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# Modulation of immune responses by commensal bacteria and intestinal helminth

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Es ist nicht genug zu wissen - man muss auch anwenden.
Es ist nicht genug zu wollen - man muss auch tun.

Johann Wolfgang von Goethe

## Summary

The dysregulation of immune responses leads to increasing prevalence of severe human diseases such as autoimmunity, allergy, and cancer and has been linked to microbiota acting to shape the immune status of its host. Two different aspects investigating the immune responses at mucosal surfaces influencing systemic immune conditions are described in this thesis.

The first part characterizes the impact of commensal bacteria on the immune response provoked by an allergic airway inflammation. To test how commensals impact on asthma, an animal model of allergic airway inflammation in germ-free (GF) mice was developed and compared to conventionally housed specific pathogen-free (SPF) mice. After systemic sensitization and local airway challenge with ovalbumin, GF mice showed exacerbated eosinophilic and lymphocyte infiltration, elevated levels of the Th2 cytokines IL-4 and IL-5, and increased production of IgE compared to SPF mice. Re-colonization of GF mice with a complex microflora could reverse the increased Th2 phenotype. Regulatory T cells and regulatory cytokines were not changed, instead increased numbers of basophils, heightened numbers and changed phenotype of conventional dendritic cells and a decreased number of alveolar macrophages and plasmacytoid dendritic cells was found. These data demonstrate that the presence of commensal bacteria is critical to ensure normal cellular maturation, recruitment, and control of allergic airway inflammation.

The second part elucidates the role of isotype-switched and affinity-matured antibodies on basophils during intestinal helminth infection. It is well appreciated that basophils are potent mediators of Th2 immunity in allergic diseases and helminth

infections. They act to amplify ongoing Th2 immune responses by the early secretion of cytokines such as IL-4 and release of granule contents like histamine. How they are regulated during these inflammatory processes remained largely unknown. In this thesis, it is shown that basophils originate mainly from the bone marrow and to a lesser extent from the spleen in an IL-3 dependent manner. The expansion of basophils following infection with the intestinal helminth *H. polygyrus bakeri* was supported by affinity-matured IgG1 and IgE, which induced autocrine IL-3 production. These findings help to understand why basophils are typically associated with Th2 immune responses and imply how antibodies contribute to Th2-mediated immunity.

# Zusammenfassung

Fehlgeleitete Immunreaktionen führten in den letzten Jahren zu steigender Prävalenz von schwerwiegenden Erkrankungen wie Autoimmunität, Allergien und Krebs. Dies konnte unter anderem mit der Veränderung des Gleichgewichts zwischen Immunmechanismen und der Mikroflora in Verbindung gebracht werden. Diese Dissertation beschäftigt sich mit der Rolle von symbiotischen Bakterien und einem Darmparasiten und deren Wirkung auf den Immunstatus des Wirtsorganismus.

Der erste Teil beschreibt die Auswirkungen der kommensalen Bakterien auf die Immunantwort in einem Modell für allergische Entzündungsreaktionen der Atemwege. Um zu testen wie kommensale Bakterien Einflauss auf die Asthmaentwicklung haben, wurde ein Modellsystem für allergisches Asthma bei sterilen (germ-free=GF) und spezifisch Pathogen-freien (SPF) Mäusen entwickelt. Nach systemischer Immunisierung mit Ovalbumin und wiederholter lokaler Antigen-Applikation von Ovalbumin wurde bei GF-Mäusen eine verstärkte allergische Reaktion mit vermehrter Infiltration von Eosinophilen und Lymphozyten, sowie erhöhte Konzentrationen der Th2 Zytokine IL-4 und IL-5, als auch gesteigerte IgE-Produktion festgestellt. Durch Re-Kolonialisierung der GF-Mäuse mit einer komplexen Mikroflora konnte diese gesteigerte Th2 Immunantwort gemildert werden. Weder regulatorische T-Zellen noch, regulatorische Zytokine, schienen an diesem Prozess massgeblich beteiligt. Hingegen konnten verringerte Mengen von plasmazytoiden dendritischen Zellen und alveolären Makrophagen, sowie erhöhte Zahlen und veränderte Aktivierungsmarker von konventionellen dendritischen Zellen gemessen werden. Unsere Daten zeigen, dass die kommensale Flora einen Einfluss auf die normale Entwicklung von Immunzellen und deren Funktion während einer

allergischen Asthmareaktion hat.

Der zweite Teil beleuchtet die Rolle von Antikörpern, welche einen Isotypen-Klassenwechsel und Affinitätsgereiftungen vollzogen haben. Deren Wirkung auf Basophile, während einer Wurminfektion des Darmtrakts, wurde in dieser Dissertation untersucht. Bis heute sind Basophile hauptsächlich für ihre Funktion während allergischen Reaktionen und Parasiteninfetkionen bekannt, bei welchen sie Th2 Immunantworten verstärken. Dieser Effekt wird hauptsächlich auf die Sekretion von IL-4 und Ausschüttung von in Granuolen befindlichen Entzündungsmediatoren zurückgeführt. Wie sie jedoch während dieser Entzündungsreaktionen kontrolliert werden, ist weitgehend unerforscht. In dieser Arbeit wird gezeigt, dass sie von Vorläuferzellen vornehmlich aus dem Knochenmark und zu geringerem Maße auch aus der Milz in Abhängigkeit von IL-3 entstehen können. Der Anstieg von Basophilenzahlen nach Infektion mit dem Darmparasiten *H. polygyrus bakeri* wurde bedingt durch Antikörper, welche einen Isotypen-Klassenwechsel zu den Klassen IgG1 und IgE sowie eine Affinitätsgereifung vollzogen hatten. Diese führten zu autokriner IL-3 Produktion bei Basophilen. Diese Beobachtungen könnten helfen zu erklären, warum Basophile meist nur in Verbindung mit Th2 Immunantworten gefunden wurden und wie Antikörper zur Entwicklung von Th2 Immunität beitragen.

# Table of Contents

# 1 Introduction

## 1.1 Mucosal Immunology

The surface of the skin, the gut, the respiratory tract and the genital tract are permanently in contact with bacteria, protozoa, viruses and fungi, which form a symbiotic relationship with the host. Intestinal bacteria have long been thought to establish a commensalistic relationship with the host. Recent evidence suggesting that these bacteria are required to maintain homeostasis and are beneficial for the host indicate that this relationship is perhaps mutualistic. However, the same bacteria pose a serious health risk in case of their dissemination into the blood stream. Hence, the mucosal immune system has developed mechanisms restricting bacterial growth to the intestinal lumen. In addition the mucosal immune system has evolved to endure the constant presence of intestinal commensals without causing overt inflammation, whilst at the same time retaining the ability to raise an inflammatory response against harmful pathogenic attacks. Although these relationships have mostly been well-studied in the intestine, similar immunological strategies are likely apply to the skin, respiratory tract and genital tract. For this purpose distinct physiological structures and protection mechanisms for the airways and the lung as well as the intestinal mucosa have developed and are being discussed below.

