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Indigenous enteric eosinophils control DCs to initiate a primary Th2 immune response in vivo

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Eosinophils natively inhabit the small intestine, but a functional role for them there has remained elusive. Here, we show that eosinophil–deficient mice were protected from induction of Th2-mediated peanut food allergy and anaphylaxis, and Th2 priming was restored by reconstitution with il4+/+ or il4−/− eosinophils. Eosinophils controlled CD103+ dendritic cell (DC) activation and migration from the intestine to draining lymph nodes, events necessary for Th2 priming. Eosinophil activation in vitro and in vivo led to degranulation of eosinophil peroxidase, a granule protein whose enzymatic activity promoted DC activation in mice and humans in vitro, and intestinal and extraintestinal mouse DC activation and mobilization to lymph nodes in vivo. Further, eosinophil peroxidase enhanced responses to ovalbumin seen after immunization. Thus, eosinophils can be critical contributors to the intestinal immune system, and granule-mediated shaping of DC responses can promote both intestinal and extraintestinal adaptive immunity.

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Abbreviations used: 3-NT, 3-nitrotyrosine; BAL, bronchoalveolar lavage; BMDC, BM-derived DC; CT, cholera toxin; EPO, heat-inactivated EPO; Ehrlich described the presence of rich magenta eosin-staining granules that characterize eosinophils over 130 yr ago (Erlich, 1879). Yet, the precise roles of eosinophils in health and disease remain to be fully defined (Rosenberg et al., 2013). Eosinophils have been identified in tissues of lower vertebrates and eosinophil-like cells in some invertebrates. Thus, eosinophils evolved before adaptive T and B cell immunity emerged (McGarry, 2013). Eosinophils have been identified in tissues of lower vertebrates and eosinophil-like cells in some invertebrates. Thus, eosinophils evolved before adaptive T and B cell immunity emerged (McGarry, 2013). Eosinophils may have evolved over 130 yr ago (Erlich, 1879). Yet, the precise roles of eosinophils in health and disease remain to be fully defined (Rosenberg et al., 2013). Eosinophils have been identified in tissues of lower vertebrates and eosinophil-like cells in some invertebrates. Thus, eosinophils evolved before adaptive T and B cell immunity emerged (McGarry, 2013). Eosinophils have been identified in tissues of lower vertebrates and eosinophil-like cells in some invertebrates. Thus, eosinophils evolved before adaptive T and B cell immunity emerged (McGarry, 2013).
In this regard, eosinophil granulocytes are classically thought of as specialized effector cells recruited from the blood and bone marrow to tissues during adaptive Th2 responses. Thus, consistently other than the intestine, most healthy tissues are devoid of eosinophils (Barnes, 2004; Rosenberg et al., 2013). Once in the tissue, recruited eosinophils are activated, produce several cytokines, and release characteristic cationic granules such as eosinophil peroxidase (EPO) in a process termed degranulation. Eosinophils have been ascribed both beneficial and harmful functions. For example, there is evidence of eosinophil attachment to helminths and subsequent degranulation, and cytotoxicity toward helminths. However, an indispensable role for eosinophils in host defense against helminths remains controversial (Cadman and Lawrence, 2010; Rosenberg et al., 2013). With respect to allergic disease, eosinophils are thought to play a pathogenic inflammatory role in diseases such as allergic asthma, atopic dermatitis, or food allergy. In response to helminths or allergens, Th2 responses in the gastrointestinal (GI) tract, and elsewhere, are characterized by the late influx of eosinophils into the tissue to mediate inflammation. However, a role for the abundant native GI eosinophil population has remained enigmatic (Drury, 1915; Duran-Jorda, 1947; Mowat and Bain, 2010).

The small intestine (SI) plays a vital role in the digestion and absorption of macronutrients. The immune system must recognize food antigens as innocuous and, hence, develop tolerance. Extensive work has shown this process to be mediated by oral antigen uptake by CD103+ DCs, which migrate to the draining mesenteric LN (MLN) in a CCR7-dependent manner to prime antigen-specific naive T cells to differentiate into T regulatory cells (Pabst and Mowat, 2012). However, a subversion of oral tolerance leads to aberrant immune responses that result in the development of diseases such as food allergies. These are characterized by an adaptive Th2 immune response that results in IgE-mediated allergic reactions, including a life-threatening systemic reaction termed anaphylaxis (Burks, 2008; Berin and Sampson, 2013). Compared with other known classes of adaptive immune responses, the innate mechanisms that drive Th2 induction are much less clear (Paul and Zhu, 2010; Pulendran et al., 2010). Indeed, the APCs involved, their activation conditions, and T cell polarization factors such as the source of the initial IL-4 for Th2 cell priming remain unclear.

In stark contrast with the classical notion that eosinophils participate in immune responses as terminal effector leukocytes whose production, migration and activation is under the control of adaptive Th2 immunity, we report just the opposite process. Here, we found a crucial role for indigenous SI eosinophils in the initiation of Th2 immunity, with critical importance to the development of peanut food allergy. Eosinophil activation during intestinal Th2 priming results in the release of EPO that controls CD103+ DC activation and CCR7-dependent migration to the MLN, where DCs then promote Th2 responses. We further show that EPO-mediated DC activation is functional in both mice and humans, is mediated by EPO’s peroxidase enzymatic activity, and that EPO is also sufficient as an adjuvant to promote extraintestinal adaptive immunity. Collectively, these data identify a critical in vivo role for indigenous eosinophils in intestinal immunity, and reveals the potential for eosinophil granule-mediated shaping of DC responses in promoting adaptive immunity.

