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Commensal bacterial–derived signals regulate basophil hematopoiesis and allergic inflammation

David A. Hill[1,2,3], **Mark C. Siracusa**[1,2,3], **Michael C. Abt**[1,2,3], **Brian S. Kim**[1,2,3], **Dmytro Kobuley**[1,2,3], **Masato Kubo**[4,5], **Taku Kambayashi**[6], **David F. LaRosa**[7], **Ellen D. Renner**[8], **Jordan S. Orange**[9], **Frederic D. Bushman**[1], and **David Artis**[1,2,3]

[1]Department of Microbiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[2]Institute for Immunology, Perelman School of Medicine, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[3]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[4]Laboratory for Signal Network, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Yokohama 230–0045

[5]Division of Molecular Pathology, Research Institute for Biological Science, Tokyo University of Science, Chiba 278–0022, Japan

[6]Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[7]Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

[8]University Children's Hospital, Dr. von Haunersches Kinderspital, Ludwig Maximilians University, Munich 80337, Germany

[9]Division of Immunology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA

Abstract

Commensal bacteria that colonize mammalian barrier surfaces are reported to influence T helper type 2 (T_H2) cytokine–dependent inflammation and susceptibility to allergic disease, although the mechanisms that underlie these observations are poorly understood. In this report, we identify that deliberate alteration of commensal bacterial populations via oral antibiotic treatment resulted in

CORRESPONDENCE: David Artis, Department of Microbiology, Institute for Immunology, Perelman School of Medicine, University of Pennsylvania, 421 Curie Boulevard, BRB II/III, room 356, Philadelphia, PA, 19104–6160, USA, dartis@mail.med.upenn.edu.

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elevated serum immunoglobulin E (IgE) levels, increased steady–state circulating basophil populations, and exaggerated basophil–mediated T_H2 cell responses and allergic inflammation. Elevated serum IgE levels correlated with increased circulating basophil populations in mice and subjects with hyperimmunoglobulinemia E syndrome. Furthermore, B cell–intrinsic expression of MyD88 was required to limit serum IgE levels and circulating basophil populations in mice. Commensal–derived signals were found to influence basophil development by limiting proliferation of bone marrow–resident precursor populations. Collectively, these results identify a previously unrecognized pathway through which commensal–derived signals influence basophil hematopoiesis and susceptibility to T_H2 cytokine–dependent inflammation and allergic disease.

Allergic diseases have reached pandemic levels[1] and represent a significant source of morbidity, mortality and healthcare cost[2]. These chronic inflammatory diseases are characterized by interleukin (IL) –4, IL–5, IL–9 and IL–13 production by $CD4^+$ T helper type 2 (T_H2) cells, immunoglobulin E (IgE) production, and the recruitment of effector cells to sites of tissue inflammation[3, 4]. It is thought that susceptibility to T_H2 cytokine–dependent allergic inflammation is influenced by both polymorphisms in mammalian genes[5] as well as environmental factors including diet and exposure to pollutants or infectious agents[6-8]. However, the specific genetic and environmental stimuli that influence allergy susceptibility, and how these factors contribute to the development of allergic disease are ongoing fields of study.

The human intestine is colonized by 100 trillion microorganisms belonging to each of the three domains of life[9]. Of these, bacteria are the most abundant; the colon is home to trillions of commensal bacteria[10] with a diversity of at least 1,000–15,000 species[11]. Epidemiologic studies have identified associations between alterations in the composition of commensal bacterial communities and the development of allergic disease. For example, infants who develop allergies display altered commensal populations early in life[12], and children who have undergone treatment with broad–spectrum antibiotics are at an increased risk of developing allergic diseases[13, 14]. Studies in animal model systems have further implicated commensal–derived signals in influencing the development of T_H2 cytokine–mediated allergic inflammation[15-18]. However, the mechanisms through which the innate immune system recognizes commensal–derived signals and regulates T_H2 cytokine responses remain poorly characterized[19].

Here, oral delivery of broad–spectrum antibiotics was employed to interrogate the influence of commensal bacterial–derived signals on innate cell populations that contribute to the development of T_H2 cytokine–dependent allergic inflammation. Depletion or deletion of bacterial communities was associated with elevated serum IgE levels, increased circulating basophil populations, and exaggerated T_H2 cell responses and allergic inflammation. Exaggerated T_H2 cell responses were reduced upon depletion of basophils, implicating this cell type in contributing to the exaggerated allergic inflammation observed in antibiotic–treated mice. IgE was found to be a critical regulator of steady–state basophil responses in mice, and subjects with hyperimmunoglobulinemia E syndrome had elevated frequencies of circulating basophils compared to controls. Additionally, B cell–intrinsic expression of MyD88 was required to limit serum IgE levels and circulating basophil populations in mice.

Finally, commensal–derived signals influenced circulating basophil populations by regulating the proliferative capacity of bone marrow–resident basophil progenitor populations. Together, these findings provide therapeutically relevant insights into the molecular and cellular mechanisms through which commensal bacterial–derived signals influence the development of T_H2 cytokine–dependent inflammation and susceptibility to allergic diseases.

RESULTS

Higher serum IgE levels and elevated circulating basophil populations following antibiotic–mediated manipulations of commensal bacteria in mice

Oral antibiotic treatment (ABX) resulted in quantitative and qualitative alterations to commensal bacteria colonizing the murine intestine including reductions in bacteria of the Firmicutes and Bacteroidetes phyla (Supplementary Fig. 1a,b) and significant increases in serum IgE levels[20, 21] (Fig. 1a). As IgE is reported to influence granulocyte homeostasis[22], we investigated whether ABX–induced elevations in IgE were associated with alterations in the frequency or number of circulating mast cells, eosinophils or basophils. ABX–treatment did not alter blood mast cell (Supplementary Fig. 2a,b) or eosinophil (Supplementary Fig. 2c,d) populations. However, frequencies and numbers of basophils (identified as non–B, non–T (NBNT), $CD117^-$, $CD49b^+$, $Fc\varepsilon RI\alpha^+$) were significantly increased in the blood (Fig. 1b,c) and spleen (Supplementary Fig. 3a,b) of ABX–treated compared to conventionally (CNV)–reared mice. Basophils from ABX–treated mice displayed increased levels of surface–bound IgE compared to controls (Fig. 1d), while expression of other surface markers (CD69, CD123, CD200R, FcεRIα, FcγR, Gr1) were unaltered (Supplementary Fig. 3c).

ABX treatment does not eliminate all commensal bacteria[23]. Therefore, to investigate whether steady–state levels of IgE or basophil populations were altered in the absence of all live microbial stimuli, we employed germ–free (GF) mice[24]. Consistent with the effects of ABX treatment, compared to CNV–reared controls, GF mice exhibited increased serum IgE levels (Fig. 1e), increased frequencies and numbers of basophils in the blood (Fig. 1f,g) and spleen (Supplementary Fig. 3d,e), and increased basophil–surface–bound IgE levels (Fig. 1h). Consistent with commensal–derived signals being sufficient to limit serum IgE levels and circulating basophil populations, conventionalization of GF mice resulted in reductions in serum IgE levels (Supplementary Fig. 3f) and blood and spleen basophil populations (Supplementary Fig. 3g,h). Collectively, these data indicate that commensal bacterial–derived signals limit serum IgE levels and circulating basophil populations in the steady–state.

Commensal bacteria influence basophil–mediated allergic inflammation

Given the role for commensal–derived signals in limiting circulating numbers of basophils, we hypothesized that antibiotic–treated mice might display exaggerated inflammation in models of basophil–associated allergic disease. It has previously been shown that basophils contribute to the inflammation that results from inhalation of house dust mite allergen (HDM)[25]. To test whether commensal–derived signals influenced the development of

HDM–induced inflammation, we exposed CNV–reared or ABX–treated IL–4/eGFP reporter mice[26] to HDM and examined innate cell responses, T_H2 cell responses, and lung inflammation. Compared to CNV–reared mice, HDM exposure in ABX–treated mice resulted in increased circulating basophil responses in the blood (Supplementary Fig. 4a), exaggerated T_H2 cell responses in the draining mediastinal lymph node (LN) (Supplementary Fig. 4b), increased LN cell–derived IL–4 and IL–13 responses (Supplementary Fig. 4c), and increased lung and bronchoalveolar lavage (BAL) eosinophil responses (Supplementary Fig. 4d). Concurrently, compared to CNV–reared controls, ABX–treated mice displayed increased HDM–elicited inflammation characterized by exaggerated alveolar infiltrates (Fig. 2a). Together, these findings indicate that commensal–derived signals limit allergic inflammation in the lung.

