's Trichrome positive stain (B, lower panels) and associated quantification (C) of overall fibrotic area performed on GF mice, eosinophil deficient GF mice (GATA-GF) and controls. GF mice, eosinophil deficient GF mice (GATA-GF) and controls were intragastrically sensitized to peanut (PN) and serum levels of PN-specific IgE (D) and IgG1 (E) were determined by ELISA. Pooled data from 2 experiments (n=6) (A, D, E) or data from 4-5 mice (B,C) represented as mean ± SEM, *P<0.05.

Intestinal eosinophils play a critical role in the initiation of allergic responses to food allergens. We have previously demonstrated that eosinophil-deficient (ΔdblGATA) mice are unable to produce peanut-specific immunoglobulins [6]. Additionally, gut dysbiosis has been associated with a higher risk of developing allergy in humans [47-50], and GF mice have been shown to be prone to generate Th2 responses [51,52]. In this context, we evaluated if the lack of microbial modulation of eosinophils may impact allergic sensitization to foods. We employed an established food allergy model using intragastric sensitization to peanut [22,30,53,54] in GF mice on a GATA or C57BL/6 backgrounds. Compared to naïve mice, both strains of GF mice develop peanut-specific IgE **(Fig. 5D)** and IgG1 **(Fig. 5E)** were elevated in both strains of GF mice. However, the production of both immunoglobulins was significantly lower in eosinophil-deficient mice. These findings raise the possibility that the microbiota influences the development of food allergic sensitization partly through its effects on intestinal eosinophils.

DISCUSSION

Mucosal eosinophils have been traditionally considered recruited proinflammatory cells, whose biological benefit is limited to defence against parasitic infections. However, new evidence has established that eosinophils also contribute the initiation, propagation, and resolution of innate and adaptive immune responses, and to homeostatic tissue repair and remodeling [4,5,55,56]. The diversity of functions ascribed to enteric eosinophils in particular has prompted the investigation of the mechanisms that influence their tissue residence and functionality. In this context, microbiota is known to be critical for the development of the SI mucosal adaptive immune system [16]; given that eosinophils natively inhabit the SI [57], we investigated the impact of the microbiota on intestinal eosinophil frequency and function.

Quantification of intestinal eosinophils was carried out by flow cytometry [6,34] and further validated by morphological analysis (**Fig. 1A-B**). The total frequency of intestinal eosinophils in either C57BL6 or BALB/c was ~2 fold higher in GF mice as compared to SPF controls (**Fig. 1C**). This is in contrast with the findings by Mishra *et al.* [44], who did not observe significant differences in eosinophils numbers in the GI of SPF and GF mice when quantified by histology. Several factors might have contributed to this discrepancy including the strain of mice (Black Swiss mice *vs* C57BL/6 and BALB/c), microbiome differences under SPF conditions, the number of mice utilized in each study (n=5 vs n=12-20) and, lastly, the technique employed for eosinophils quantification, as it is likely that flow cytometry allowed for a more comprehensive and precise quantification than immunohistochemistry using the eosinophil granule protein, major basic protein (MBP) [58]. Further, we show that intestinal eosinophils have a propensity for degranulation, which may lead to eosinophil underdetection when using MBP-based methods.

Consistent with previous reports [6,44], eosinophils were pre-eminently localized in the proximal end of the SI and decreased in frequency distally. However, the proportion of eosinophils in each section was significantly higher in GF mice compared to SPF controls (**Fig. 1D**). Importantly, colonization of GF mice with a complex microbiota reduced enteric eosinophils to a frequency comparable to SPF mice. This shows that enteric eosinophilia is greatly influenced by the host intestinal microbiome (**Fig. 1E**).

We next considered whether enteric hypereosinophilia could be an innate response to compensate for the immaturity of the adaptive immune system that occurs in GF mice. It is known that feeding SPF mice with an elemental diet results in an immature immune system [59]. Our data show that the administration of an elemental diet for 3 generations resulted in features indicative of immune immaturity, such as a reduced number of Peyer's patches and lower $CD4^+$ T lymphocytes. However, it did not lead to the hypereosinophilia identified in GF mice fed with a conventional diet (**Fig. 2**), which suggests that enteric eosinophilia is independent of the maturity of the adaptive immune system and thus dependent on the microbiota.

The relative hypereosinophilia observed in GF mice, its regulation by complex microbial colonization and its independence of the maturity of the adaptive immune system, along with the

findings of unperturbed eosinopoiesis suggest that the microbiota directly regulates enteric eosinophils through interactions with cells resident in the mucosal compartment. The increased expression of signals involved in the attraction, retention and activation of eosinophils such as RANTES, CCR5, CCL24, GM-CSF and TLSP [42-45] in the SI of GF mice compared to SPF supports this notion (**Fig. 4**).