RESULTS
Definition of indigenous eosinophils along the intestinal tract
Eosinophils along the intestinal tract were histologically quantified in 1999 (Mishra et al., 1999), and Carlens et al. (2009) recently established a sensitive flow cytometric method to evaluate intestinal eosinophils. Here, we took advantage of flow cytometry’s capability for precise quantitation and multiparameter analysis to better define eosinophils along the intestinal tract. Eosinophils were most prevalent within the SI, ranging from ~15–25% of all lamina propria (LP) leukocytes in the duodenum, ~15–35% in the jejunum, and ~5–15% in the ileum (Fig. 1 A). The frequency of eosinophils was lower in the large intestine (LI), ~3–5% eosinophils in the cecum, ~7% in the colon, and ~1% in the rectum (Fig. 1 A). Quantifying eosinophil numbers revealed that they decreased along the length of the intestinal tract (Fig. 1 A), potentially suggesting negative regulation by the microbiota. Phenotypically, although both SI and LI eosinophils were Siglec-F+, CD11b+, CD44+, CD103+, and ~30% Ly6G+, SI and LI eosinophils also displayed distinct cell surface phenotypes (Fig. 1 B). More SI eosinophils expressed CD11c, and at higher levels, than LI eosinophils. As well, a greater proportion of SI eosinophils expressed ST2 (IL-33 receptor), CD69, and Ly6C, compared with LI eosinophils. Therefore, different compartments of the intestine harbor different proportions, numbers and phenotypes of eosinophils at baseline. Given the prominence of eosinophils in the SI, we next sought to evaluate the impact of eosinophil ablation on SI function and immunity.

Indigenous enteric eosinophils promote induction of Th2 immunity in vivo independently of their ability to produce IL-4
ΔdblGATA1 mice (Yu et al., 2002; Humbles et al., 2004; Fattouh et al., 2011), lack a high-affinity GATA-binding site in the GATA-1 promoter, resulting in strong, but submaximal GATA-1 promoter activity and, ultimately, complete ablation of the eosinophil lineage (Yu et al., 2002). Other GATA-1–dependent lineages, namely erythrocytes, megakaryocytes and mast cells, are not affected as their lineages do not require maximal GATA-1 promoter activity. A recent publication suggested that ΔdblGATA1 mice may have slightly reduced basophils (Nei et al., 2013).

Intragastric (i.g.) immunization to the common food allergen peanut (PN) with the classical oral Th2-inducing adjuvant cholera toxin (CT) induces basophil-independent, OX40L- and IL-4–dependent SI Th2 priming, such that subsequent allergen challenge elicits systemic anaphylaxis (Chu et al., 2013) similar to human peanut allergy (Li et al., 2000; Leung et al., 2003; Vadas et al., 2008; Arias et al., 2009, 2011). Whereas WT
possibility that eosinophils were required during SI priming to provide IL-4 to promote Th2 differentiation. However, mixed bone marrow chimeras showed that reconstitution of the eosinophil compartment (Fig. 2 C) with IL-4–deficient eosinophils fully restored Th2 priming to i.g. PN+CT. This was borne out by full restoration of antigen-specific IgE production, clinical anaphylaxis, and cytokine production (Fig. 2 B). Thus, normally resident enteric eosinophils are critical to initiate SI Th2 immunity in vivo, independent of their ability to produce IL-4.

Conventional parameters of intestinal and adaptive immunity are intact in eosinophil-deficient mice
A series of control experiments revealed that eosinophil deficiency did not impair conventional parameters of intestinal and adaptive immunity, including Peyer’s patch organogenesis (Fig. 3 A), intestinal IgA levels (Fig. 3 B), macronutrient uptake (Fig. 3 C), antigen-specific IgE production in response to intraperitoneal immunization, or oral tolerance induction (Fig. 3 D). These data show that eosinophil-deficient mice are not inherently defective in basal intestinal immunity and macronutrient absorption, nor are they defective in their capability to elicit parenteral adaptive Th2 immunity and IgE responses, as well as, under basal conditions, to induce CD103+ DC, CCR7, and T regulatory cell–dependent oral tolerogenic immunity (Pabst and Mowat, 2012). Consistent with reports of intact Th2 priming to parenteral immunization in eosinophil-deficient mice (Humbles et al., 2004; Swartz et al., 2006; Knott et al., 2007; Fattouh et al., 2011; Svensson et al., 2011), we also found that the requirement for eosinophils in initiating Th2 adaptive immunity was site-dependent as immunization via the peritoneum, skin, or rectum, tissues with little to no eosinophils at baseline, resulted in robust Th2 priming (Fig. 3, E–G). These data comprehensively show that the immunological machinery to initiate Th2 responses is intact in eosinophil-deficient mice. They also show that eosinophils are not required to induce anaphylaxis, per se. Thus, eosinophils are required for SI Th2 priming, but not baseline intestinal immunity or parenteral Th2 priming.