### 1.1.1 Cellular and non-cellular components of the mucosal immune system

The mucosal immune system is comprised of both innate and adaptive immune components. In addition mucosal surfaces are separated from the environment by a single layer of epithelial cells. In the intestine this cell layer is composed of distinct cell types including enterocytes, goblet cells and Paneth cells. Goblet cells secrete

mucins to form the mucus layer overlying the epithelium. Paneth cells reside in intestinal crypts and produce anti-microbial peptides such as α-defensins (1, 2). The mucosal immune system can be separated into specialized mucosal-associated lymphoid tissue (MALT) which acts as an inductive site and the lamina propria which contains effector cells (3). Within the MALT dedicated microfold or membraneous (M) cells sample antigen from the gut lumen and present it to antigen presenting cells (APC), which in turn activate naive T and B cells (4). The inductive sites of the gut-associated lymphoid tissue (GALT) comprise Peyers's patches (PP) and isolated lymphoid follicles (ILF). These lack afferent lymphatics but have efferent lymphatics, which drain to the associated mesenteric lymph nodes. From here activated cells move to the thoracic duct and are released into the blood stream. By virtue of their expression of specific adhesion molecules and chemokine receptors, these cells then exit the blood stream and enter the intestinal lamina propria (4, 5). In the PPs induced immunoglobulin (Ig) M+ B220+ and IgA+ B220+ B cells serve as precursors for plasmablasts, which can develop into plasma cells in a T cell dependent manner to produce high-affinity IgA. The plasma cells reside in the LP, where they produce polymeric forms of IgA joined by a J-chain, called secretory IgA (SIgA), which can bind to polymeric Ig receptors (pIgR) on epithelial cells. With the help of pIgR these high-affinity IgA antibodies pass through the epithelial cells to be released at the apical surface as sIgA into the mucus layer, where they can neutralize toxins (6, 7). Low-affinity IgA can be induced in a T cell-independent manner and serves to prevent the penetration of microorganisms through the epithelium, thereby contributing to protective mucosal immunity (6, 8).

### 1.1.2 T helper cell subsets

CD4+ T helper (Th) cells are part of the adaptive immunity and can generally be divided into 4 different subsets including Th1, Th2, Th17 and T regulatory (Treg) cells (9). Th1 and Th2 cells play major roles as effector cells against foreign antigens and pathogens and were first defined by Mosmann and Coffmann in 1989 (10). These authors distinguished the two subsets according to their characteristic secretion of cytokines: While Th1 secrete IFN-γ and are important for responses against intracellular microorganisms, Th2 cells stably express and secrete interleukin (IL-) 4 and are essential for the rejection of helminth parasites (11, 12). More recently Th17 cells were defined as another distinct T-cell population, producing IL-17. These cells take action during autoimmunity, clearance of extracellular bacteria and immune responses against fungi (13, 14). T regulatory cells counteract all the above-described reactions to dampen ongoing immune responses and maintain peripheral tolerance (15, 16). More recent publications indicate the existence of another subset named, T follicular helper cells (Tfh) (17, 18). These cells represent a distinct subset, which is required for promoting antibody isotype switching and germinal center reactions. They are also the main IL-4 producing cell type residing in lymphoid organs following helminth infection (19). Lastly, it has recently been suggested that T cells secreting IL-9 could represent a distinct subset (Th9) that might be involved in immunological responses in the context of allergy or helminth infections (20).

## 1.2 Type 2 immunity

In this section the initiation and effector functions of Th2 cells are first described in general before the different aspects of Th2 immunity in the context of allergic disorders and helminth infections are addressed in more detail.

### 1.2.1 Initiation and effector functions of type 2 immune responses

Immune responses associated with allergic diseases and helminth infections are mainly of the T helper 2 type, characterized by $CD4^+$ T cells secreting the cytokines IL-3, IL-4, IL-5, IL-9 and IL-13 (21). The initiation of Th2 reactions involves innate immune cells, which can sense danger signals and direct naïve T helper cells to develop into Th2 effector cells (Figure 1). The sites of first encounter are epithelial cells of the skin, gut and lung, where contact to invading microorganisms or danger signals takes place. Lipopolysaccharides (LPS) can be sensed by toll-like receptor (TLR) 4, expressed on epithelial cells and lead to secretion of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). In fact, it has been shown that in the absence of TLR signaling, such as in mice deficient for TLR4 or MyD88 (adapter molecule for TLR signaling) the intestinal homeostasis is imbalanced (22). IL-25, IL-33 and TSLP, secreted by mucosal epithelial cells, act on basophils, dendritic cells (DC) and non-T non-B cells, which leads to the activation of these cells and subsequent release of cytokines like IL-4, IL-5 and IL-13. When antigen is presented to naïve CD4+ T cell in the presence of IL-4, Th2 priming can occur.

Signal transducer and activator of transcription 6 (STAT6) and GATA binding protein 3 (GATA-3) have been shown to be essential for the development of Th2 cells. STAT6 is a transcription factor downsteam of the IL-4 receptor (IL-4R) (23). Signaling through STAT6 induces the up-regulation of GATA-3. There are different pathways how GATA-3 contributes to the development of a Th2 response (24). It has been shown that IL-2 activates STAT5, which can lead to Th2 differentiation in an IL-4 independent manner, but the presence of GATA-3 is required for this process (25). This was confirmed by a conditional knock out mouse cell line of GATA-3, which showed defective responses for both IL-4-dependent and IL-4-independent Th2

priming (26). Additionally, it has been revealed that GATA-3 expression is essential for the production of IL-5 and IL-13 by Th2 cells and that it acts to suppress Th1 responses (26).

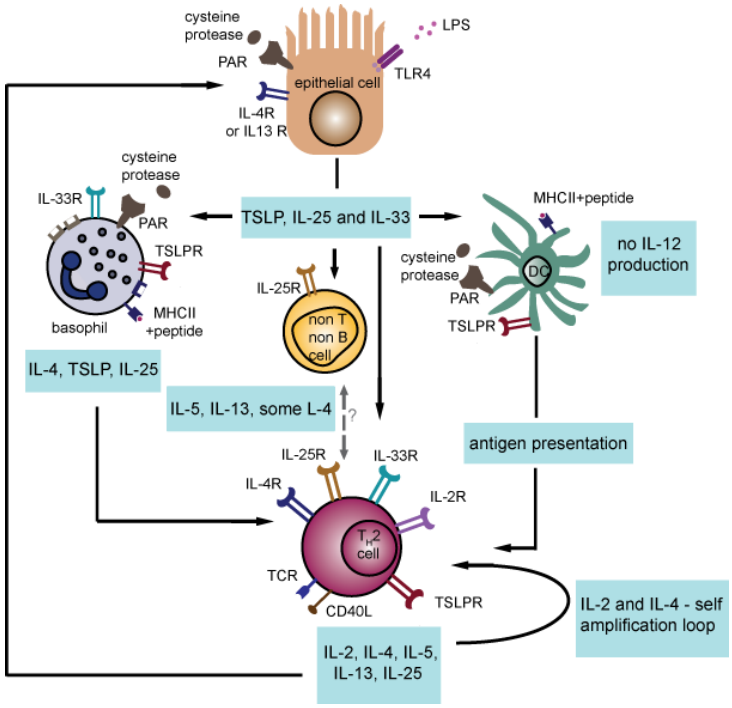


**Figure 1:** Role of cytokines in the initiation and amplification of Th2 immune responses modified from William E. Paul and Jinfang Zhu (21).