Basophils are recruited to draining LNs early in the response to infectious[27] or allergic[28, 29] stimuli where they may cooperate with dendritic cells (DCs) to promote optimal T_H2 cell responses[27, 29-31]. To test whether ABX–treated mice exhibited altered allergen–induced T_H2 cell responses, we exposed CNV–reared or ABX–treated IL–4/eGFP reporter mice to PBS or papain, a cysteine protease associated with occupational allergy in humans[32] and T_H2 cell responses in mice[28]. Following PBS–exposure, similar frequencies of basophils (Fig. 2b) and IL–4/eGFP$^+$CD4$^+$ T_H2 cells (Fig. 2c) were observed in the popliteal LNs of CNV–reared or ABX–treated mice. However, following exposure to papain, ABX–treated mice exhibited increased frequencies of LN basophils (Fig. 2d) and IL–4/eGFP$^+$CD4$^+$ T_H2 cells (Fig. 2e) compared to CNV–reared controls. Depletion of basophils by administration of anti–FcεRIα antibody[28] resulted in a reduction in the frequency of LN basophils (Fig. 2f), and reduced frequencies (Fig. 2g) and numbers (Fig. 2h) of papain–elicited IL–4/eGFP$^+$CD4$^+$ T_H2 cells, indicating that basophils contribute to the exaggerated T_H2 cell responses observed in ABX–treated mice.

DCs are critically important for the development of optimal T_H2 cell responses to some allergens and helminth parasites[25, 33]. To investigate the influence of commensal–derived signals on steady–state DC populations, we examined DCs in CNV–reared or ABX–treated mice. DC frequencies in the spleen were similar between CNV–reared or ABX–treated mice (data not shown) as was DC expression of MHC–I, MHC–II, CD40, CD80, CD86, and FcεRIα (Supplementary Fig. 5a–c), indicating that commensal–derived signals do not significantly influence splenic DC populations in the steady–state. Non–basophil FcεRIα$^+$ cells are important for the development of inflammatory responses to some allergens[25]. Papain–elicited, non–basophil FcεRIα$^+$ cell frequencies in the LN were similar between CNV–reared or ABX–treated mice (Supplementary Fig. 5d–f) indicating that non–basophil FcεRIα$^+$ cell responses are not significantly influenced by commensal–derived signals in this setting. As FcεRIα$^+$ cells are influenced by anti–FcεRIα antibody treatment[25], we examined the effect of anti–FcεRIα antibody treatment on non–basophil FcεRIα$^+$ cell populations. Compared to controls, anti–FcεRIα antibody treatment did not significantly influence the frequency of papain–elicited, non–basophil FcεRIα$^+$ LN cells (Supplementary Fig. 5d–f). Together, these findings suggest that neither steady–state DC responses nor papain–elicited non–basophil FcεRIα$^+$ cell responses are significantly dysregulated in ABX–treated mice.

To further examine the contributions of basophils to the exaggerated T_H2 cell responses observed in ABX–treated mice, we adopted a loss–of–function approach complementary to anti–FcεRIα antibody treatment by utilizing a basophil–specific diphtheria toxin (DT)–dependent depletion mouse system (BaS–TRECK)[34]. ABX–treatment resulted in increased frequencies of circulating basophils (Supplementary Fig. 5g) and exaggerated frequencies (Supplementary Fig. 5h) and numbers (Supplementary Fig. 5i) of papain–elicited LN T_H2 cells. DT–treatment of CNV-reared or ABX–treated BaS–TRECK mice, but not littermate controls, resulted in efficient depletion of LN basophils (Supplementary Fig. 5g) and reductions in the frequency (Supplementary Fig. 5h) and number (Supplementary Fig. 5i) of T_H2 cells. Together with the results of anti–FcεRIα antibody–mediated basophil depletion, DT receptor–mediated deletion indicates that basophils contribute to the exaggerated allergen–induced T_H2 cell responses observed in ABX–treated mice.

Signals derived from commensal bacteria influence circulating basophil populations via an IgE–dependent mechanism

We next sought to determine the mechanisms by which commensal–derived signals regulate steady–state circulating basophil populations. The epithelial cell–derived cytokine thymic stromal lymphopoietin (TSLP) was recently found to expand circulating basophil populations[34]. To test whether TSLP was required for the elevated serum IgE levels or circulating basophil populations observed in ABX–treated mice, we utilized TSLP–deficient (*Tslp*$^{-/-}$) mice. As in WT mice, antibiotic treatment of *Tslp*$^{-/-}$ mice resulted in elevated serum IgE levels (Supplementary Fig. 6a), and increased circulating basophil frequencies and numbers (Supplementary Fig. 6b,c), indicating that TSLP is not necessary for these responses to commensal alteration.

As IgE regulates mast cell homeostasis[22] and has been hypothesized to have similar roles in basophils[35], we examined the relationship between elevated IgE levels and increased circulating basophil populations in ABX–treated mice. Serum IgE levels significantly correlated with circulating basophil numbers in CNV–reared or ABX–treated mice (Fig. 3a), suggesting that IgE may influence basophil homeostasis. To test whether the increase in circulating basophils observed in ABX–treated mice was dependent on lymphocytes, we employed recombination activating gene 1–deficient mice (*Rag1*$^{-/-}$)[36]. Consistent with a role for IgE in regulating basophil homeostasis, blood basophil frequencies and numbers were similar between CNV–reared and ABX–treated (Fig. 3b,c) or GF *Rag1*$^{-/-}$ mice (Supplementary Fig. 7a,b). As IL–4 is necessary for IgE production[37], we utilized IL–4–deficient mice (*Il4*$^{-/-}$) to test the role of IL–4 in mediating the elevated serum IgE levels and basophil responses observed in ABX–treated mice. While ABX–treatment of wild–type (WT) mice resulted in increased serum IgE levels (Supplementary Fig. 7c) and blood basophils (Supplementary Fig. 7d), IgE was not detected in *Il4*$^{-/-}$ mice (Supplementary Fig. 7c) and ABX–treatment of *Il4*$^{-/-}$ mice did not significantly influence the frequency or number of blood basophils (Supplementary Fig. 7d,e). Together, these results suggest that IL–4–induced IgE production contributes to the increased circulating basophil responses observed in ABX–treated mice.

To directly test the role of IgE in mediating commensal bacterial–dependent regulation of basophil responses, we utilized IgE–deficient (*Igh–7*$^{-/-}$) mice. While ABX–treatment of WT mice resulted in increased serum IgE levels (Fig. 3d) and blood basophils (Fig. 3e,f), IgE was not detected in *Igh–7*$^{-/-}$ mice (Fig. 3d) and blood basophil populations were similar between CNV–reared or ABX–treated *Igh–7*$^{-/-}$ mice (Fig. 3e,f). Together, these results indicate that IgE is necessary to mediate the exaggerated steady–state circulating basophil responses observed in ABX–treated mice. To test whether IgE was sufficient to promote the population expansion of circulating basophils, we treated *Rag1*$^{-/-}$ mice with IgE and examined blood basophil populations. Compared to IgG, transfer of IgE into *Rag1*$^{-/-}$ mice resulted in recovery of serum IgE levels (Fig. 3g) and significantly increased frequencies and numbers of blood basophils (Fig. 3h,i). Collectively, these gain– and loss–of–function approaches identify IgE as one regulator of peripheral basophil responses in mice.