CD11c+ cells are required for intestinal Th2 priming
Aside from being a potential source of IL-4, there is also evidence that granulocytes such as basophils may supplant DCs in their requirement as APCs to launch Th2 immune responses (Perrigoue et al., 2009; Sokol et al., 2009; Akuthota et al., 2010) and eosinophils have been recognized as potential APCs under certain conditions (Shi et al., 2000; Akuthota et al., 2010). CD11c-diphtheria toxin receptor (DTR) transgenic mice are commonly used to probe the function of DCs in a given process through selective depletion of CD11c+ cells. However, the use of CD11c-targeted strategies (e.g., DTR, GFP/YFP-reporters, or MACS) to analyze the intestinal immune system is potentially complicated by non-DC CD11c+ populations such as GI eosinophils (Carlens et al., 2009; Fig. 1 B). Thus, to clarify whether the CD11c-DTR transgenic system depleted SI eosinophils, DCs, or both, we generated CD11c-DTR...
transgenic → WT BM chimeras, which allow for the depletion of CD11c⁺ cells with repeated injection of DT. Importantly, such chimeras treated with DT lacked SI DCs but not CD11c⁺ eosinophils (Fig. 4, A and B), and this was associated with ∼10–100-fold higher expression of CD11c (and therefore likely the DTR transgene) by DCs (Fig. 4 C). Depletion of DCs throughout sensitization protected CD11c-DTR → WT chimeras from i.p. PN+CT sensitization and anaphylaxis (Fig. 4, D–F) as indicated by a lack of antigen-specific IgG1 and IgE, IL-4 production, and clinical anaphylactic hypothermia and hemoconcentration. Altogether, these data show that this CD11c-DTR system can be used to selectively deplete intestinal DCs and not CD11c⁺ SI eosinophils. These also show a differential critical contribution of CD11c⁺ DCs and eosinophils to initiating intestinal Th2 priming.

**MLN CD103⁺ DCs, not eosinophils, act as APCs during Th2 priming to oral antigen**

Adaptive immunity is primed in the draining LNs, where naive T cells interact with APCs that have migrated from peripheral sites carrying antigen and are equipped with a particular package of immunological instructions (Ritz et al., 2002). Thus, we further evaluated whether eosinophils or DCs acted as APCs by migrating from the SI to the MLN to activate CD4⁺ T cells. CD103⁺ intestinal migratory DCs are elicited from the SI LP after parenteral LPS i.p. administration (Turnbull et al., 2005;
to oral antigen is unclear, especially given reports that mice deficient in CCR7 signaling mount augmented Th2, IgE, and inflammatory responses in various systems (Grinnan et al., 2006; Kawakami et al., 2012; Moschovakis et al., 2012). We found that CCR7 controlled homing of CD103+ DCs, but not eosinophils, to the MLN and that this was required to drive Th2 responses to i.g. PN+CT (Fig. 5, E and F). Likewise, IRF8 (R249C) mutant mice, which are deficient in the SI CD103+ DC subset because of a block in differentiation (Edelson et al., 2010), were sufficient in eosinophils (Fig. 5 E) and protected from allergic sensitization (Fig. 5 F). These data illustrate a differential contribution of eosinophils and DCs to i.g. priming, whereby antigen-carrying CD103+ DCs are elicited from the SI to the MLN in a CCR7-dependent manner and these

Schulz et al., 2009), and we used this as a positive control in our experiments. In response to PN+CT, CD103+ DCs decreased in the SI LP (Fig. 5 A), up-regulated CCR7 (Fig. 5 B), and accumulated within the MLN (Fig. 5 C), suggesting DC migration. Although CD103+ DCs increased within the MLN, eosinophils did not (Fig. 5 C). Similarly, MLN DCs, but not eosinophils, expressed MHC II, CCR7, and the co-stimulatory molecule CD86 (Fig. 5 D). These data suggest that DCs, not eosinophils, are the primary migratory APCs in this system.

Under basal conditions, SI CD103+ DCs preferentially capture ingested food antigens and then home to the draining MLN in a CCR7-dependent fashion to induce oral tolerance and T regulatory cells (Worbs et al., 2006; Schulz et al., 2009). Whether similar mechanisms take place during Th2 priming
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in LP eosinophils in vivo, including the development of cytoplasmic protrusions and cell membrane ruffling (Fig. 6 E, top row), marked decreases in granule density (Fig. 6 E, top row) and the development of degranulation chambers, a sign of exocytosis (Fig. 6 E, top right). We also observed membrane-bound granules and semidissolved granules amid extracellular cell debris (Fig. 6 E, bottom left). Together, these data illustrate that Th2 priming with PN+CT induces eosinophil degranulation by means of both active granule release and eosinophil cytosis (Fig. 6 E). Notably, several granules lost their electron-lucent matrix (Fig. 6 E, bottom right), which contains the eosinophil-specific granule protein, EPO. To test if degranulation could result from direct encounter with allergen, eosinophils were activated in vitro with lyso-platelet–activating factor (L-PAF) as a positive control (Dyer et al., 2010), or PN+CT. Similar to L-PAF, PN+CT induced release of EPO (Fig. 6 F). Thus, eosinophils control CD103+ DC migration to the MLN in response to i.g. PN+CT, and this is associated with degranulation of EPO in vitro and in vivo.

EPO activates mouse and human DCs

EPO is a peroxidase enzyme that oxidizes substrates through catalysis of hydrogen peroxide (H2O2). EPO-mediated oxidation of proteins generates 3-nitrotyrosine (3-NT; Duguet et al., 2001). As expected (Ain et al., 2002), recombinant EPO oxidative activity is abolished upon heat-induced denaturation, direct enzyme inhibition by resorcinol, or if H2O2 substrate is depleted with exogenous catalase (Fig. 7 A).

To test if EPO could directly activate mouse DCs, we generated BMDCs and incubated them with media, increasing migratory DCs act as a critically required APC to initiate adaptive Th2 immunity.