Another crucial element for Th2 differentiation and subsequent IL-4 production is STAT5, which can be activated through downstream signaling of IL-2, IL-7 and TSLP (27). Both GATA-3 and STAT5 bind directly to Th2 cytokine loci (27). Valuable insights came from the use of an IL-4 reporter mouse, which expresses the enhanced green fluorescent protein (EGFP) thereby allowing for the tracking of IL-4 competent cells *in vivo*. These studies confirmed that the expansion of IL-4 expressing Th2 cells was STAT6-dependent (28). With these tools other innate immune cells, called nuocytes, innate type 2 helper cells, and natural helper cells could be identified, which aid to initiate and maintain Th2 responses (29). Indeed, these cells show the same characteristics of the previously described non-T non-B cell

populations as they are also responsive to epithelial cell derived IL-25 and IL-33 (29-31).

### 1.2.2 Type 2 immunity during allergic airway reactions

Allergic asthma is a chronic disease of the airways characterized by airway hyperresponsiveness (AHR), which is driven by a Th2 response against otherwise harmless environmental antigens. Other features include goblet cell hyperplasia, airway smooth muscle cell proliferation (and thickening of the bronchial mucosa), broncho-constriction, and deposition of fibronectin, leading to airway remodeling (32). The sensitization occurs when dendritic cells sample allergens from the airway lumen in the presence of other danger signals and pathogen-associated molecular patterns, like LPS (33). In the case of repetitive or constant exposure to the allergen, a chronic inflammatory state develops (Figure 2). During chronic airway inflammation the recruitment of activated mast cells, eosinophils, and basophils into the lung and airway lumen is triggered by exaggerated Th2 responses, involving the secretion of IL-3, IL-4, IL-5, IL-9, and IL-13 and the production of IgE (32). More recent studies report on the contribution of epithelial cells to Th2 immunity by their orchestration of eosinophilic airway inflammation through the secretion of IL-25, IL-33, and TSLP leading to subsequent maturation of DCs. (34, 35)

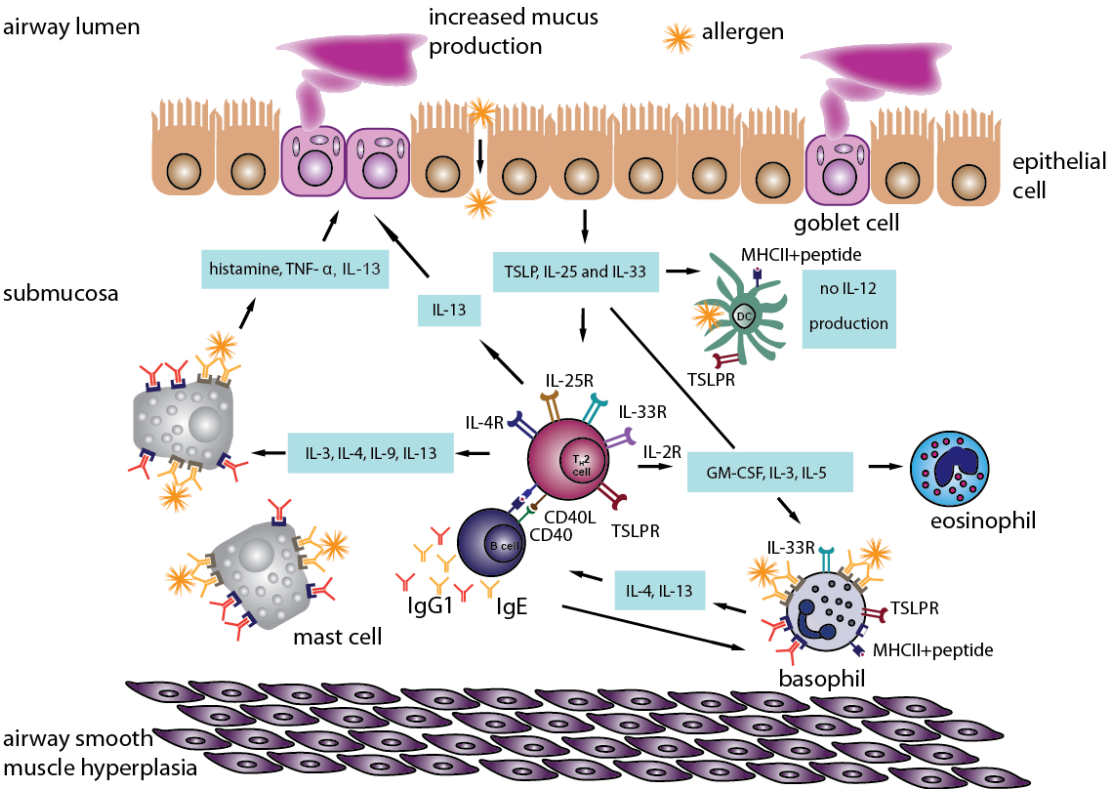


**Figure 2:** Chronic airway inflammation upon repetitive exposure to allergen modified from Galli et al. (36). Features of a chronic allergic inflammation are goblet cell hyperplasia with increased mucus production, proliferation of airway smooth muscle cells, infiltration of eosinophils, basophils, mast cells, cytokine secreting Th2 cells, and elevated levels of IgE from B-cells.

In addition to effector cytokines, regulatory mechanisms in asthma comprise cytokines as IL-10, transforming growth factor (TGF) beta, as well as cellular components as alveolar macrophages (AM) and regulatory T (Treg) cells (37-39).

Recently, Rubtsov et al. reported that even though IL-10 is not required for control of systemic autoimmunity at mucosal sites like the lung, it contributes to dampen AHR and inflammation after exposure to inhaled allergens. Additionally, the authors of this study could show that mice developed enhanced inflammation and AHR after inhaled exposure to allergens, if IL-10 was specifically deleted in Treg cells (40). Previously to this report, other groups showed, that Treg cells were involved in the control of AHR and allergic airway inflammation both before and after allergen sensitization using adoptive transfer experiments as well as depletion studies. (41-45)

Alveolar macrophages are known to balance immune responses at mucosal sites in the lung (46). In asthma AM act mainly to suppress airway hyperresponsiveness (39). Thepen et al. showed that the depletion of AM leads to enhanced antigen presentation by dendritic cells and also affects the humoral immune response to inhaled antigens (47). Additionally, tissue resident lung interstitial macrophages have been reported to interact with local dendritic cells to prevent the development of allergic airway inflammation (48). Currently, treatment of allergic asthma includes corticosteroids and bronchodilators, acting against symptoms, but not tackling the underlying causes of chronic airway inflammation (33). To develop more specific therapies, it is required to further elucidate the interplay of the above-mentioned players and their interaction with environmental factors.