Serum IgE levels correlate with blood basophil frequencies in humans

As IgE was found to regulate circulating basophil populations in mice, we hypothesized that IgE would have a similar role in humans. Subjects with loss–of–function polymorphisms in the dedicator of cytokinesis 8 gene (*DOCK8*−) exhibit an autosomal recessive form of hyperimmunoglobulinemia E syndrome[38, 39] characterized by recurrent infections, increased susceptibility to atopic eczema, and average serum IgE levels 10 times higher than those found in control subjects[38, 39]. Based on the identification of a role for IgE in regulating basophil responses in mice, we hypothesized that elevated serum IgE levels in *DOCK8*− subjects may be associated with increased circulating basophil populations. As previously reported, *DOCK8*− subjects displayed elevated serum IgE levels compared to control subjects[38, 39] (Fig. 4a). In control subjects, human blood basophils (identified as TCRβ^{-}, CD11c^{-}, CD19^{-}, CD117^{-}, CD123^{+}, FcεRIα^{+}) comprised approximately 1% of the NBNT cell compartment (Fig. 4b,d). In contrast, in four of five *DOCK8*− subjects examined, basophils comprised approximately 4% of the NBNT compartment (Fig. 4c,d). Further, basophils isolated from *DOCK8*− subjects exhibited increased surface–bound IgE levels compared to those of control subjects (Fig. 4e). These data are consistent with our findings in animal models and suggest that IgE may influence peripheral basophil homeostasis and the development of basophil–mediated allergic inflammation in humans.

Given the potential role for IgE in regulating circulating basophil populations in mice and human subjects, we hypothesized that therapeutic depletion of serum IgE levels may reduce circulating basophil populations. Omalizumab (IgE–specific antibody) is a monoclonal, humanized mouse anti–human IgE–specific antibody and an FDA approved anti–allergy drug[40, 41]. To test whether IgE–specific antibody treatment influenced serum IgE levels or circulating basophil populations, we treated CNV–reared or GF mice with control or IgE–specific antibody and examined serum IgE levels and blood basophil populations. As IgE–specific antibody treatment decreases FcεRIα expression on basophils[42], we identified basophils as NBNT, CD117^{-}, CD49b^{+}, CD200R^{+} cells[43]. IgE–specific antibody treatment of GF mice resulted in reductions in serum IgE levels (Fig. 4f), and reduced frequencies and numbers of blood basophils (Fig. 4g,h). Together, these results identify IgE–specific antibody treatment as a potential therapeutic intervention to limit IgE–mediated increases in circulating basophil populations.

MyD88–dependent signaling in B cells limits serum IgE levels and circulating basophil populations

It has been hypothesized that commensal bacterial–derived signals influence allergic responses by signaling through pattern recognition receptors (PRRs)[15, 17]. To interrogate the molecular mechanisms through which commensal–derived signals influence serum IgE levels and circulating basophil responses, we utilized mice deficient in myeloid differentiation primary response gene 88 (*Myd88*$^{-/-}$) which encodes a critical adaptor molecule that regulates signaling through multiple PRRs[44]. Compared to littermate controls, CNV–reared *Myd88*$^{-/-}$ mice exhibited increased serum IgE levels[44] (Fig. 5a), increased frequencies and numbers of blood basophils (Fig. 5b,c) and increased basophil–surface–bound IgE levels (Fig. 5d) indicating that MyD88 signaling pathways limit these responses.

Nucleotide–binding oligomerization domain–containing protein 1 (NOD1) is a MyD88–independent intracellular PRR that mediates innate and adaptive immunity by recognizing commensal bacterial–derived signals[45]. To test whether NOD1 influenced steady–state circulating basophil populations, we examined serum IgE levels and circulating basophil populations in control or NOD1–deficient (*Nod1*$^{-/-}$) mice. Neither serum IgE levels (Supplementary Fig. 8a) nor blood basophils populations were altered in *Nod1*$^{-/-}$ compared to control mice (Supplementary Fig. 8b,c), indicating that NOD1–dependent signaling does not significantly influence steady–state serum IgE levels or circulating basophil responses in this setting. To examine whether MyD88–dependent bacterial–derived signals were sufficient to limit serum IgE levels or circulating basophil populations, we treated CNV–reared or ABX–treated mice with CpG[15, 46] and examined serum IgE levels and circulating basophil populations. CpG treatment of ABX–treated mice resulted in reductions in serum IgE levels (Supplementary Fig. 8d) and reduced frequencies and numbers of blood basophil populations (Supplementary Fig. 8e,f). Together, these findings identify MyD88–dependent signaling pathways as important regulators of steady–state serum IgE levels and circulating basophil populations in mice.

MyD88–dependent signaling in B cells can inhibit IgE class switching *in vitro*[47]. To test whether B cell–intrinsic expression of MyD88 influenced serum IgE levels or basophil homeostasis *in vivo*, we generated chimeras by sorting and adoptively transferring B or T cells from *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ mice into *Rag1*$^{-/-}$ recipients (Fig. 5e). Compared to controls, mice that received *Myd88*$^{-/-}$ B cells with *Myd88*$^{+/+}$ T cells displayed elevated serum IgE levels (Fig. 5f), increased frequencies (Fig. 5g) and numbers (Fig. 5h) of blood basophils, and increased basophil–surface–bound IgE levels (Fig. 5i). Together, these results indicate that B cell–intrinsic MyD88 expression limits steady–state serum IgE levels and circulating basophil populations *in vivo*.

Commensal bacteria influence basophil development by limiting IgE–mediated proliferation of a bone marrow–resident precursor population

We hypothesized that commensal–derived signals may regulate circulating basophil populations by influencing basophil survival. To test this, we sort–purified basophils from CNV–reared or ABX–treated mice and cultured them in the presence of IL–3. Basophils isolated from CNV–reared or ABX–treated mice exhibited similar survival (Supplementary

Fig. 9a), suggesting that commensal–derived signals do not readily influence basophil survival in this setting. As IgE was found to regulate commensal–dependent alterations in circulating basophil populations, we sought to investigate whether IgE influenced basophil survival in this assay. Basophils isolated from control or *Igh–7*$^{-/-}$ mice exhibited comparable survival rates (Supplementary Fig. 9b), suggesting that IgE does not readily influence basophil survival in this setting.

Rather than influencing survival, we hypothesized that commensal–derived signals may influence basophil development from the bone marrow. To test whether proliferation of basophils from bone marrow cells was influenced by commensal–derived signals, we CFSE labeled bone marrow from CNV–reared or ABX–treated mice, cultured it in the presence of IL–3, and examined mature basophil populations[48]. Compared to basophils derived from bone marrow of CNV–reared mice, a greater proportion of basophils derived from bone marrow of GF mice had diluted CFSE fluorescence and expressed the cell proliferation marker Ki67 (Fig. 6a) indicating that they originated from precursors that had undergone more rounds of cell division. Consistent with this, cultures of bone marrow cells from GF (Fig. 6b) or ABX–treated (Supplementary Fig. 9c) mice exhibited a greater population expansion of basophils compared to equal numbers of bone marrow cells isolated from CNV–reared mice. Together, these results indicate that commensal–derived signals limit the proliferative capacity of bone marrow–resident basophil precursor populations.

Bone marrow–resident basophils precursors (BaPs) are characterized by expression of FcεRIα and the hematopoietic progenitor adhesion molecule CD34[49]. To determine whether BaPs were influenced by alterations in commensal–derived signals, we isolated bone marrow from CNV–reared or GF mice and examined BaP populations. Frequencies of BaPs (identified as NBNT, CD117^{-}, FcεRIα^{+}, CD34^{+}) were significantly increased in the bone marrow of GF compared to CNV–reared mice (Fig. 6c,d). Having identified a role for IgE in mediating the influence of commensal–derived signals on circulating basophil populations, we hypothesized that IgE might mediate commensal–dependent effects on bone marrow BaP populations. To test this, we isolated bone marrow from CNV–reared or ABX–treated *Igh–7*$^{-/-}$ mice and examined BaP populations. Frequencies of BaPs were similar between CNV–reared or ABX–treated *Igh–7*$^{-/-}$ mice (Supplementary Fig. 9d) suggesting that IgE contributes to the increased numbers of bone marrow BaP cells observed following ABX–treatment.