Eosinophil control of DC activation and migration is associated with degranulation in vitro and in vivo

That ablation of either eosinophils or CD103+ DCs prevented the induction of Th2 responses suggested that these two cell types acted in series rather than in parallel. The capability to induce adaptive immunity by nonoral immunization routes (Fig. 3) suggested that the defect in immunizing ΔdblGATA1 mice i.e. was upstream of T or B cell activation and differentiation. Consistent with this reasoning, we found that eosinophils were required for CD103+ DC mobilization to the MLN in response to i.g. PN+CT (Fig. 6 A). Although CD103+ DCs were comparable in their baseline activation and expression of CCR7 and CD86, only CD103+ DCs from WT or eosinophil-reconstituted ΔdblGATA1 mice up-regulated CCR7 and CD86 (Fig. 6 A) and emigrated from the LP in response to intestinal priming with PN+CT (Fig. 6 B). Likewise, activated CD103+ DCs accumulated in the MLN of WT, but not eosinophil-deficient mice, and this was corrected with eosinophil reconstitution (Fig. 6 C). The ability to mobilize SI DCs was not inherently disabled in eosinophil-deficient mice because CD103+ DCs migrated to the MLN in response to i.p. LPS (Fig. 6 C).

To better understand how eosinophils control DC activation and migration, we undertook ultrastructural analysis of intestinal eosinophils within 24 h after i.g. PN+CT priming. Electron microscopy revealed that compared with normal SI eosinophils (Fig. 6 D) i.g. PN+CT caused marked morphological changes in LP eosinophils in vivo, including the development of cytoplasmic protrusions and cell membrane ruffling (Fig. 6 E, top row), marked decreases in granule density (Fig. 6 E, top row) and the development of degranulation chambers, a sign of exocytosis (Fig. 6 E, top right). We also observed membrane-bound granules and semidissolved granules amid extracellular cell debris (Fig. 6 E, bottom left). Together, these data illustrate that Th2 priming with PN+CT induces eosinophil degranulation.

Figure 4. CD11c-DTR → WT chimeras show that DCs are required for induction of Th2 immunity to oral antigen. CD11c-DTR → WT chimeras received i.p. PBS or DT 24h before each i.g. PN+CT, weekly, for four weeks. (A and B) Depletion of SI DCs but not eosinophils. (C) Comparison of CD11c MFI ± SD between SI eosinophils and CD103+ DCs. (D) Serum PN-specific PN-IgE at week 5. (E) Clinical anaphylaxis assessment of hypothermia and hemoconcentration after i.p. challenge at week 5. (F) Th2 cytokine production from PN-stimulated splenocytes. Mean ± SEM unless otherwise stated; n = 5–10 from 2 experiments. *, P < 0.05 versus (B and F) -DT or (C) DCs. ns, not statistically different.
Th2 responses through modulation of DC IL-12 production. To accomplish this, mouse DCs were conditioned with media, EPO, or CT as a positive control (Braun et al., 1999; Chu et al., 2013) before activation with LPS. Compared with mock conditioning, EPO significantly inhibited DC IL-12p40 production (Fig. 7 D). Thus, EPO directly activates mouse DC co-stimulatory molecule expression and proinflammatory cytokine production through its enzymatic activity, while also suppressing IL-12p40 production.

We next tested if this pathway was conserved in humans by incubating EPO with monocyte-derived DCs from healthy adult donors. EPO consistently activated human DCs at concentrations of EPO, or a replication-deficient vesicular stomatitis virus (VSV) as a positive control (Boudreau et al., 2009). Compared with media controls, mouse DCs stimulated with EPO expressed higher levels of CD80, CD86, CCR7, and OX40L (Fig. 7 B), a co-stimulatory molecule that we have previously shown to be critical for driving intestinal Th2 immunization (Chu et al., 2013; Chu et al., 2014a). This effect was inhibited by heat-inactivation of EPO or co-incubation of EPO with resorcinol (Fig. 7, A and B). Consistent with this, DCs activated with EPO secreted high levels of IL-6 (Fig. 7 C) and TNF (not depicted), and this was inhibited by resorcinol or heat inactivation. We also tested if EPO selectively promoted Th2 responses through modulation of DC IL-12 production. To accomplish this, mouse DCs were conditioned with media, EPO, or CT as a positive control (Braun et al., 1999; Chu et al., 2013) before activation with LPS. Compared with mock conditioning, EPO significantly inhibited DC IL-12p40 production (Fig. 7 D). Thus, EPO directly activates mouse DC co-stimulatory molecule expression and proinflammatory cytokine production through its enzymatic activity, while also suppressing IL-12p40 production.

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We next tested if this EPO–DC pathway was important for intestinal priming in vivo. In response to i.g. PN+CT, EPO-deficient mice phenocopied ΔdblGATA1 mice. Indeed, a lack of EPO resulted in the inability of DCs to be mobilized from the SI LP (Fig. 8, A and B) to the MLN (Fig. 8 C) and become activated, including up-regulation of CCR7, CD86, and OX40L (Fig. 8 D). As it would then be expected, EPO deficiency resulted in a lack of induction of antigen-specific IgE (Fig. 8 E) and full protection from anaphylaxis (Fig. 8 F), peritoneal delayed-type hypersensitivity eosinophilic inflammation (Fig. 8 G), and Th2 cytokine production (Fig. 8 H).