### 1.2.3 Type 2 immunity during helminth infections

Protective host responses against intestinal helminths involve the development of a Th2 response. When infected with these large invading parasites, the host is faced with the challenge to keep the balance between developing effective immunity while maintaining its integrity during the state of chronic helminth infection. The cellular players in response to helminth infections are depicted in Figure 3. In brief, the activated Th2 cells secrete IL-3, IL-4, IL-5, IL-9, and IL-13. These cytokines attract various innate cell types, including eosinophils, mast cells, and basophils, as has been described in chapter 1.2.1. Alternatively activated macrophages are also thought to play an important role in protective immunity and tissue repair (Figure 3) (49).

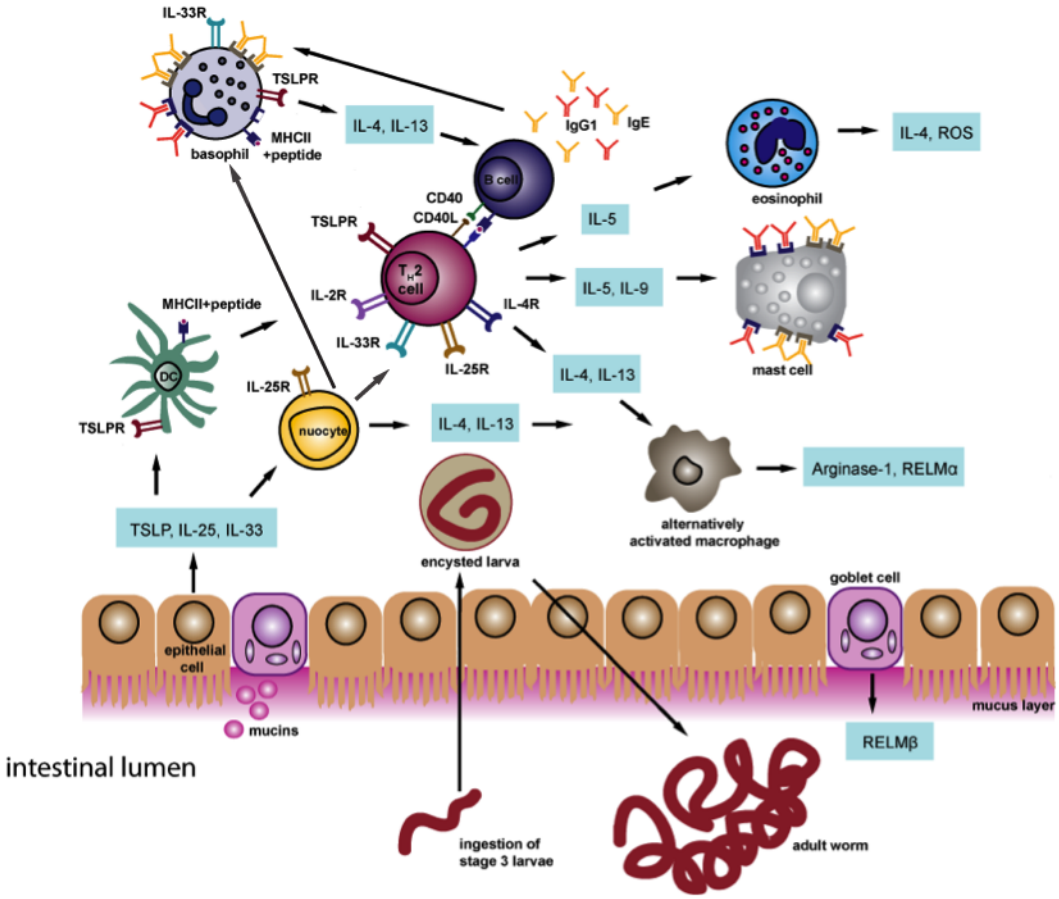


**Figure 3:** Type 2 immunity during helminth infections modified from Anthony et al. (11). The host ingests stage 3 larvae of the helminth parasite, which enter the submucosa form a cyst before they reenter the intestinal lumen as adult parasites. The main player of the host response against the helminth are T helper type 2 (Th2) cells, which produce cytokines like IL-3, IL-4, IL-5, IL-9, and IL-13. However many other innate cells, like nuocytes, basophils, eosinophils, and alternatively activated macrophages also play a role.

Due to the multi-cellular structure and the many different developmental stages of the parasite, the immune response that has co-evolved between host and parasite represents very distinct features. Already helminth secretory/excretory (50) products as such provoke the activation of immune cells to switch from a pro- to an anti-inflammatory phenotype (51). Further mechanisms that are important for the protective immunity against helminth infections are described in chapter 3.4.

## 1.3 Hygiene Hypothesis

In 1989, Strachan published a study named “Hayfever, hygiene and household size” describing the interaction of infectious agents and the development of allergy in westernized countries (52). Later, this theory came to be known as the “hygiene hypothesis” and many epidemiological studies confirmed the correlation of increasing incidences of allergic asthma with a decrease in bacterial exposure, changing life style (including diet), early childhood treatment with antibiotics and better overall hygiene status (53-55). Initially, it was believed that the increased rate of allergies was caused by an imbalance of Th1 versus Th2 immune responses, due to a lack of IL-12 production, which would favor the Th1 arm (56). More recently, it was proposed to shift the focus of the classical hygiene hypothesis more towards the possible mutualism of commensal bacteria and the host immune system to a so called “microbiota hypothesis” (57). However, not only bacteria can alter the host immune response but also enteric helminths have demonstrated immunomodulatory capacities in *in vivo*-studies and helminths and their products are thus being tested in clinical trials (58).

### 1.3.1 Effect of the microbiota on the immune system and chronic diseases

Epidemiological studies indicated that the initiation of allergic disease takes place in early infancy (59-61). Other studies provide evidence for an increased incidence of asthma in the industrialized countries over the past decades as a consequence of environmental changes. Proposed aspects include a change of diet, improved hygienic cleanliness, widespread use of antibiotics, and therefore altered diversity and density of the human microbiota (57, 60, 61). Many other immune disorders and autoimmune diseases such as type 1 diabetes (62), inflammatory bowel disease (5, 63-65), multiple

sclerosis (66), and obesity (62) have been reported to be related to altered microbial compositions in affected patients. Deciphering the exact interplay of the microbiota and the host immune system would provide valuable insights of how commensal bacteria and their host co-evolved to preserve protective mechanisms and to find better therapies.

In fact, until today no experimental models were available to elucidate the direct link between the manifestation of allergic airway disease and the impact of the commensal microbiota.