Commensal bacteria limit BaP surface expression of CD123 and responsiveness to IL–3

As the development of basophils from bone marrow–resident precursors is regulated in part by IL–3–IL–3 receptor (IL–3R) signaling[50], we examined whether commensal–derived signals influenced BaP expression of the IL–3R subunit CD123. Compared to CNV–reared controls, BaPs in the bone marrow of ABX–treated or GF mice displayed increased surface–bound CD123 (Fig. 6e). Based on elevated surface-bound CD123, we hypothesized that BaPs from ABX–treated mice may be more responsive to IL–3 compared to BaPs from CNV–reared mice. To test this, we sort–purified BaPs from CNV–reared or ABX–treated mice and cultured them in equal numbers in the presence or absence of IL–3 (Supplementary Fig. 9e). In the presence of IL–3, BaPs from both CNV–reared or ABX–

treated mice developed into mature basophil populations as indicated by reduced expression of CD34 (Supplementary Fig. 9f), expression of CD49b and FcεRIα (Supplementary Fig. 9g), and mature cellular morphology (Supplementary Fig. 9h,i). However, culture of BaPs isolated from ABX–treated mice resulted in the development of a greater number of mature basophils compared to culture of an equal number of BaPs isolated from CNV–reared mice (Supplementary Fig. 9j). Together, these results indicate that commensal–derived signals limit BaP populations in the bone marrow, limit surface–bound CD123 on BaPs, and regulate responsiveness of BaPs to IL–3.

Due to the increased surface–bound CD123 on BaPs of ABX–treated mice, and the increased responsiveness of these BaPs to IL–3, we hypothesized that the elevations in circulating basophils observed upon ABX–treatment of mice would be dependent on IL–3R–dependent signaling. To test this, we examined basophil responses in CNV–reared or ABX–treated mice deficient in both IL–3Rβil3 and IL–3Rβc (*Csf2rb*$^{-/-}$*Csf2rb*$^{-/-}$). ABX–treatment of *Csf2rb*$^{-/-}$*Csf2rb*$^{-/-}$ mice did not significantly alter the frequency of blood basophil populations (Fig. 6f), suggesting that IL–3R signaling is necessary for ABX–induced elevations in circulating basophil populations. Therefore, the effects of commensal–derived signals on basophils are dependent in part on both IgE and IL–3R signaling.

IgE mediates increased surface–bound CD123 on BaP cells isolated from germ–free mice

Based on our findings that ABX–induced elevations in circulating basophils were dependent on IgE and IL–3R signaling, and correlated with bone marrow BaP surface–bound CD123, we hypothesized that IgE may regulate the increased CD123 surface expression on BaPs of GF mice. To test this hypothesis, we treated CNV–reared or GF mice with control or IgE–specific antibody and examined both surface–bound CD123 on BaPs and total bone marrow BaP frequencies. Compared to BaPs in the bone marrow of CNV–reared mice, BaPs in the bone marrow of GF mice exhibited significantly elevated expression of CD123 (Fig. 6g,h). Treatment of GF mice with IgE–specific antibody, but not control antibody, significantly reduced surface–bound CD123 on GF bone marrow BaPs (Fig. 6g,h) and reduced frequencies of BaPs in the bone marrow of GF mice (Fig. 6i). Collectively, these results indicate that commensal–derived signals influence bone marrow BaP populations in part by regulating IgE–mediated expression of CD123.

Discussion

In this study, oral delivery of broad–spectrum antibiotics was employed to interrogate the influence of commensal bacterial–derived signals on innate immune cell populations known to contribute to the development of T_H2 cytokine–dependent allergic inflammation. Antibiotic treatment resulted in elevated serum IgE levels, increased circulating basophil populations, and exaggerated basophil–associated T_H2 cell responses and allergic inflammation. IgE was identified as a critical regulator of steady–state circulating basophil populations in mice. These findings are consistent with studies that indicate that IgE can influence mast cell survival and function and suggest a broader immunoregulatory role for IgE in settings of infection and allergy [22]. Additionally, serum IgE levels were found to correlate with increased circulating basophil frequencies in subjects with

hyperimmunoglobulinemia E syndrome implicating IgE–mediated regulation of basophil populations in contributing to the increased susceptibility to allergies observed in these and other subjects with genetic or pathogenic elevations in circulating IgE levels[38, 39].

Previous reports that utilized genomic deletions in *Myd88* identified MyD88–dependent signaling as an important regulator of T_H2 cytokine–associated inflammation[44, 51]. Here, B cell–intrinsic expression of MyD88 was identified as one regulatory pathway that limits steady–state serum IgE levels and circulating basophil populations in mice. This finding is supported by *in vitro* studies that indicate that CpG can inhibit IgE class switching by B cells[47]. Consistent with this, human subjects with deficiencies in MyD88 or IRAK–4 signaling pathways exhibit elevated serum IgE levels[52]. Given these associations, further examination of circulating basophil populations or susceptibility to allergic inflammation in these subject populations may be of value.

Commensal bacterial–derived signals were found to regulate basophil development from bone marrow–resident precursor populations by influencing the responsiveness of these precursors to IL–3. These findings suggest that in addition to regulating immune cells in the periphery, commensal–derived signals can alter mammalian hematopoietic programs in the bone marrow to promote or protect against the development of allergic responses. The identification of a role for commensal–derived signals in regulating hematopoiesis could have implications beyond allergy to other chronic inflammatory disease states that are associated with alterations in commensal bacteria including cancer, infection or autoimmunity.

While the focus of the present report has been on the influence of commensal–bacterial derived signals on steady–state basophil populations and basophil–associated allergic inflammation, these findings do not exclude a potential role for DCs or regulatory T cells (Tregs) in influencing aspects of commensal regulation of allergic responses[25, 53]. Additionally, the findings described here do not exclude a synergistic role for other mediators of allergic inflammation such as thymic stromal lymphopoietin, a cytokine that is regulated by IgE and elevated in the intestines of germ-free mice[54, 55]. Further analysis of the effects of selective manipulation of commensal bacteria on DCs and Tregs (including inducible LAP^+ cell populations[56]) in murine models and human subjects is therefore warranted.

In summary, the data presented here indicate that commensal bacterial–derived signals limit steady–state serum IgE levels, circulating basophil populations, and basophil–associated allergic inflammation. We propose that in addition to influencing mature basophils in the periphery, IgE binds to bone marrow–resident basophil precursors increasing their responsiveness to IL–3 and resulting in both the population expansion of mature circulating basophils in the steady–state and exaggerated allergen–induced inflammation (Supplementary Fig. 10). These mechanistic insights into the influence of the commensal–IgE–basophil axis on the development of allergic inflammation may have utility in the design of new strategies to prevent or treat allergic diseases in humans.

METHODS

Mice

BALB/c, Swiss–Webster, C57BL/6, *Rag1*$^{-/-}$, BaS–TRECK, *Csf2rb*$^{-/-}$*Csf2rb*$^{-/-}$, *Igh–7*$^{-/-}$, IL–4/eGFP reporter, *Il4*$^{-/-}$, *Myd88*$^{-/-}$, *Nod1*$^{-/-}$ or *Tslp*$^{-/-}$ mice (8–24 weeks of age) were bred at the University of Pennsylvania, purchased from Jackson, Taconic or Charles River Laboratories, or provided by Amgen. Germ–free mice were provided by the Penn Gnotobiotic Mouse Facility. Conventional animals were housed in specific pathogen–free conditions. Experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the guidelines of the IACUC of the University of Pennsylvania.