Similar to eosinophil-deficient mice, parenteral immunization did not rely on EPO for DC mobilization and activation, IgE or cytokine production, or induction of allergy and in a dose-dependent manner, and this was inhibited by heat inactivation or resorcinol inhibition (Fig. 7 E). An end-product of EPO activity is the nitration of proteins, most readily detected as 3-Nitrotyrosine (Duguet et al., 2001; Ulrich et al., 2008), and we questioned if 3-Nitrotyrosine mediated the activation of DCs by EPO. However, 3-Nitrotyrosine failed to stimulate human DCs over media controls (Fig. 7 E), suggesting that EPO activates DCs independently of 3-Nitrotyrosine generation. Similar to mouse DCs, human DCs activated with EPO also secreted high levels of IL-6 (Fig. 7 F) and TNF (not depicted). Lastly, we evaluated potential toxicity of EPO-mediated activation of DCs. Notably, cell viability was not impaired after incubation of mouse and human DCs with EPO (Fig. 7 G). Thus, EPO can be a nontoxic direct activator of multiple DCs parameters through its oxidant enzyme activity in both mice and humans.

Figure 6. Eosinophil control of CD103+ DC migration and activation is associated with degranulation in vitro and in vivo.

WT, ΔdblGATA1 (GATA), or eosinophil–reconstituted (GATA+Eos) mice were kept naive or received PN+CT i.g. daily for three consecutive days, or i.p. LPS 16 h before analysis. (A) Expression of CCR7 and CD86 on SI DCs. (B) LP DC cell number. (C) Proportion, number, and activation status of CD103+ DCs in MLN. (D, top and bottom) Normal SI eosinophil transmission electron microscopy ultrastructure, showing bi-lobed nuclei and a high density of granules composed of an electron-dense core surrounded by an electron-lucent matrix rich in EPO (arrowhead). (E, top left) PN+CT-stimulated intact eosinophil with marked membrane protrusions and ruffling with loss of granule density. (E, top right) Formation of degranulation chambers (arrowheads). (E, bottom left) Extracellular free granules amid apoptotic/necrotic eosinophil debris (arrowheads). (E, bottom right) Dissolution of eosinophil granule matrix. (F) Purified eosinophils were incubated for 24 h with L-PAF or increasing amounts of PN+CT and assayed for EPO activity. Mean ± SEM, n = 3–8 from 2–4 experiments. *, P < 0.05 versus WT. ns, not statistically different versus WT.
inflammatory cellular responses, including total cells, eosinophils, DCs, and Th2 cells (Fig. 10 E). Both IL-5 and IL-13 were elevated in the lungs of mice immunized with OVA+EPO compared with those immunized with OVA alone (Fig. 10 F). Consistent with this, histological analysis of lungs revealed greater cellular inflammation and goblet cell hypertrophy and hyperplasia (Fig. 10 G). These enhanced responses were maintained during recall cytokine production, suggesting enhanced priming in the OVA+EPO group over those immunized with OVA alone. Thus, although EPO is necessary for enteral, but not parenteral, immune priming, these data show that EPO is sufficient for extraintestinal DC activation and promotion of adaptive immunity in vivo.

DISCUSSION
Recruited eosinophil granulocytes are a hallmark of Th2-mediated immune responses. However, in some tissue, particularly in the SI, eosinophils are constitutively present. In contrast to the conventional view of eosinophils as effector cells during anaphylaxis (Fig. 9), thus, DCs rely on eosinophil cues, at least in the form of EPO, to become activated and migrate to the MLN to initiate intestinal adaptive immunity in vivo.

EPO promotes extraintestinal DC activation and adaptive immunity
Lastly, we evaluated whether EPO-mediated DC activation could be functional outside of the intestine in vivo. In response to the administration of EPO s.c. or i.p., DCs became activated and migrated to draining LNs (Fig. 10, A–C), suggesting that EPO could promote extraintestinal innate-to-adaptive immune activation.

To test if EPO could act as an adjuvant in vivo, we used a standard model involving i.p. immunization, followed by recall i.n. challenge to induce airway inflammation. Compared with i.p. OVA alone, i.p. combination OVA with EPO (OVA+EPO) induced enhanced levels of OVA-specific IgG1 and IgE (Fig. 10 D). Further, when these mice were challenged with OVA i.n., OVA+EPO-immunized mice mounted greater inflammatory cellular responses, including total cells, eosinophils, DCs, and Th2 cells (Fig. 10 E). Both IL-5 and IL-13 were elevated in the lungs of mice immunized with OVA+EPO compared with those immunized with OVA alone (Fig. 10 F). Consistent with this, histological analysis of lungs revealed greater cellular inflammation and goblet cell hypertrophy and hyperplasia (Fig. 10 G). These enhanced responses were maintained during recall cytokine production, suggesting enhanced priming in the OVA+EPO group over those immunized with OVA alone. Thus, although EPO is necessary for enteral, but not parenteral, immune priming, these data show that EPO is sufficient for extraintestinal DC activation and promotion of adaptive immunity in vivo.
Th2 responses, here we show that intestinal eosinophils play a critical role in the initiation of Th2 immunity with direct relevance to the development of food allergy. Eosinophil activation during intestinal immunization results in the release of EPO that triggers CD103+ DC activation and CCR7-dependent migration to the MLN, where DC CD86 and OX40L then control induction of Th2 responses. In support of these data, human intestinal eosinophils also degranulate during infection with CT-producing *Vibrio cholera* (Qadri et al., 2004), patients with food allergy exhibit increased intestinal eosinophil activation and degranulation compared to non-allergic controls (Vandeza et al., 1999; Schwab et al., 2003), and in vitro co-cultures of human DCs and eosinophils result in enhanced DC activation to CpG-ODN stimulation (Lotfi and Lotze, 2008). Indeed, here we also show that EPO activates mouse and human DCs through its enzymatic activity, and this pathway is sufficient for EPO to act as an adjuvant in vivo. Thus, eosinophil-granule-DC shaping of adaptive immune responses may represent a therapeutic target in food allergy, and may also be a useful adjuvant or immunomodulator for other diseases.