### 1.3.2 Germ-free mouse models

All mammals are sterile until birth and thereafter are gradually colonized with commensal bacteria (67-70). Ever since these observations have been made in the late 19$^{th}$ century, the aim was to create a model system to study the mutualism between the mammalian host and its commensal microorganisms. The development of sterile isolators harboring axenic (germ-free) mice and rats has become a valuable tool to investigate certain aspects of the host and its commensals (71). For this purpose animals are housed in sterile flexible isolators and are given autoclaved food and water (59). These animals show major defects in the development of GALT with a decreased size of mesenteric lymph nodes and PPs and an absence of mature ILFs (72). The foremost immunological difference was found to be a decrease in secretory IgA levels in the intestine of germ-free mice (73). A possible explanation for this phenomenon has recently been provided by studies describing the induction of IgA-producing B cells by dendritic cells, which have sampled antigen from commensal bacteria (74, 75). This mechanism was shown to be important to prevent the bacteria from crossing the epithelial barriers (8). Thus, evidence of gut commensals shaping the host immune system is increasing constantly. A great maen allowing for the

detailed study of this interaction is the selective introduction of specific bacterial strains into an "empty" model system, which will be colonized within days (74). Recently, Hapfelmeier et al. introduced a reversible colonization protocol, which allows the investigation of the dynamics of immune responses induced by a specific *E.coli* strain (76).

### 1.3.3 Murine model for allergic airway inflammation

Murine models for asthma should display many features of the human disease, like allergen specific immunoglobulin production, acute and chronic inflammation, airway remodeling, and airway hyperressponsiveness. So far, no murine model sytem is able to provide all of the above features. The outcome of animal models is dependent on the route of allergen exposure, the timing/frequency of allergen-challenges, the type of adjuvant used, and the genetic background of the mouse strain. Therefore, a variety of murine models, addressing different aspects of allergic airway inflammation, are being used today. The most common model of the past decades takes advantage of a strong systemic sensitization involving chicken egg ovalbumin (OVA) (77) together with aluminum hydroxide (alum). The systemic priming is carried out by single or repetitive intraperitoneal injections of OVA/alum, followed by a local airway challenge with the same antigen. This leads to strong eosinophilic airway infiltration, subsequent airway hyperresponsiveness together with an increased secretion of the Th2 cytokines IL-4, IL-5, IL-13, and elevated levels of IgG1 and IgE. More recently, models which utilize more physiological approaches to common allergenic substances like house dust mite (HDM), birch pollen, cat allergy, and bee / wasp venom allergy are being used more widely (78). Here, both the sensitization and challenge are carried out via the airways, providing a valuable tool to study allergic airway inflammation in a more realistic model system (79).

## 1.4 Protective immunity against helminth infections

Over one quarter of the worlds population is infected with helminth parasites (80). In 2008 about 2 billion people suffered from soil-transmitted nematode infections, including *Ascariasis, Trichuriasis* and Hookworms (81). These infections are usually not fatal but are major causes of morbidity, leading to anemia and malnutrition during chronic infections (82). In order to develop effective therapies and potent vaccines a more detailed understanding of the host-parasite interaction is desirable. The current understanding of protective immunity against enteric helminth infections will be described in the following.

### 1.4.1 Role of innate immunity during helminth infection

Innate immune cells, such as dendritic cells, neutrophils, eosinophils, basophils, mast cells and macrophages are the first to respond to invading pathogens. Their role exceeds the function of first effector cells as they can also sense the danger and pathogen-associated molecular patterns, and activate the adaptive arm of immunity. Depending on the cytokines secreted, innate immune cells direct the immune response toward a Th1 or Th2 phenotype, but they also act to sustain ongoing immune responses. Neutrophils and macrophages are typically the first responders during helminth infections. Macrophages can develop into different subsets when activated. Upon exposure to IFN-γ (often in conjuction with LPS), polarization towards classically activated macrophages occurs, whereas Th2 cytokine environment leads to alternatively activated macrophages (AAM) (83). AAM are characterized by the expression of arginase-1, mannose receptor (CD206), and high expression levels of the IL-4 receptor (84, 85). AAM accumulate during challenge – but not primary – infections with the enteric parasite *H. polygyrus bakeri* (86). Their main functions are to regulate immune responses, the induction of wound healing, and conferring

resistance against parasite infections, by releasing defense factors such as chitinase, RELMα (also known as FIZZ1), and matrix metalloproteinase (MMP) 12 (87, 88). During submucosal invasion of stage 3 larval parasites, neutrophils get activated and are rapidly recruited to the sites of infection (11). A direct effect of neutrophils on damaging the parasite *Strongyloides stercoralis* has been demonstrated *in vitro* and an important role for host resistance to this helminth has been proposed *in vivo* when acting in concert with eosinophils (89). Other granulocytes associated with Th2 immune responses are eosinophils, basophils and mast cells. Numbers of blood eosinophils and basophils strongly increase during helminth infections (90, 91). Eosinophils are recruited to the site of infection and release their granule contents, which are able to damage the parasite especially during challenge infections (92). Eosinophils and basophils have also been proposed to promote Th2 development by secreting IL-4 during early inflammatory responses (91). Recently, basophils have gained significant attention in the context of Th2 immune responses, including allergic diseases and helminth infections (93, 94). Antibodies bound to Fc receptors, cytokines, TLR ligands and proteases can activate basophils, which in turn release preformed mediators like histamine, heparin or antimicrobial peptides as well as de novo formed lipid mediators such as leukotriene C4 or prostaglandin D2 (PMID:21712025) (95). Upon activation basophils also synthesize and release cytokines, like IL-4, IL-5, IL-13 and TSLP (91, 95). The expression of high affinity IgE receptor (FcεR), expression of toll-like receptors (TLR) 2 and TLR4, degranulation and release of granule contents upon activation, as well as the secretion of IL-4 are characteristics shared by both basophils and mast cells. Nevertheless, they appear to play independent roles in the immune response. For instance during anaphylaxis, IgE largely evokes mast cell-mediated responses whilst IgG causes

systemic anaphylaxis by activating circulating basophils in response to blood borne allergens (96, 97).

While mast cells are not necessary for clearance of challenge infections with *H. polygyrus bakeri*, it has been proposed that they play a potential role in reducing the fecundity of this helminth species (98). In addition to granulocytes and mast cells, innate lymphocyte populations have been reported to contribute to protective immunity against helminths (30). Recently described other innate sources of IL-4, IL-5, and IL-13 are nuocytes, natural helper cells (NHC), innate helper 2 (IH2) cells, and multi-potent progenitor $(MPP)^{type2}$ cells (31, 99, 100). Even though these cells seem very similar with respect to their function and cytokine production, they express slightly different levels of the surface marker stem cell factor (SCF) receptor, also known as c-kit as well as Sca-1, also known as Ly-6a, which could be related to their distinct sites of localization (29, 30). In this context, also non-lymphoid cells like intestinal epithelial cells are known to impact on immune homeostasis and inflammatory responses (101). IL-25 and IL-33 produced by intestinal epithelial cells can impact on helminth immunity by activating other immune components such as nuocytes, NHCs, IH2, and $MPP^{type2}$ cells (29, 30). In contrast, TSLP which is also released from intestinal epithelial cells has been shown to impact on DCs (80, 102).

### 1.4.2 Role of adaptive immunity during helminth infection

Helminths elicit a potent Th2 response, as described in section 1.2.3, which includes the activity of CD4+ Th2 effector cells and the activation of IgG1 and IgE secreting plasma cells (103). The activated Th2 cell produces IL-3, IL-4, IL-5, IL-9, and IL-13 (104). IL-4 and IL-13 alter epithelial and smooth muscle cell function, leading to the "weep and sweep" (increased secretion and motility) mechanism, which enables the host not to kill but, to clear the adult helminth from the intestinal lumen (105).