Reagents and treatments

We fed mice autoclaved water +/− ampicillin (0.5 mg ml^{-1}), gentamicin (0.5 mg ml^{-1}), metronidazole (0.5 mg ml^{-1}), neomycin (0.5 mg ml^{-1}), and vancomycin (0.25 mg ml^{-1}) continuously via water bottle for four weeks. In the event of poor animal hydration, we supplemented control and antibiotic water with artificial sweetener. We conventionalized germ–free mice by housing with conventionally–reared mice for 4–8 weeks. We inoculated mice intranasally on D–17, D–14, D–7 with 50 μl of PBS +/− 100 μg of *D. pteronyssinus* extract (Greer)[25]. We injected mice subcutaneously in contralateral footpads with 50 μl of PBS +/− 50 μg of papain (Calbiochem)[28] and sacrificed on D3 or D4 post injection for basophil or T_H2 cell analyses. We treated mice by intraperitoneal injection (i.p.) with 10 μg of MAR1 or isotype antibody (eBioscience) on D–3, –2, –1. We treated littermate control or BaS–TRECK mice i.p with 750 ng DT on D–2 and D0. We treated *Rag1*$^{-/-}$ mice i.p. with 50 μg of IgG or IgE antibody (BD Bioscience) daily for seven days. We treated mice i.p. with 200 μg of control IgG or Omalizumab (IgE–specific) antibody (Genentech) daily for 7–9 days. We treated mice i.p. weekly with 100 μl of PBS containing 100 μg of GpC–Phos (5'-ZOOFZEFEOZZOOZEFEZOZT-3') or CpG–Phos (5'-ZOOFZEFOEZZOOZEFOEZZT-3').

Flow cytometry, cell sorting and adoptive transfer

We collected blood, spleen, bone marrow, lung, bronchoalveolar lavage or lymph node tissues, digested with collagenase and dispase (lung), homogenized, purified of red blood cells by histopaque (Sigma–Aldrich) or RBC lysis, and stained with mouse fluorochrome–conjugated monoclonal antibodies against B220, CD3ε, CD4, CD8, CD11c, CD19, CD34, CD40, CD49b, CD69, CD80, CD86, CD117, CD123, CD200R, FcεRIα, FcγR, Gr–1, IgE, IgM, IL–4, Ki67, MHC–I, MHC–II or Siglec F (BD Bioscience, BioLegend, eBioscience). We obtained samples from Hyperimmunoglobulinemia E or control subjects with participant consent, assent, and/or parental consent under protocols approved by the Children's Hospital of Philadelphia and the Ludwig Maximilians University Institutional Review Boards. We isolated PBMCs by Ficoll (GE) gradient, stained with antibodies against human CD11c, CD19, CD117, CD123, FcεRIα, IgE or TCRβ (BD Bioscience, eBioscience) and fixed with 4% PFA. We acquired/sorted cells with a FACSCanto II, LSR II or FACSAria with DiVa software (BD Bioscience) and analyzed with FlowJo software (version 8.7.1; Tree Star). We

transferred purified B or T cells i.p. in equal ratios and allowed to reconstitute for 8–10 weeks.

Culture, ELISA and histology

We cultured single–cell LNs suspensions for 2–4 days at 200–250,000 cells in 200 μl of complete media +/− anti–CD3 and anti–CD28 (0.5–1.0 μg ml^{-1}), stimulated for 4 hours with 50 ng ml^{-1} phorbol 12–myristate 13–acetate, 750 ng ml^{-1} ionomycin, 10 μg ml^{-1} Brefeldin A (Sigma–Aldrich) and treated with Fix/Perm (eBioscience). We CFSE labeled and cultured bone marrow cells for 4–5 days at 2–2.5×10^6 cells per ml +/− 10 ng ml^{-1} of IL–3 (R&D Systems). We cultured sort–purified BaPs for 4–5 days at 50,000 cells per ml +/− 10 ng ml^{-1} of IL–3 (R&D Systems). We assayed IgE, IL–4 or IL–13 levels by sandwich ELISA (BD Bioscience) or chart review. We fixed lung tissue sections in 4% paraformaldehyde, embedded in paraffin, cut 5 μm sections and stained with hematoxylin and eosin.

16S rDNA sample acquisition and quantification of 16S rDNA

We collected stool samples and extracted DNA with the QIAamp DNA Stool Mini Kit (Qiagen) as described previously[23]. We quantified 16S rDNA by real–time RT–PCR with degenerate bacterial 16S rDNA–specific primers (5′-AGAGTTTGATCCTGGCTCAG-3′; forward), (5′-CTGCTGCCTYCCGTA-3′; reverse), (5′-FAM-TA+ACA+CATG+CA +AGTC+GA-BHQ1-3′; probe; + precedes position of LNA base).

DNA manipulations, sequencing and bioinformatic analysis

We obtained 16S rRNA gene fragments as described previously[23]. We carried out PCR reactions (50 μl) with the AmpliTaq System (Applied Biosystems). We gel purified PCR products with the QIAquick Gel extraction kit (Qiagen). We pooled and pyrosequenced amplicons (100 ng). We assessed sequence quality, and samples with <100 sequences were discarded, and sequences were inserted into a 16S rRNA gene tree using parsimony insertion implemented in ARB. We obtained taxonomic assignments using RDP Classifier.

Statistical analysis

Results are shown as mean ± standard deviation for individual animals. We determined significance by non–parametric two–tailed Mann–Whitney test, unweighted means analysis two–way ANOVA, or linear regression analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acronyms/Abbreviations

IL	Interleukin
T_H2	T Helper Type 2
IgE	Immunoglobulin E
CNV	Conventional
ABX	Antibiotic
GF	Germ–Free
NBNT	Non–B Non–T
LN	Lymph Node
WT	Wild–Type

PRR	pattern recognition receptor
DOCK8	Dedicator of Cytokinesis 8

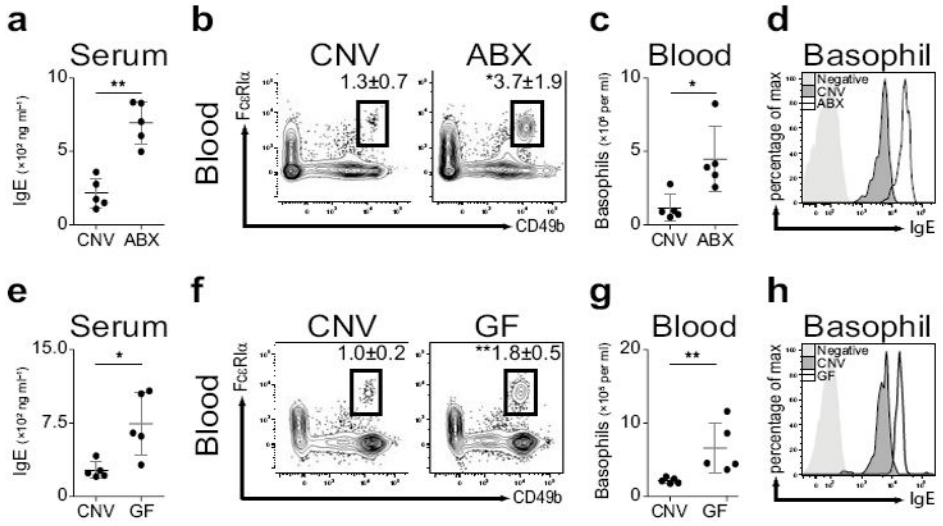

Figure 1.

Elevated steady–state serum IgE levels and circulating basophils in antibiotic–treated or germ–free mice. (**a**) Serum IgE from conventionally–reared (CNV) or antibiotic–treated (ABX) mice as measured by ELISA. (**b**) Flow cytometric analysis of blood basophils from CNV or ABX mice. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD117^-$ cells. (**c**) Number of basophils per ml of blood from CNV or ABX mice. (**d**) Mean fluorescence intensity of surface–bound IgE on blood basophils from CNV or ABX mice as determined by flow cytometry. (**e**) Serum IgE from CNV or germ–free (GF) mice as measured by ELISA. (**f**) Flow cytometric analysis of blood basophils from CNV or GF mice. (**g**) Number of basophils per ml of blood from CNV or GF mice. (**h**) Mean fluorescence intensity of surface–bound IgE on blood basophils from CNV or GF mice as determined by flow cytometry. Data representative of three or more independent experiments, results shown as mean ± s.d., significance determined by Mann–Whitney test (CNV, n=5; ABX, n=5; GF, n=5; *, $P \leq 0.05$; **, $P \leq 0.01$).