The healthy intestinal tract has long been recognized as the most abundant site of normally resident eosinophils. Histological analyses of genetically deficient mice have shown that this baseline eosinophilia is partially dependent on eotaxin (Mishra et al., 1999) and independent of β7-integrin (Artis et al., 2000). More recently, Carlens et al. (2009) developed an eosinophil-sensitive intestinal isolation and flow cytometry method. They showed that SI eosinophils have a markedly longer lifespan compared with circulating eosinophils, and that regulation of cell survival rather than constant influx of newly generated eosinophils likely determines baseline SI eosinophilia (Carlens et al., 2009). Although additional factors that regulate homeostatic intestinal eosinophilia remain to be determined, herein we sought to better define the number of phenotype along the intestinal tract. We found that the SI, particularly the duodenum and jejunum, is enriched in eosinophils compared with the LI. Further, SI and LI eosinophils differed in expression of ST2, CD69, Ly6C, and CD11c. That CD11c is correlated with eosinophil longevity (Carlens et al., 2009) suggests that LI eosinophils may have shorter lives and hence, rely more on influx from the blood to maintain homeostatic tissue levels. This further supports the notion that SI and LI eosinophils may differ phenotypically and functionally. Indeed, priming through tissues with little to no baseline eosinophilia, such as the skin, peritoneum, or rectum, resulted in unimpaired Th2 priming in eosinophil deficient mice, which is consistent with
reports of largely intact Th2 responses to the helminths Schistosoma mansoni (Swartz et al., 2006), Nippostrongylus brasiliensis (Knott et al., 2007), or Trichuris muris (Svensson et al., 2011) in the absence of eosinophils. Nevertheless, it remains to be tested whether the eosinophil–DC activation pathway identified here might also be operative in other tissues with abundant indigenous eosinophil populations, such as the uterus or thymus, or those in a pathological (hyper)eosinophilic state.

Aside from their classical cytotoxic/destructive inflammatory functions, eosinophils have more recently been ascribed various immunomodulatory and tissue remodeling activities (Rosenberg et al., 2013; Wong et al., 2014). Notably, several of these reports stem from in vitro work or data on eosinophils infiltrating helminth infected or allergen exposed tissues such as the lung, skin, or peritoneum. First, direct APC activity for T cell priming has been shown for eosinophils after they were Ag-pulsed in vitro, and then placed in co-culture or transferred to otherwise naive mice (Shi et al., 2000; Akuthota et al., 2010). Second, eosinophils have been shown to express IL-4 (Lacy and Moqbel, 2000; Shinkai et al., 2002; Voehringer et al., 2004; Svensson et al., 2011), and as the initial source of IL-4 required for naive CD4+ T to Th2 differentiation remains contentious, eosinophils have been speculated to be a potential candidate for this. In support of these data, eosinophils have long been recognized to localize to the T cell zone of LNs (Litt, 1964). Here, we found that intestinal LN eosinophils did not express MHC II, CCR7, or CD86 or increase in number under naive or immunized conditions in vivo and, further, that selective deletion of IL-4 in eosinophils did not impair the induction of Th2–cytokine mediated immunity. The defect in intestinal immunization of eosinophil-deficient mice was instead more proximal to the level of T or B cell activation as these mice could fully mount cellular and humoral Th2 responses to priming through the lung (Fattouh et al., 2011), skin, or peritoneum. These data highlight novel and essential contributions of indigenous eosinophils to shaping adaptive immunity. During the review of our article, a study was published that suggests a role for eosinophils in regulating B cells, particularly intestinal IgA (Chu et al., 2014b). These and our data also raise questions as to how eosinophils might regulate commensal microbes and vice versa.

Tolerance and immunity are viewed as opposing outcomes after intestinal antigen encounter with the immune system (Pabst and Mowat, 2012). DCs are also thought to be central to this process, with antigen-bearing CD103+ DCs migrating in a CCR7-dependent manner to the MLN to induce T regulatory cells. It is less clear how Th2 responses are induced. Indeed, Th2 responses to the intestinal helminth T. muris require basophils to act as APCs instead of DCs (Perrigoue et al., 2009). Although we have previously shown that basophils are not required for Th2 priming to oral antigen (Chu et al., 2013), we questioned the importance of DCs in this system. Indeed, although Fahlen-Yrlid et al. (2009) showed that CD11c-DTR–mediated depletion of CD11c+ cells prevented OVA+CT–induced IgG and IgA titers, it remained unclear to what extent this was due to depletion of DCs or eosinophils and whether or not this truly impacted Th2 immunity. Here, we clarify that at least this CD11c-DTR system can selectively deplete DCs.
Eosinophils and EPO induce adaptive immunity via DCs | Chu et al.