Additionally T regulatory cells are induced during helminth infection and contribute to the down-modulation of inflammatory responses and therefore act contrary to the clearance of the parasite (106). The interaction of T cells with B cells in the context of IL-4 receptor signaling leads to B cell class switching to IgG1 and IgE (107). During helminth infections increased levels of the antibodies of the IgE, IgG, and IgM subclasses are produced (108). IgE can bind to FcεR on mast cells and basophils, which leads to their activation and the release of granule content and cytokines. However, it has been shown that IgE is not crucial for the protective immunity against parasites like *H. polygyrus bakeri* or *Nippostrongylus brasiliensis* (77, 109). Nevertheless, in the absence of B cells or in AID deficient mice, which lack the ability to undergo isotype-switching, the parasite burden is increased after challenge infection with *H. polygyrus bakeri* (109). Besides their role as antibody producers, B cells also secrete cytokines, which impact on the ongoing Th2 response (110). The protective effect of antibodies could be confirmed by serum or purified IgG transfer in the parasite infection model with *H. polygyrus bakeri* (109, 111). Although, the exact mechanisms of antibody-mediated immunity remains elusive, possible mechanisms include direct neutralization of parasitic enzymes inhibiting feeding or migration as well as the activation of cellular components via antibody receptor crosslinking (109, 111).

### 1.4.3 Role of Fc-Receptors and Antibody-dependent cellular Cytotoxicity

As mentioned for IgE in section 1.4.1 and 1.4.2, antibodies can bind to Fc-receptors, which activates innate cells, mediates antibody-dependent cellular cytotoxicity (ADCC), and also serves to modulate immune responses (112). Additionally, the receptors for the Fc fragment of immunoglobulins have also been described to serve as potent regulators in autoimmunity (113, 114). Therefore understanding the role of

Fc receptors in health and disease provides valuable insights for the crosstalk of the two arms of innate and adaptive immunity. The most abundant antibody isotype is IgG, which comprises four subclasses in mice (IgG1, IgG2a, IgG2b, IgG3) and binds to Fcγ-receptors (FcγR), of which four different forms are known, namely FcγRI, FcγRIIB, FcγRIII, and FcγRIV (112). While FcγRI, FcγRIII, and FcγRIV are activating members, associating with the common γ-chain carrying immunoreceptor tyrosine based activating motifs (ITAM), FcγRIIB is the one inhibitory member with an immunoreceptor tyrosine based inhibitory motif (ITIM) in its cytosolic domain (115). The only high affinity receptor FcγRI binds preferentially monomeric IgG, whereas the other FcγRs show only low to medium affinity and especially signal in the context of complexed IgG ligation (116). The protective role of antibodies in general is mediated by Fc receptor crosslinking on innate cells, which leads to enhanced phagocytosis, degranulation, release of inflammatory mediators, and cytokines, as well as to antibody-dependent cellular cytotoxicity (115). Important insights came from the use of specific mouse knockout studies, for instance Fc common γ-chain knockout ($FcR\gamma^{-/-}$), which lack the receptors FcγRI, FcγRIII, FcεRI, and FcαRI (117). It could be demonstrated that both FcγR ligation as well as complement activation assist during host antibody-mediated protective immunity against *H. polygyrus bakeri* (109).

### 1.4.4 Murine models of helminth infections

To study the nature of the Th2 responses of the host organism against helminth parasites, rodent models are used widely. In our studies, we focus on the rodent roundworm species *Heligmosomoides polygyrus bakeri* and *Nipposprongylus*

*brasiliensis*, which can be used as an animal model that reflects human hookworm infections (80).

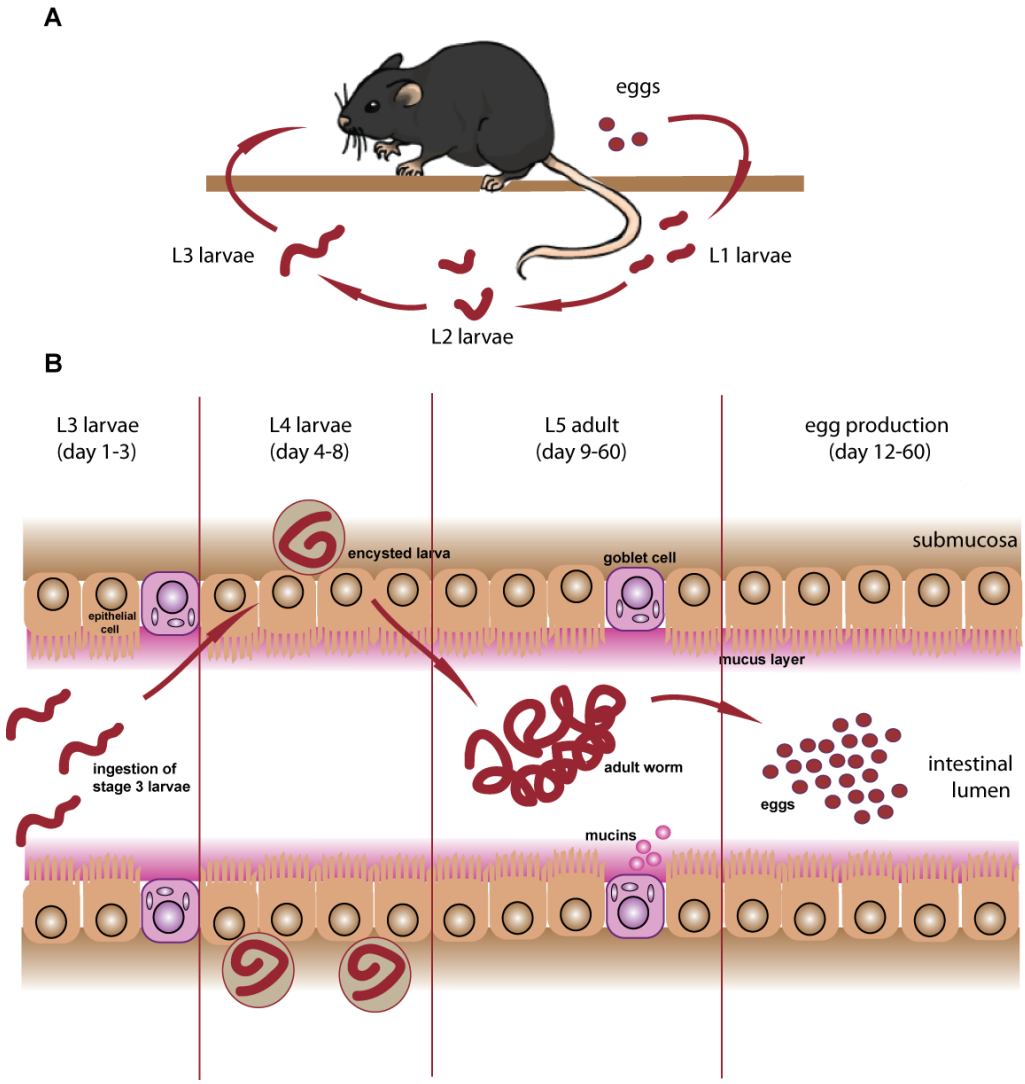


**Figure 4:** Life cycle of *Heligmosomoides polygyrus bakeri*. A) The eggs hatch outside the host and develop through several larval stages into infective L3. B) The life cycle of the parasite inside the rodent host involves ingestion of stage 3 larvae, which enter the submucosa and pass through several molts before maturing to the L4 stage. After 9 days L4 leave the submucosa and mature into adult worms in the lumen of the small intestine, where they mate and produce eggs (86).