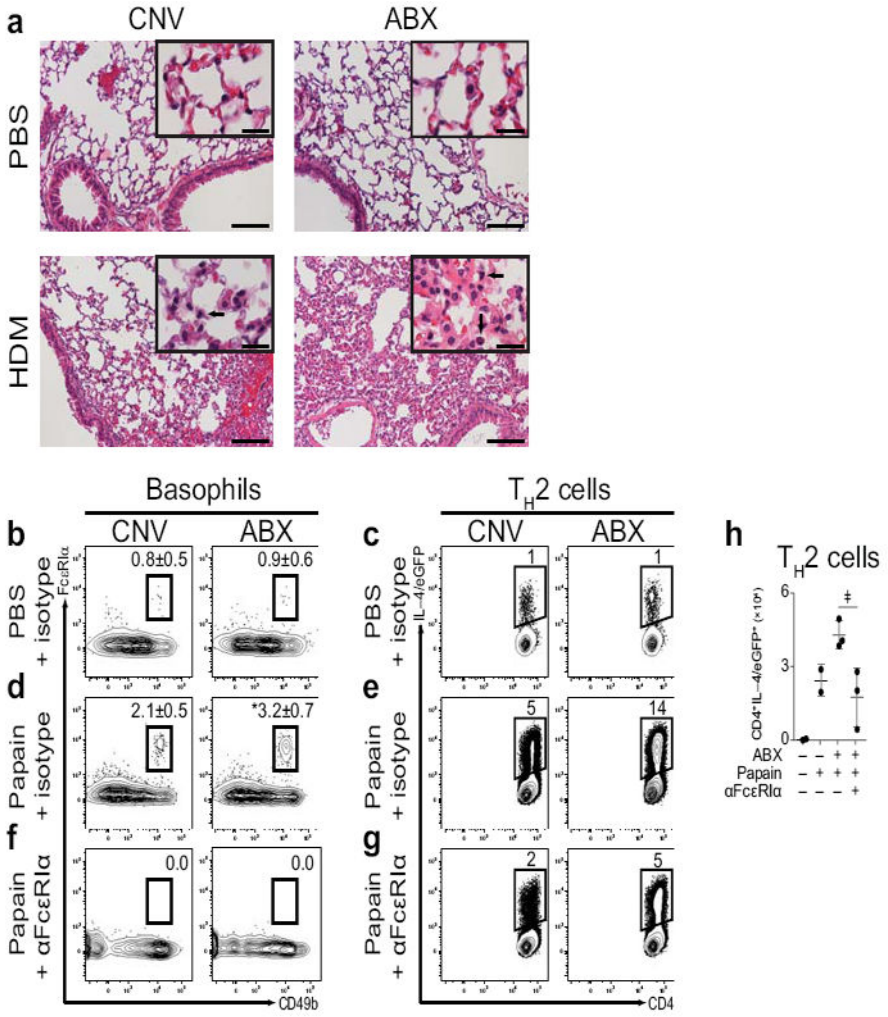

Figure 2.

Exaggerated basophil–mediated allergic airway inflammation and T_H2 cell responses in antibiotic–treated mice. (**a**) Histological sections of lungs from CNV or ABX mice exposed to PBS or HDM and stained with H&E (Large Bar = 100 μm; Small Bar = 20 μm; Arrows indicate eosinophilic infiltrates; CNV–PBS, *n*=2; ABX–PBS, *n*=1; CNV–HDM, *n*=3; ABX–HDM, *n*=2). (**b**) Flow cytometric analysis of day 3 popliteal LN (pLN) basophils from CNV or ABX mice treated with PBS and isotype control antibody. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD117^-$ cells. (**c**) Flow cytometric analysis of day 4 pLN IL–4/$eGFP^+CD4^+$ T_H2 cells from CNV or ABX mice treated with PBS and isotype control antibody. Gated on $CD8^-$, $CD19^-$, $CD4^+$ cells. (**d**) Flow cytometric analysis of day 3 pLN basophils from CNV or ABX mice treated with papain and isotype control antibody. (**e**) Flow cytometric analysis of day 4 pLN IL–4/$eGFP^+CD4^+$ T_H2 cells from CNV or ABX mice treated with papain and isotype control antibody. (**f**) Flow cytometric analysis of day 3 pLN basophils from CNV or ABX mice treated with papain and anti–FcεRIα (αFcεRIα) antibody. (**g**) Flow cytometric analysis of day 4 pLN IL–4/$eGFP^+CD4^+$ T_H2 cells from CNV or ABX mice treated with papain and αFcεRIα antibody (CNV–PBS–ISO, *n*=2–5; ABX–PBS–ISO, *n*=2–5; CNV–PAP–ISO, *n*=5;

ABX–PAP–ISO, *n*=5; CNV–PAP–αFcεRIα, *n*=2; ABX–PAP–αFcεRIα, *n*=2; significance determined by Mann–Whitney test; *, $P \leq 0.05$). (**h**) Number of day 4 pLN IL–4/eGFP^{+}CD4^{+} T_H2 cells from CNV or ABX mice treated with PBS or papain and isotype control (−) or αFcεRIα (+) antibody (means of three experiments ± s.d.; CNV–PBS–ISO, *n*=4; CNV–PAP–ISO, *n*=4; ABX–PAP–ISO, *n*=8; ABX–PAP–αFcεRIα, *n*=6; significance determined by 2 way ANOVA; ‡, $P \leq 0.06$). Data representative of two or more independent experiments, results shown as mean ± s.d.

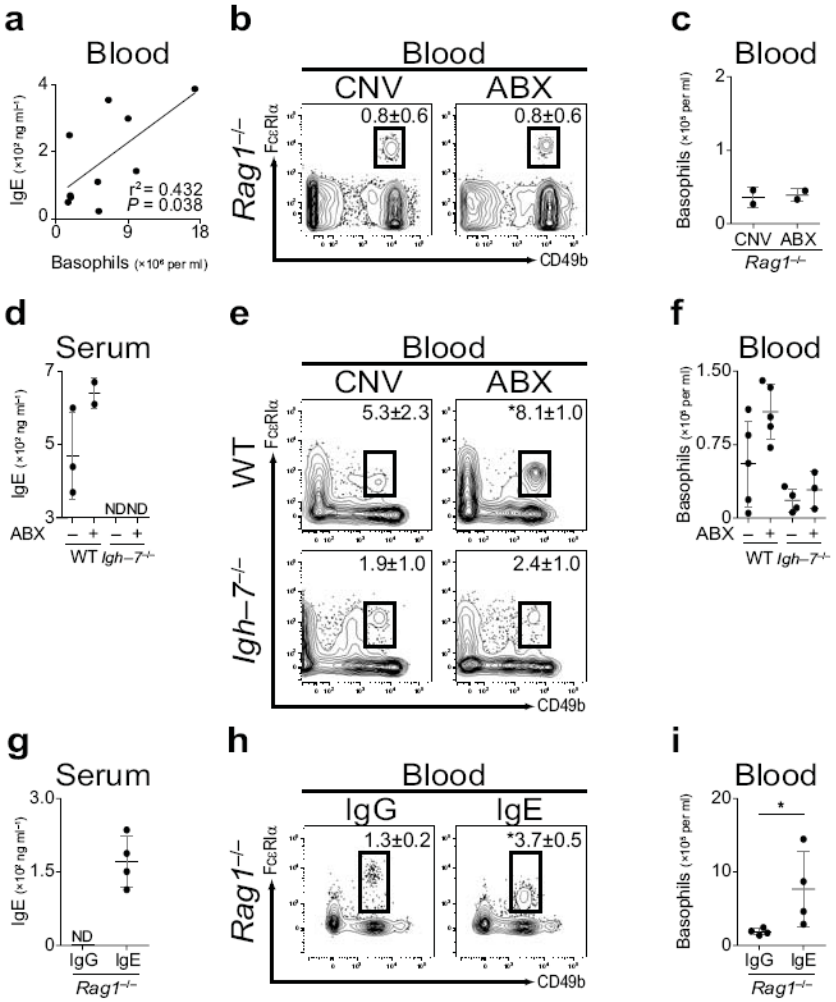

Figure 3.