We found that EPO oxidant enzymatic activity induced mouse and human DCs to up-regulate multiple co-stimulatory molecules, produce cytokines such as IL-6, and suppress IL-12p40 expression, without inducing toxicity. Although a hallmark end-product of EPO activity is production of 3-NT, stimulation of DCs with this failed to mimic the effects of EPO. Nonetheless, these data fortify the notion that DCs may heavily rely upon signals from the microenvironment, now including eosinophil cues, to initiate specific classes of adaptive immunity. Consistent with this, we found that eosinophil deficiency prevented Th2 induction, but did not impact T regulatory cell-mediated oral tolerance. Thus, the initiation and articulation of adaptive immunity is not only determined by direct DC sensing of antigen, but over eosinophils, likely due to the 10–100-fold higher expression of CD11c on DCs and transgenic system setup. We further show that ablation of oral tolerance-associated CD103+ DCs or CCR7 prevented Th2 immunization. Thus, these data identify DCs as being critical for Th2 immunity in this system and further illustrate the potential Janus nature of intestinal CD103+ DCs as inducers of either tolerance or immunity to oral antigen (Laffont et al., 2010).

Granulocyte-DC cooperation has been reported in pain-induced Th2 responses, with antigen-presenting DCs cooperating with IL-4+ basophils during T cell priming (Tang et al., 2010). Herein, we identify a different level of granulocyte-DC cooperation: eosinophils and EPO mediate activation of DCs, and these activated DCs then migrate to the LN to prime T cells. We found that EPO oxidant enzymatic activity induced mouse and human DCs to up-regulate multiple co-stimulatory molecules, produce cytokines such as IL-6, and suppress IL-12p40 expression, without inducing toxicity. Although a hallmark end-product of EPO activity is production of 3-NT, stimulation of DCs with this failed to mimic the effects of EPO. Nonetheless, these data fortify the notion that DCs may heavily rely upon signals from the microenvironment, now including eosinophil cues, to initiate specific classes of adaptive immunity. Consistent with this, we found that eosinophil deficiency prevented Th2 induction, but did not impact T regulatory cell-mediated oral tolerance. Thus, the initiation and articulation of adaptive immunity is not only determined by direct DC sensing of antigen, but
also by the demographics and activity of the cells neighboring those DCs.

We have analyzed the contribution of eosinophils to multiple parameters of intestinal innate and adaptive immunity and have found that normally resident SI eosinophils initiate primary adaptive immune responses through a pathway conserved in mice and humans that is dependent on EPO oxidant enzymatic activity. These data identify a role for indigenous eosinophil populations in influencing the initiation of adaptive immunity, as well as a Th2 induction pathway whereby the hallmark effector cell type also acts as an incipient early inducer of the very same immune cascade. That we identify a molecular link between food, eosinophils, DCs and T cell priming, suggests that eosinophil activation in the intestine may represent a primitive digestive-immune mechanism that has been coopted during evolution to aid in the initiation of adaptive immunity. Indeed, DC activation required degradation of the microbi-cidal peroxidase EPO, consistent with an innate defense role for intestinal eosinophils (Yousefi et al., 2008). While this pathway is necessary to promote adaptive immunity in the SI, it is can be fully instigated extraintestinally. Therefore, the identification of a mechanism that shapes adaptive immunity through cooperative eosinophil-DC interaction should broadly encourage investigation into the role of eosinophils, EPO, or other eosinophil products in the (patho)genesis or treatment of immune mediated processes such as chronic inflammation, allergy, autoimmunity, cancer, vaccines and infectious disease.

MATERIALS AND METHODS

Mice. Age-, sex-, vendor-, and strain-matched controls were used in all experiments. AbiGATA1 (C57Bl/6-Tg(CAG-CAT)10Hew) and CD11c-DTR (B6.FVB-Tg(R249C)1Rfor/J, IRF8(R249C); BXH2/Tfj), and EPO-deficient (Duguid et al., 2001; NZW/Lacj) mice were purchased from The Jackson Laboratory. IL-5 transgenic (IL-5Tg) mice were bred in-house. IL-4 KO (BALB/c-Il4tm2Nnt/J; Jung et al., 2002) mice were bred in-house. IL-4 KO animals were obtained from Charles River. All procedures were approved by the McMaster University Research Ethics Board.

Intestinal cell isolation. Intestines were opened longitudinally, cut into 2–3 mm pieces and incubated in 1 mM DTT (Sigma-Aldrich) in PBS for 15 min, and then 3 rounds of 15 min in 10% FBS in PBS and 2 mM EDTA before digestion in 0.239 mg/ml Collagenase A (Roche) with DNase (Roche) for 50–60 mm and 40/70% Percoll (GE Healthcare) separation (Carlens et al., 2009).

Flow cytometry. In all assays, cells were incubated with anti-FcγRII/IIIb before incubation with fluorochrome-conjugated antibodies. Dead cells were excluded by propidium iodide (PI) uptake or fixable viability dye eFlour780 (eBioscience), and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets. Whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and gated on singlets.

Cytokine production. 800,000 live splenocytes were cultured for 120 h in complete RPMI alone or supplemented with 250 µg/ml CPE and cytokines in cell-free supernatants were quantified using LumineX (Millipore) or ELISA (R&D Systems).

Bone marrow chimeras. Mice irradiated twice with 5.5 Gy (Ct15 source) received 5 × 106 T cell depleted BM cells i.v., rested for 8 wk, and then were used in experiments. 100 µg DT was injected i.p. 24 h before each gavage for CD11c-DTR experiments. For mixed BM chimeras, i.p. injections were composed of 80% WT or AbiGATA1 BM mixed with 20% IL-4 KO BM.