A hallmark feature of eosinophils activation is the release of granular contents such as MBP or EPO [56]. We detected a significant reduction of EPO activity in enteric eosinophils from GF mice, as compared to SPF controls, which is indicative of degranulation (**Fig. 5A**). While eosinophils degranulation is often associated with tissue damage, eosinophil-derived granule proteins can also participate in tissue homeostasis [60]. For example, eosinophil-derived neurotoxin and EPO promote fibroblast proliferation and collagen biosynthesis respectively [61,62]. However, the effect may be dependent on tissue location as *ex vivo* experiments with eosinophil lysates induced opposite effects in terms of collagen production in dermal and lung fibroblasts [63]. In the gut, increased eosinophil activation has been detected in patients with ulcerative colitis (UC), as compared to controls. Interestingly, the number activated eosinophils was larger during the remission phase of UC [64], which may evince a dual role in intestinal inflammation and repair. We found that in mice lacking both eosinophils and microbiota, collagen deposition in the submucosal layer was double that of mice deficient in either eosinophils or microbiota alone (**Fig. 5B & C**), suggesting that at least certain components of tissue remodeling are regulated by interactions between the microbiota and eosinophils.

Several lines of evidence have indicated that dysbiosis in humans is associated with an increased prevalence of allergic sensitization [47,65-67]. However, the mechanisms underlying this association remain to be fully elucidated. It was recently shown that GF and antibiotic-treated mice developed increased allergic sensitization as compared to SPF mice [52]. On the other hand, we have shown that enteric eosinophils are essential to the induction of allergic sensitization in SPF mice[6]. These observations raise the question of whether the microbiota and enteric eosinophils synergize in the induction of allergic sensitization. Here, having demonstrated that GF mice exhibit hypereosinophilia, we show that the absence of eosinophils in GF mice results in an attenuation of allergic sensitization. Thus, these data support the concept that the microbiota influences the capacity to develop allergic sensitization, at least in part, through its effects on enteric eosinophils.

In summary, this study demonstrates that eosinophil frequency and activation in the intestinal mucosa is regulated by the microbiota. It also shows that processes such as tissue repair and the induction of allergic sensitization appear to be regulated by an interplay between the commensal microbiota and intestinal eosinophils. Given that the tissue microenvironment crucially shapes the nature and evolution of subsequent antigen-host interactions, these data have fundamental implications to understanding the role of the microbiota and eosinophils in health and disease.

AUTHOR CONTRIBUTIONS

RJS conceptualized the project and designed experiments. RJS and VA performed experiments, analyzed the data and wrote the manuscript. TW, MEG, TSM, RC, JFK and YE, helped with experiments. HJG conducted experiments in the Axenic and Gnotobiotic Unit. EFV generated GATA-GF mice. SA and KA performed HALO analysis. AH performed genetic analyses. EFV, KA and DKC provided scientific input and revised the manuscript. MJ obtained funding, oversaw the project and edited the manuscript.

FUNDING

This work was supported by Food Allergy Canada, the Delaney family, the Zych family, the Walter and Maria Schroeder Foundation and AllerGen NCE.

ACKNOWLEDGMENTS

We acknowledge Dr. Puja Bagri for critical review of the manuscript. We thank all the members of the Jordana Lab for technical help and scientific input.

REFERENCES

1 Lee, J. J., Jacobsen, E. A., McGarry, M. P., Schleimer, R. P. & Lee, N. A. Eosinophils in health and disease: the LIAR hypothesis. *Clin Exp Allergy* **40**, 563-575, doi:10.1111/j.1365-2222.2010.03484.x (2010).

2 Rosenberg, H. F., Dyer, K. D. & Foster, P. S. Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol* **13**, 9-22, doi:10.1038/nri3341 nri3341 [pii] (2013).

3 Arnold, I. C. *et al.* Eosinophils suppress Th1 responses and restrict bacterially induced gastrointestinal inflammation. *J Exp Med*, doi:10.1084/jem.20172049 (2018).

4 Abdala-Valencia, H. *et al.* Shaping eosinophil identity in the tissue contexts of development, homeostasis, and disease. *J Leukoc Biol* **104**, 95-108, doi:10.1002/JLB.1MR1117-442RR (2018).

5 Xenakis, J. J. *et al.* Resident intestinal eosinophils constitutively express antigen presentation markers and include two phenotypically distinct subsets of eosinophils. *Immunology* **154**, 298-308, doi:doi:10.1111/imm.12885 (2018).