IgE correlates with circulating basophil populations in mice. (**a**) Statistical correlation of blood basophil number and serum IgE concentration (significance determined by linear regression analysis; $r^2 = 0.432$; $P \leq 0.038$). (**b**) Flow cytometric analysis of blood basophils from CNV or ABX *Rag1*$^{-/-}$ mice. Numbers adjacent to outlined areas indicate percent cells in each gate (CNV, *n*=4; ABX, *n*=5). Gated on CD3$^-$, CD4$^-$, CD8$^-$, CD19$^-$, CD117$^-$ cells. (**c**) Number of basophils per ml of blood from CNV or ABX *Rag1*$^{-/-}$ mice (CNV, *n*=2; ABX, *n*=2). (**d**) Serum IgE from CNV or ABX wild–type (WT) or *Igh–7*$^{-/-}$ mice as measured by ELISA (ND, not detected). (**e**) Flow cytometric analysis of blood basophils from CNV or ABX WT or *Igh–7*$^{-/-}$ mice (CNV–WT, *n*=5; ABX–WT, *n*=5; CNV–*Igh–7*$^{-/-}$, *n*=4; ABX–*Igh–7*$^{-/-}$, *n*=3). (**f**) Number of basophils per ml of blood from CNV or ABX WT or *Igh–7*$^{-/-}$ mice (CNV–WT, *n*=5; ABX–WT, *n*=5; CNV–*Igh–7*$^{-/-}$, *n*=4; ABX–*Igh–7*$^{-/-}$, *n*=3). (**g**) Serum IgE levels from IgG or IgE treated *Rag1*$^{-/-}$ mice as measured by ELISA (IgG, *n*=4; IgE, *n*=4; ND, not detected). (**h**) Flow cytometric analysis of blood basophils from IgG or IgE treated *Rag1*$^{-/-}$ mice (IgG, *n*=4; IgE, *n*=4). (**i**) Number of basophils per ml of blood from IgG or IgE treated *Rag1*$^{-/-}$ mice (IgG, *n*=4; IgE, *n*=4). Data representative of

three or more independent experiments, results shown as mean ± s.d., significance determined by Mann–Whitney test unless otherwise indicated (*, $P \leq 0.05$).

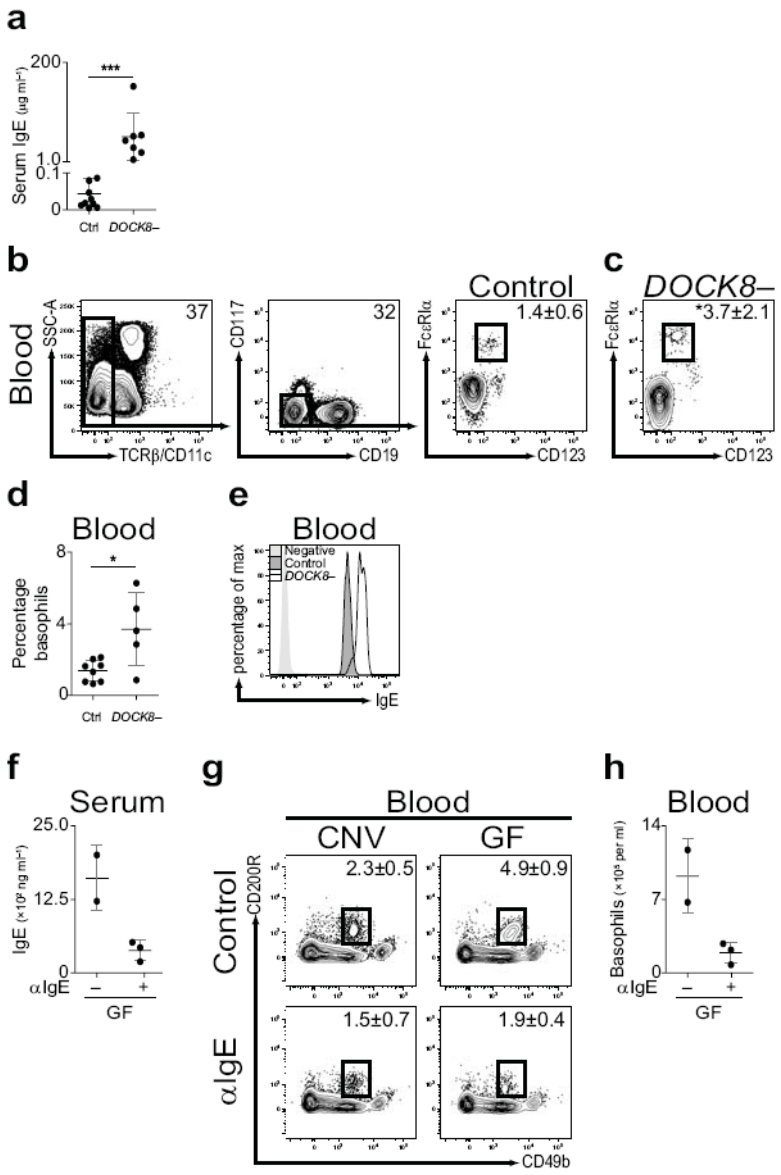

Figure 4.

Serum IgE levels correlate with circulating basophil populations in human subjects with hyperimmunoglobulinemia E syndrome. (**a**) Serum IgE levels from control (Ctrl) or subjects with polymorphisms in DOCK8 (*DOCK8*–) (Ctrl, *n*=15; *DOCK8*–, *n*=7). (**b**) Identification of blood basophils from control subjects by flow cytometric analysis as TCRβ^{-}, CD11c^{-}, CD19^{-}, CD117^{-}, FcεRIα^{+}, CD123^{+} cells. (**c**) Flow cytometric analysis of blood basophils from *DOCK8*– subjects. (**d**) Frequency of basophils in blood from control or *DOCK8*– subjects (Ctrl, *n*=8; *DOCK8*–, *n*=5). (**e**) Mean fluorescence intensity of surface–bound IgE on blood basophils from control or *DOCK8*– subjects as determined by flow cytometry. (**f**) Serum IgE from germ–free (GF) mice treated with control (−) or IgE–specific antibody (αIgE; +) antibody as measured by ELISA (control, *n*=2; αIgE, *n*=3). (**g**) Flow cytometric analysis of blood basophils from CNV or GF mice treated with control or αIgE antibody.

Numbers adjacent to outlined areas indicate percent cells in each gate (CNV–control, *n*=2; GF–control, *n*=2; CNV–αIgE, *n*=3; GF–αIgE, *n*=3). Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD117^-$ cells. (**h**) Number of basophils per ml of blood from GF mice treated with control or αIgE antibody (control, *n*=2; αIgE, *n*=3). Data representative of three or more independent experiments, results shown as mean ± s.d., significance determined by Mann–Whitney test (**, $P \leq 0.01$; ***, $P \leq 0.001$).

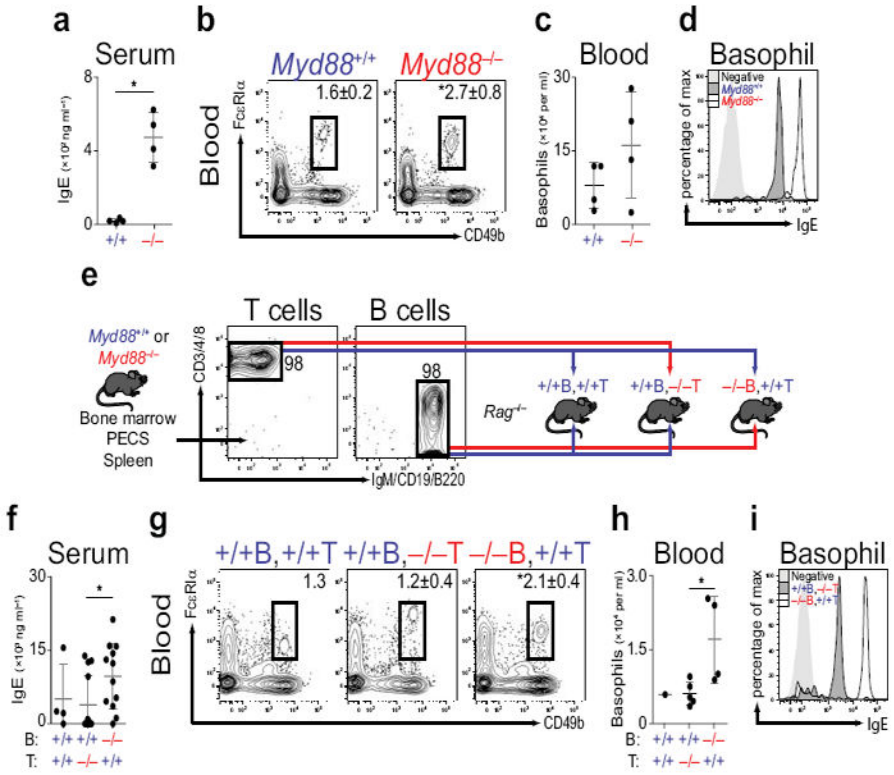

Figure 5.