The intestinal nematode *Heligmosomoides polygyrus* has undergone several name changes in the past 4 decades and is referred to in this thesis as *H. polygyrus bakeri*. Unlike *Nippostrongylus brasiliensis*, *H. polygyrus bakeri* induces chronic infections in rodents, which last for over 30 weeks (118). The life cycle of *Heligmosomoides*

*polygyrus bakeri* in the murine infection model can be described in two different parts (86). The stage 3 larval parasite enters the rodent host through the gastrointestinal tract, migrates into the submucosa of the small intestine, where it undergoes two molts and can be found in the intestinal lumen as an adult worm after about 9 days following infection (Figure 4 B). The adult parasite mates in the intestinal lumen and produces eggs from day 12 until at least day 60 following infection. To complete its lifecycle stage 1 larvae hatch from the eggs outside of the rodent host (Figure 4 A). The primary infection is chronic but can be cleared by treatment with an antihelmintic drug. The immunocompetent host can then expel the worm after 2 weeks of challenge infection, rendering this model system particularly interesting for the study of memory responses (119).

Another helminth, commonly used in mouse models, and occuring naturally in rats is the parasite *Nippostrongylus brasiliensis*. In contrast to *H. polygyrus bakeri* , larvae of *Nippostrongylus brasiliensis* enter through the skin, move deep through loose subcutaneous tissue, where they invade blood vessels and enter their tissue-invasive phase in the lung and rather than the small intestine. From the lung larvae migrate via the trachea into the gut, where they develop into adult worms, mate and produce eggs beginning 6 days following infection. Worm expulsion usually occurs between days 10 and 13 post infection (120).

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# 2 Dysregulation of allergic airway inflammation in the absence of microbial colonization

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## 2.1 Abstract

The incidence of allergic disorders is increasing in developed countries and has been associated with reduced exposure to microbes and alterations in the commensal bacterial flora. To ascertain the relevance of commensal bacteria upon the development of an allergic response, we utilized a model of allergic airway inflammation in germ-free (GF) mice that lack any exposure to pathogenic or non-pathogenic microorganisms. Allergic airway inflammation was induced in GF, specific pathogen free (SPF) or recolonized mice by sensitization and challenge with ovalbumin (OVA). The resulting cellular infiltrate and cytokine production were measured. Our results show that the total number of infiltrating lymphocytes and eosinophils were elevated in the airways of allergic GF mice as compared to control SPF mice, and that this increase could be reversed by re-colonization of GF mice with the complex commensal flora of SPF mice. Exaggerated airway eosinophilia correlated with increased local production of Th2 associated cytokines and elevated IgE. These data demonstrate that the presence of commensal bacteria is critical for control of allergic airway inflammation.

## 2.2 Introduction

Variations in exposure to environmental microbes during the early years of life have been implicated in protecting against, or enhancing susceptibility to allergic diseases (2). An increased prevalence of allergies in populations living within developed countries has clearly been documented over the last few decades; a phenomenon that has been hypothesized to be a result of decreased exposure to infectious microorganisms (3). More recently, dysbiosis of commensal bacteria has also been postulated to modulate the development of allergic disease based on findings of

distinct compositions of bacterial communities found within the stool samples of allergic and non-allergic infants (4-6).

Commensal bacteria colonize the mucus membranes and the skin of humans soon after birth with the greatest density found within the intestine (with approximately $10^{12}$ organisms per gram of intestinal content, represented by approximately 1,000 different species) (7). The exact number and diversity of an individual's community of commensal bacteria appears to be determined by factors occurring in early childhood, including the type of birth (natural versus caesarean) (8), diet (formula versus breast-milk) (9), early use of antibiotics and environmental conditions (10). Although the bulk of bacteria reside within the intestine, the upper respiratory tract also harbours bacteria (11) and is likely to be repetitively inoculated with the normal bacterial flora of the pharynx. In addition it has been suggested that immune responses in the intestine and lung are linked through yet undefined mechanisms (12). Thus, microbial colonization and exposure to microbial products or metabolites in the intestine may have profound effects upon the lung and vice versa.

Understanding whether and how commensal bacteria can modulate allergic diseases is crucial for the development of preventative strategies based on the use of bacteria and their products, including pro-biotics and pre-biotics. To address this question we used in-bred GF mice as an experimental model, allowing us to circumvent the impact that varied nutritional status, diverse genetics and lifestyles, and individual histories of medical care including antibiotic treatment, can have on epidemiological studies. GF mice were housed and maintained under strict conditions to ensure a complete lack of exposure to environmental microorganisms (bacteria, fungi, viruses). The impact of commensal bacteria was then investigated by comparison of allergic airway inflammation in GF mice, SPF mice and GF mice re-colonized with a complex SPF

microbiota. Age and sex matched mice were maintained under the same environmental conditions and fed an identical diet for the entire duration of these studies.

Using this model we demonstrate, for the first time, that commensal bacteria play a fundamental role in shaping the type and extent of Th2 inflammatory responses in the lung. The absence of a commensal microbiota leads to an overall exaggerated allergic airway inflammation.

## 2.3 Materials and Methods

Germ-free C57BL/6 mice were kindly provided by the Institute of Laboratory Animal Science, University of Zurich or from the Clean Animal Facility, University of Bern, Switzerland. Germ-free mice were housed in flexible isolators until the day of sacrifice. Fecal samples of the germ-free mice and swabs of the inner wall of the isolator were cultured under aerobic and anaerobic conditions. Additionally, gram stains and DNA stains were performed of fecal samples collected immediately before export from the isolators. SPF C57BL/6 mice (Harlan Laboratories, Füllinsdorf, Switzerland) were maintained in a pathogen-free animal facility and were given the same food as germ-free mice and water *ad libitum*. Re-colonization of 5-6 weeks old germ-free mice was established by putting an SPF mouse into the same cage for at least 3 weeks prior to the first immunization. Studies were carried out with mice aged 9-12 weeks. Animal experiments were performed according to institutional guidelines and to Swiss federal and cantonal laws on animal protection. Experiments involving germ-free and SPF JHD mice were performed at McMaster University in Hamilton, Canada and were conducted with approval from the McMaster University Animal Care Committee.