6 Chu, D. K. *et al.* Indigenous enteric eosinophils control DCs to initiate a primary Th2 immune response in vivo. *J Exp Med* **211**, 1657-1672, doi:10.1084/jem.20131800 (2014).

7 Cañas, J. A. *et al.* Eosinophil-derived exosomes contribute to asthma remodelling by activating structural lung cells. *Clinical & Experimental Allergy* **48**, 1173-1185, doi:doi:10.1111/cea.13122 (2018).

8 Takemura, N. *et al.* Eosinophil depletion suppresses radiation-induced small intestinal fibrosis. *Sci Transl Med* **10**, doi:10.1126/scitranslmed.aan0333 (2018).

9 Bordon, Y. Mucosal immunology: Eosinophils get the party started. *Nat Rev Immunol* **14**, 580-581, doi:10.1038/nri3731 (2014).

10 Yang, B. G., Seoh, J. Y. & Jang, M. H. Regulatory Eosinophils in Inflammation and Metabolic Disorders. *Immune Netw* **17**, 41-47, doi:10.4110/in.2017.17.1.41 (2017).

11 Sugawara, R. *et al.* Small intestinal eosinophils regulate Th17 cells by producing IL-1 receptor antagonist. *J Exp Med* **213**, 555-567, doi:10.1084/jem.20141388 (2016).

12 Matsumoto, K., Fukuda, S., Nakamura, Y. & Saito, H. Amphiregulin production by human eosinophils. *Int Arch Allergy Immunol* **149 Suppl 1**, 39-44, doi:10.1159/000210652 (2009).
13 Wen, T. & Rothenberg, M. E. The Regulatory Function of Eosinophils. *Microbiol Spectr* **4**, doi:10.1128/microbiolspec.MCHD-0020-2015 (2016).
14 Shi, N., Li, N., Duan, X. & Niu, H. Interaction between the gut microbiome and mucosal immune system. *Mil Med Res* **4**, 14, doi:10.1186/s40779-017-0122-9 (2017).
15 McDermott, A. J. & Huffnagle, G. B. The microbiome and regulation of mucosal immunity. *Immunology* **142**, 24-31, doi:10.1111/imm.12231 (2014).
16 Smith, K., McCoy, K. D. & Macpherson, A. J. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol.* **19**, 59-69, doi:10.1016/j.smim.2006.10.002 (2007).
17 Crabbe, P. A., Bazin, H., Eyssen, H. & Heremans, J. F. The normal microbial flora as a major stimulus for proliferation of plasma cells synthesizing IgA in the gut. The germ-free intestinal tract. *Int Arch Allergy Appl Immunol* **34**, 362-375 (1968).
18 Bandeira, A. *et al.* Localization of gamma/delta T cells to the intestinal epithelium is independent of normal microbial colonization. *J Exp Med* **172**, 239-244 (1990).
19 Rosenberg, H. F., Masterson, J. C. & Furuta, G. T. Eosinophils, probiotics, and the microbiome. *J Leukoc Biol* **100**, 881-888, doi:10.1189/jlb.3RI0416-202R (2016).
20 Slack, E. *et al.* Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science* **325**, 617-620, doi:10.1126/science.1172747 (2009).
21 Galipeau, H. J. *et al.* Intestinal microbiota modulates gluten-induced immunopathology in humanized mice. *Am J Pathol* **185**, 2969-2982, doi:10.1016/j.ajpath.2015.07.018 (2015).
22 Jimenez-Saiz, R. *et al.* Lifelong memory responses perpetuate humoral TH2 immunity and anaphylaxis in food allergy. *J Allergy Clin Immunol* **140**, 1604-1615 e1605, doi:10.1016/j.jaci.2017.01.018 (2017).
23 Carlens, J. *et al.* Common gamma-chain-dependent signals confer selective survival of eosinophils in the murine small intestine. *J Immunol* **183**, 5600-5607, doi:10.4049/jimmunol.0801581 (2009).
24 Jimenez-Saiz, R., Rupa, P. & Mine, Y. Immunomodulatory effects of heated ovomucoid-depleted egg white in a BALB/c mouse model of egg allergy. *J Agric Food Chem* **59**, 13195-13202, doi:10.1021/jf202963r (2011).
25 Anipindi, V. C. *et al.* Estradiol Enhances CD4+ T-Cell Anti-Viral Immunity by Priming Vaginal DCs to Induce Th17 Responses via an IL-1-Dependent Pathway. *PLoS Pathog* **12**, e1005589, doi:10.1371/journal.ppat.1005589 (2016).
26 Humbles, A. A. *et al.* Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo. *J Exp Med* **186**, 601-612 (1997).
27 Al-Garawi, A. *et al.* Shifting of immune responsiveness to house dust mite by influenza A infection: genomic insights. *J Immunol* **188**, 832-843, doi:10.4049/jimmunol.1102349 (2012).
28 Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnology Letters* **26**, 509-515, doi:10.1023/b:Bile.0000019559.84305.47 (2004).