LN processing. LNs were triturated between frosted slides in HBSS, washed and filtered (40 µm).

Transmission electron microscopy. Immediately after excision, tissues were immersed in fixative consisting of 3% formaldehyde and 1% glutaraldehyde in 0.1-M phosphate buffer (pH 7.2). After the initial fixation, samples were post-fixed in 1% osmium tetroxide for 1 h, dehydrated in graded acetone solutions, and embedded in Polybed 812 (Polysciences, Inc.). Ultrathin sections (60–80 nm) were cut on an LKB MK III ultratome and routinely contrasted with uranyl acetate and lead citrate. The sections were examined using a FEI Tecnai Spirit BioTWIN transmission electron microscope (FEI).

EPO assay. With minor modifications from previously described EPO assays (Humbles et al., 2002; Dyer et al., 2010), eosinophils were isolated as described above, and incubated for 24 h with media, L-PAF (Sigma-Aldrich), and 1 or 10 µg/ml PN+CT and assayed for EPO activity by oxidation of o-phenylenediamine (Sigma-Aldrich), stopped with sulfuric acid and absorbance measured at 492 nm. Results were normalized to maximal activity from Triton-X 100 lysed eosinophils. EPO was heat denatured by incubation at 90°C for 15–60 min and inhibited with resorcinol or catalase (both Sigma-Aldrich).
Mouse BM-derived DCs (BMDCs) generation, stimulation, and conditioning. As previously reported (Chu et al., 2013), GM-CSF–derived BMDCs were generated and then incubated for 24 h with media, increasing concentrations of EPO with or without resorcinol, heat-inactivated EPO, or 25 multiplicity of infection VSV-ΔM51, which transduces DCs without significant progeny virus production or effect on viability. Some DCs were conditioned for 16 h with media, EPO, or 10 µg/ml CT before stimulation for 12 h with or without 10 ng/ml E. coli 0111:B4 LPS (Cell culture tested; Sigma–Aldrich). For intracellular cytokine staining, DCs were incubated with 10 µg/ml brefeldin A (eBioscience) for the last 6 h. Supernatants were analyzed for IL-6 and TNF by duoset ELISA (R&D).

Generation of human monocye-derived DCs (MoDCs) and immunophenotyping. Peripheral blood was obtained from adult healthy human donors after informed consent and approved by the McMaster Research Ethics Board. PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation using standard techniques and monocytes were enriched by adherence. Monocytes were differentiated for 8 d in X-VIVO 10 media (Lonza), supplemented with 5% pooled human AB serum (Corning) and 50 ng/ml IL-4 and GM-CSF (BioLegend), replacing with fresh media on day 4.

For immunophenotyping, the following conjugated antibodies were used: CD303-FITC, OX40L-PE (BD); CD1c-Brilliant Violet 421, CD14-Pacific Blue or -Brilliant Violet 605, CD3-Alexa Fluor 700, CD19-Alexa Fluor 700, CD56-Alexa Fluor 700, CD40-APC Cy7, CD80-PE Cy7, CD83-APC, CD86-Brilliant Violet 510, HLA-DR-PerCp Cy5.5, CCR7-PerCp eFluor710 (BioLegend); CD209-FITC (ABD Serotec). Blood DCs were defined as: lineage negative (CD3, CD19, CD56), HLA-DR positive, CD14 negative, and CD1c (myeloid DCs) or CD303 positive (plasmacytoid DCs). MoDCs were defined as CD14 low or negative and CD209 positive.

All stimulations were performed in X-VIVO 10 media with 5% human AB serum for 48 h. Some MoDCs were stimulated with 100 µM 3-NT (Eiserich et al., 1999; Sigma–Aldrich). MoDCs were stimulated at 5.0–7.5 × 10^6 cells/ml, whereas PBMCs were stimulated at 4.0 × 10^6 cells/ml. Cytokine secretion was measured using Ready-Set Go ELISAs (eBioscience) according to manufacturer’s instructions.

Airway inflammation model. Mice were immunized i.p. with 100 µg OVA with or without 5 µg EPO on day 0 and 14, before i.n. challenge on days 28–30 and tissue collection on day 31. Lungs were homogenized in 500 µl PBS, incubated with 1% Triton-X 100 for 1 h under constant agitation, centrifuged at 13,000 rpm for 10 min and stored at −20°C. Bronchoalveolar lavage (BAL) was performed with sequential instillation and recovery of 300 and 200 µl PBS, respectively, before being spun at 2,000 rpm for 10 min, supernatants stored at 400 µl PBS, respectively, before being spun at 2,000 rpm for 10 min, supernatants stored at 20°C and cell pellets resuspended in 150 µl PBS and counted with Turks. Spleens were stimulated with 40 µg/ml OVA for 5 d (Fattouh et al., 2005). Cytokines were analyzed by Luminex.

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D.K. Chu designed and performed experiments, analyzed data and wrote the manuscript. T.D. Walker, S. Boncharova, A. Ulop-Guevara, N.G. Barra, J.D. Bassett, J. Kong, M.E. Gordon, R. Jimenez-Saiz, R. Fatouh, and P. Shen performed experiments. C.P. Verschoor performed and analyzed human DC experiments. J.S. Erjefält performed TEM experiments and analysis. R. Fatouh, R. Kolbeck, A.A. Humbles, K.D. McCoy, D.M. Bowdish and O. Palit provided reagents and input. S. Waserman and M. Jordan obtained funding. M. Jordan oversaw the project and edited the manuscript.

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