Elevated serum IgE levels and circulating basophil populations in mice lacking B cell–intrinsic MyD88 signaling. (**a**) Serum IgE from conventionally–reared (CNV) *Myd88*$^{+/+}$ (+/+) or *Myd88*$^{-/-}$ (−/−) mice as measured by ELISA (*Myd88*$^{+/+}$, *n*=4; *Myd88*$^{-/-}$, *n*=4). (**b**) Flow cytometric analysis of blood basophils from CNV *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ mice. Numbers adjacent to outlined areas indicate percent cells in each gate (*Myd88*$^{+/+}$, *n*=4; *Myd88*$^{-/-}$, *n*=4). Gated on CD3^{-}, CD4^{-}, CD8^{-}, CD19^{-}, CD117^{-} cells. (**c**) Number of basophils per ml of blood from CNV *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ mice (*Myd88*$^{+/+}$, *n*=4; *Myd88*$^{-/-}$, *n*=4). (**d**) Mean fluorescence intensity of surface–bound IgE on blood basophils from CNV *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ mice as determined by flow cytometry. (**e**) Experimental diagram of sort and transfer of *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ B or T cells into *Rag1*$^{-/-}$ recipients. (**f**) Serum IgE from *Rag1*$^{-/-}$ mice reconstituted with *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ B or T cells as measured by ELISA (+/+B +/+T, *n*=4; +/+B −/−T, *n*=12; −/−B +/+T, *n*=12). (**g**) Flow cytometric analysis of blood basophils from *Rag1*$^{-/-}$ mice reconstituted with *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ B or T cells (+/+B +/+T, *n*=1; +/+B −/−T, *n*=5; −/−B +/+T, *n*=4). (**h**) Number of basophils per ml of blood from *Rag1*$^{-/-}$ mice reconstituted with *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ B or T cells (+/+B +/+T, *n*=1; +/+B −/−T, *n*=5; −/−B +/+T, *n*=4). (**i**) Mean fluorescence intensity of surface–bound IgE on blood basophils from *Rag1*$^{-/-}$ mice reconstituted with *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ B or T cells as determined by flow cytometry. Data representative of three or more independent experiments, results shown as mean ± s.d., significance determined by Mann–Whitney test (*, $P \leq 0.05$).

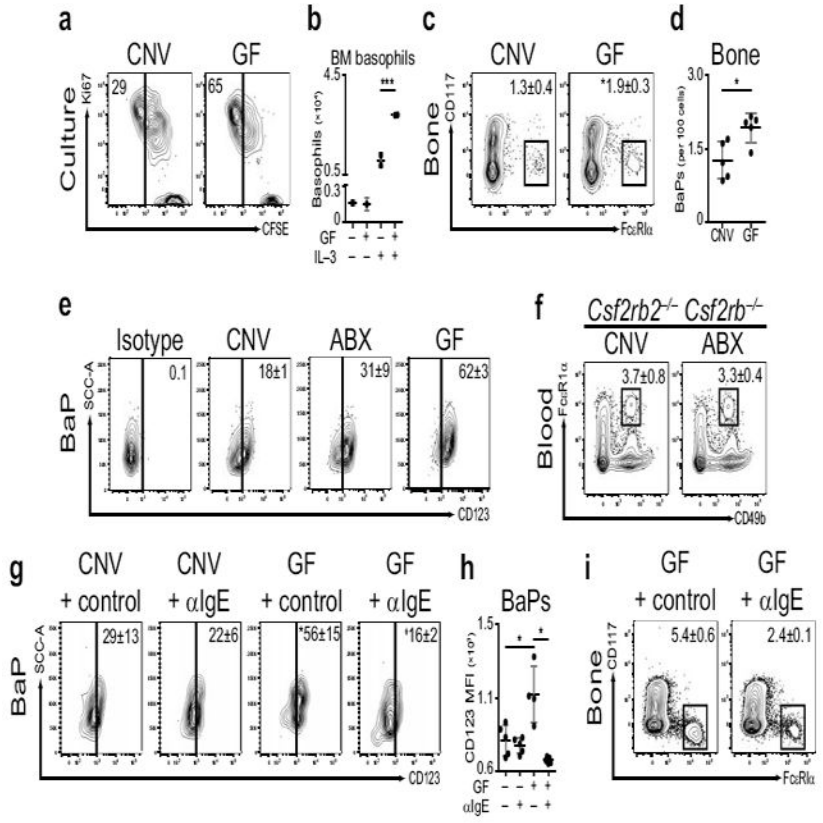

Figure 6.

Dysregulated basophil development in germ–free or antibiotic–treated mice. (**a**) Bone marrow of conventionally–reared (CNV) or germ–free (GF) mice was CFSE labeled, cultured in the presence of IL–3, and basophil populations were examined by flow cytometry. Numbers adjacent to outlined areas indicate percent cells in each gate. Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD117^-$, $CD49b^+$, $Fc\varepsilon R1\alpha^+$ cells. (**b**) Bone marrow of CNV (−) or GF (+) mice was cultured in the absence (−) or presence (+) of IL–3 and basophil numbers were determined (means of two experiments ± s.d.; CNV–media, *n*=5; GF–media, *n*=3; CNV–IL–3, *n*=10; GF–IL–3, *n*=8; significance determined by 2 way ANOVA; ***, $P \leq 0.001$). (**c**) Flow cytometric analysis of BaPs in bone marrow of CNV or GF mice. Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD34^+$ cells (CNV, *n*=5; GF, *n*=5). (**d**) Number of BaPs in bone marrow of CNV or GF mice (CNV, *n*=5; GF, *n*=5). (**e**) Frequency of $CD123^+$ BaPs in bone marrow of CNV, antibiotic–treated (ABX) or GF mice as determined by flow cytometry. Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD117^-$, $CD34^+$, $Fc\varepsilon R1\alpha^+$ cells (CNV, *n*=2; ABX, *n*=2; GF, *n*=2). (**f**) Flow cytometric analysis of blood basophils from CNV or ABX $Csf2rb^{-/-}Csf2rb^{-/-}$ mice. Gated on $CD3^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD117^-$ cells (CNV, *n*=3; ABX, *n*=3). (**g**) Frequency of $CD123^+$ BaPs in bone marrow of CNV or GF mice treated with control or IgE–specific antibody (αIgE) (CNV–control, *n*=5; CNV–αIgE, *n*=5; GF–control, *n*=4; GF–αIgE, *n*=5; CNV–control vs. GF–control, *, $P \leq 0.05$; GF–control vs. GF–αIgE, ‡, $P \leq 0.05$). (**h**) Mean fluorescence intensity of CD123 on BaPs in bone marrow of CNV or GF mice treated with control or αIgE antibody (CNV–control, *n*=5; CNV–αIgE, *n*=5; GF–control, *n*=4; GF–αIgE, *n*=5; *, $P \leq 0.05$). (**i**) Flow cytometric

analysis of BaPs in bone marrow of GF mice treated with control or αIgE antibody (GF–control, *n*=2; GF–αIgE, *n*=3). Data representative of two or more independent experiments, results shown as mean ± s.d., significance determined by Mann–Whitney test unless otherwise indicated (*, $P \leq 0.05$).