29 Swirski, F. K. *et al.* Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor. *J Immunol* **169**, 3499-3506 (2002).
30 Chu, D. K. *et al.* T helper cell IL-4 drives intestinal Th2 priming to oral peanut antigen, under the control of OX40L and independent of innate-like lymphocytes. *Mucosal Immunol* **7**, 1395-1404, doi:10.1038/mi.2014.29 (2014).
31 Jimenez-Saiz, R. *et al.* IgG1(+) B-cell immunity predates IgE responses in epicutaneous sensitization to foods. *Allergy*, doi:10.1111/all.13481 (2018).
32 Goldner, J. A modification of the masson trichrome technique for routine laboratory purposes. *Am J Pathol* **14**, 237-243 (1938).
33 Fischer, A. H., Jacobson, K. A., Rose, J. & Zeller, R. Hematoxylin and eosin staining of tissue and cell sections. *CSH Protoc* **2008**, pdb prot4986, doi:10.1101/pdb.prot4986 (2008).
34 Dyer, K. D., Garcia-Crespo, K. E., Killoran, K. E. & Rosenberg, H. F. Antigen profiles for the quantitative assessment of eosinophils in mouse tissues by flow cytometry. *J Immunol Methods* **369**, 91-97, doi:10.1016/j.jim.2011.04.009 (2011).
35 Sellers, R. S., Clifford, C. B., Treuting, P. M. & Brayton, C. Immunological variation between inbred laboratory mouse strains: points to consider in phenotyping genetically immunomodified mice. *Vet Pathol* **49**, 32-43, doi:10.1177/0300985811429314 (2012).
36 Perez-Muñoz, M. E., Arrieta, M.-C., Ramer-Tait, A. E. & Walter, J. A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: implications for research on the pioneer infant microbiome. *Microbiome* **5**, 48, doi:10.1186/s40168-017-0268-4 (2017).
37 Smith, K., McCoy, K. D. & Macpherson, A. J. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol* **19**, 59-69, doi:10.1016/j.smim.2006.10.002 (2007).
38 Macpherson, A. J. & Harris, N. L. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* **4**, 478-485, doi:10.1038/nri1373 (2004).
39 Round, J. L. & Mazmanian, S. K. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* **9**, 313-323, doi:10.1038/nri2515 (2009).
40 Faria, A. M. *et al.* Food components and the immune system: from tonic agents to allergens. *Front Immunol* **4**, 102, doi:10.3389/fimmu.2013.00102 (2013).
41 Griseri, T. *et al.* Granulocyte Macrophage Colony-Stimulating Factor-Activated Eosinophils Promote Interleukin-23 Driven Chronic Colitis. *Immunity* **43**, 187-199, doi:10.1016/j.immuni.2015.07.008 (2015).
42 Lee, J. J. *et al.* Human versus mouse eosinophils: "that which we call an eosinophil, by any other name would stain as red". *J Allergy Clin Immunol* **130**, 572-584, doi:10.1016/j.jaci.2012.07.025 (2012).
43 Vancheri, C. *et al.* Human lung fibroblast-derived granulocyte-macrophage colony stimulating factor (GM-CSF) mediates eosinophil survival in vitro. *Am J Respir Cell Mol Biol* **1**, 289-295, doi:10.1165/ajrcmb/1.4.289 (1989).
44 Mishra, A., Hogan, S. P., Lee, J. J., Foster, P. S. & Rothenberg, M. E. Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. *J Clin Invest* **103**, 1719-1727, doi:10.1172/JCI6560 (1999).
45 Wong, C. K., Hu, S., Cheung, P. F. & Lam, C. W. Thymic stromal lymphopoietin induces chemotactic and prosurvival effects in eosinophils: implications in allergic

inflammation. *Am J Respir Cell Mol Biol* **43**, 305-315, doi:10.1165/rcmb.2009-0168OC (2010).
46 White, S. R., Kulp, G. V., Spaethe, S. M., Van Alstyne, E. & Leff, A. R. A kinetic assay for eosinophil peroxidase activity in eosinophils and eosinophil conditioned media. *J Immunol Methods* **144**, 257-263 (1991).
47 Plunkett, C. H. & Nagler, C. R. The Influence of the Microbiome on Allergic Sensitization to Food. *J Immunol* **198**, 581-589, doi:10.4049/jimmunol.1601266 (2017).
48 Pascal, M. *et al.* Microbiome and Allergic Diseases. *Front Immunol* **9**, doi:10.3389/fimmu.2018.01584 (2018).
49 Zimmermann, P., Messina, N., Mohn, W. W., Finlay, B. B. & Curtis, N. Association between the intestinal microbiota and allergic sensitization, eczema, and asthma: A systematic review. *Journal of Allergy and Clinical Immunology*, doi:10.1016/j.jaci.2018.09.025.
50 Feehley, T. *et al.* Healthy infants harbor intestinal bacteria that protect against food allergy. *Nat. Med.*, doi:10.1038/s41591-018-0324-z (2019).
51 McCoy, K. D., Ignacio, A. & Geuking, M. B. Microbiota and Type 2 immune responses. *Curr Opin Immunol* **54**, 20-27, doi:10.1016/j.coi.2018.05.009 (2018).
52 Stefka, A. T. *et al.* Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci U S A*, doi:10.1073/pnas.1412008111 (2014).
53 Arias, K. *et al.* Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice. *J Allergy Clin Immunol* **127**, 1552-1561 e1551, doi:10.1016/j.jaci.2011.03.044 (2011).
54 Kong, J. *et al.* Comprehensive metabolomics identifies the alarmin uric acid as a critical signal for the induction of peanut allergy. *Allergy* **70**, 495-505, doi:10.1111/all.12579 (2015).
55 Travers, J. & Rothenberg, M. E. Eosinophils in mucosal immune responses. *Mucosal Immunol* **8**, 464-475, doi:10.1038/mi.2015.2 (2015).
56 Kita, H. Eosinophils: multifaceted biological properties and roles in health and disease. *Immunol Rev* **242**, 161-177, doi:10.1111/j.1600-065X.2011.01026.x (2011).
57 Mowat, A. M. & Bain, C. C. News & Highlights. *Mucosal Immunology* **3**, 420, doi:10.1038/mi.2010.24 (2010).
58 Szantho, E. *et al.* Comparative Analysis of Multicolor Flow Cytometry and Immunohistochemistry for the Detection of Disseminated Tumor Cells. *Appl Immunohistochem Mol Morphol* **26**, 305-315, doi:10.1097/PAI.0000000000000519 (2018).
59 Menezes, J. S. *et al.* Immunological activities are modulated by enteral administration of an elemental diet in mice. *Clin Nutr* **25**, 643-652, doi:10.1016/j.clnu.2006.01.005 (2006).
60 Shamri, R., Xenakis, J. J. & Spencer, L. A. Eosinophils in innate immunity: an evolving story. *Cell Tissue Res.* **343**, 57-83, doi:10.1007/s00441-010-1049-6 (2011).
61 Puxeddu, I., Ribatti, D., Crivellato, E. & Levi-Schaffer, F. Mast cells and eosinophils: a novel link between inflammation and angiogenesis in allergic diseases. *J Allergy Clin Immunol* **116**, 531-536, doi:10.1016/j.jaci.2005.06.007 (2005).
62 DeNichilo, M. O. *et al.* Peroxidase enzymes regulate collagen extracellular matrix biosynthesis. *Am J Pathol* **185**, 1372-1384, doi:10.1016/j.ajpath.2015.01.013 (2015).
63 Levi-Schaffer, F. *et al.* Human eosinophils regulate human lung- and skin-derived fibroblast properties in vitro: A role for transforming growth factor β (TGF-

β). *Proceedings of the National Academy of Sciences* **96**, 9660-9665, doi:10.1073/pnas.96.17.9660 (1999).

64 Lampinen, M. *et al.* Eosinophil granulocytes are activated during the remission phase of ulcerative colitis. *Gut* **54**, 1714-1720, doi:10.1136/gut.2005.066423 (2005).

65 Risnes, K. R., Belanger, K., Murk, W. & Bracken, M. B. Antibiotic exposure by 6 months and asthma and allergy at 6 years: Findings in a cohort of 1,401 US children. *Am J Epidemiol* **173**, 310-318, doi:10.1093/aje/kwq400 (2011).

66 Russell, S. L. *et al.* Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep* **13**, 440-447, doi:10.1038/embor.2012.32 (2012).

67 Ellenbogen, Y. *et al.* The Initiation of Th2 Immunity Towards Food Allergens. *Int J Mol Sci* **19**, doi:10.3390/ijms19051447 (2018).