**Protocol for experimental allergic airway inflammation**

Mice were immunized with 100 μg OVA (Sigma-Aldrich, Steinheim, Germany) and 200 μl 2% aluminum hydroxide (SERVA Electrophoresis GmbH, Heidelberg, Germany) via the intraperitoneal route. On day 9 and 10, post immunization mice were challenged intranasally with 100 μg OVA suspended in 50 μl sterile PBS. Control mice either received immunization with aluminum hydroxide only and were challenged with OVA in sterile PBS, according to the protocol used for allergic mice, or were untreated. Both control groups showed comparable results. Airway hyperresponsiveness was assessed on day 3 after the last intranasal challenge using whole body plethysmography (Buxco Electronics, Inc., Petersfield, United Kingdom). Mice were placed in individual unrestrained chambers where airflow obstruction was induced with aerosolized methacholine-chloride (MetCh, Aldrich Chemie, Steinheim, Germany). This procedure estimates total pulmonary airflow in the upper and lower respiratory tracts. The chamber pressure was used as a measure of the difference between thoracic expansion (or contraction) and air volume removed from (or added to) the chamber during inspiration (or expiration). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics, Petersfield, United Kingdom). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh and monitoring the breathing pattern for 5 min after initiation of aerosol dose. Germ-free mice were exported from isolators one day before sacrifice to perform airway hyperresponsiveness tests. All mice were sacrificed on day 4 after the last intranasal challenge, the day after airway hyperresponsiveness test. To analyze the cellular compartment of the airway lumen broncho-alveolar lavage (BAL) was performed. For this purpose a small incision on the trachea was made, through which a catheter was

introduced. The lungs were flushed three times with 1 ml PBS. BAL cells were harvested by centrifugation. Total cell numbers per BAL were determined by using Coulter Counter (IG Instrumenten Gesellschaft AG, Basel, Switzerland). Differential cell counts were performed on cytospins stained with QuickDiff (Dade Behring, Siemens Healthcare Diagnostics, Deerfield, USA) and the percentage of eosinophils, neutrophils, macrophages, and lymphocytes within a total population of 200 cells was determined.

**Histology**

Lungs were harvested after broncho-alveolar lavage and perfused with PBS to remove excess blood. Lungs then were inflated with 1 ml of 10% formalin and embedded into paraffin. Prepared sections (4 μm) were stained with hematoxylin and eosin (H&E) and periodic acid Schiff reaction (PAS) using standardized protocols and analyzed with Axioskop 2 plus microscope equipped with AxioCam HRc (Carl Zeiss Microimaging GmbH, Jena, Germany).

**Flow Cytometry**

For analysis of cytokine production, 2.5-5 x $10^5$ cells from BAL cells were stimulated with PMA, ionomycin, and monensin (Sigma–Aldrich, Steinheim, Germany) for 3 h at 37 °C in complete Iscove's Modified Dulbecco's Media (IMDM) medium containing 10% fetal calf serum (FCS). Thereafter, cells were stained with PerCP-labeled anti-CD4 mAb, fixed with 2% paraformaldehyde, permeabilized in saponin buffer then stained with anti-cytokine antibodies (IL-4, IL-5, IL-10 and IFN-γ; BioLegend, San Diego, USA). All cells were recorded using BD FACSCalibur or BD LSR II (BD Biosciences, San Jose, USA). Samples were analyzed using FlowJo 8.8.6 software (Tree Star Inc., Ashland, USA).

**ELISA and Multiplex**

Antibody titers of IgA and IgE in BAL fluid and serum were tested using sandwich ELISA as described (13). In brief, 96 well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with unlabeled goat anti-mouse antibodies to either IgA (Southern Biotech, Birmingham, USA) or IgE (clone 6HD5) in ELISA coating buffer (5.88 g/l NaHCO3, 3.18 g/l Na2CO3 (Fluka, Sigma-Aldrich, Steinheim, Germany) in ddH2O, pH 9.6) overnight at 4°C. Plates were washed with PBS/0.05% Tween H20 (Fluka, Sigma-Aldrich, Steinheim, Germany) and blocked with PBS/1% BSA for 2 h at room temperature (RT). Serum was serially diluted in PBS/0.1% BSA starting with a 10 fold dilution. Broncho-alveolar lavage supernatant was used undiluted. Purified mouse IgA (BD Pharmingen, New Jersey, USA), or mouse IgE (clone TIB141) served as standards and were serially diluted in parallel to the samples starting from a concentration of 3 mg/ml and according to the isotype of the coating antibody. Samples and standards were incubated for 2 h at RT then washed extensively and incubated with alkaline- phosphatase-labeled goat anti-mouse antibodies to IgA (Southern Biotech, Birmingham, USA) or using biotin-conjugated anti-IgE (clone RIE-4) followed by a streptavidin conjugated alkaline-phosphatase antibody diluted into PBS/0.1% BSA, according to the isotype of the coating antibody. Plates were incubated for a further 1 h at RT, washed extensively, then developed using the substrate p-nitrophenyl phosphate (Sigma-Aldrich, Steinheim, Germany). ODs were measured on an ELISA reader (Bucher Biotec, Basel, Switzerland) at 405 nm. OVA-specific titers of IgA, IgE and IgG1 were determined by ELISA coated with 50 μg/ml OVA in ELISA coating buffer and detected in the same manner as for the total IgA, IgE. OVA-specific IgG1 of serum samples was determined with a starting dilution of

100 fold. Quantification of IL-4, IL-5, IL-10, IL-13 and IFNγ in BAL fluid and serum was performed using BioPlex Kit (BioLegend, San Diego, USA) multiplex assays, following the manufacturer's instructions. For analyzing total TGF-β in supernatant of BAL samples the human/mouse TGF-β 1 ELISA Ready-Set-Go! (eBioscience, San Diego, USA) assay was used, following the manufacturer's instructions.

**Statistical analysis**

Student's t test (unpaired, two-tailed) was used to calculate significance levels between treatment groups. *P* values of less than 0.05 were considered significant and are depicted as *p<0.05; **p<0.01; ***p<0.001. Graph generation and statistical analysis was performed using Prism version 4.0c software (GraphPad, La Jolla, USA).

## 2.4 Results

**Regulation of allergic airway inflammation by the commensal microbiota**

To directly assess the impact of commensal bacteria on allergic airway inflammation we induced an OVA-specific Th2 inflammatory response in the lungs of SPF mice (SPF), germ-free mice (GF) and GF mice re-colonized with a complex SPF microbiota (Figure 1 A). GF mice exhibited increased airway hyper-responsiveness (AHR) upon methacholine challenge compared to SPF mice as determined using a whole body unrestrained plethysmograph (Figure 1 B). Although this is not a direct invasive measurement of lung function, these data are highly indicative of exaggerated airway constriction and Th2 inflammation. Increased AHR in the absence of commensal bacteria was concomitant with an increase in the total number of cells infiltrating the airways (Figure 1 C), which could be accounted for by elevated numbers of eosinophils (Figure 1 D) and lymphocytes (Figure 1 E). Prior re-

colonization of GF mice was sufficient to ensure that this exaggerated Th2 response did not develop (Figure 1 C-E). These changes could also be observed in histological sections where OVA sensitized and airway challenged GF mice exhibited increased goblet cell hyperplasia and increased peri-vascular and peri-bronchial inflammatory cell infiltration as compared to SPF mice (Figure 1 F).

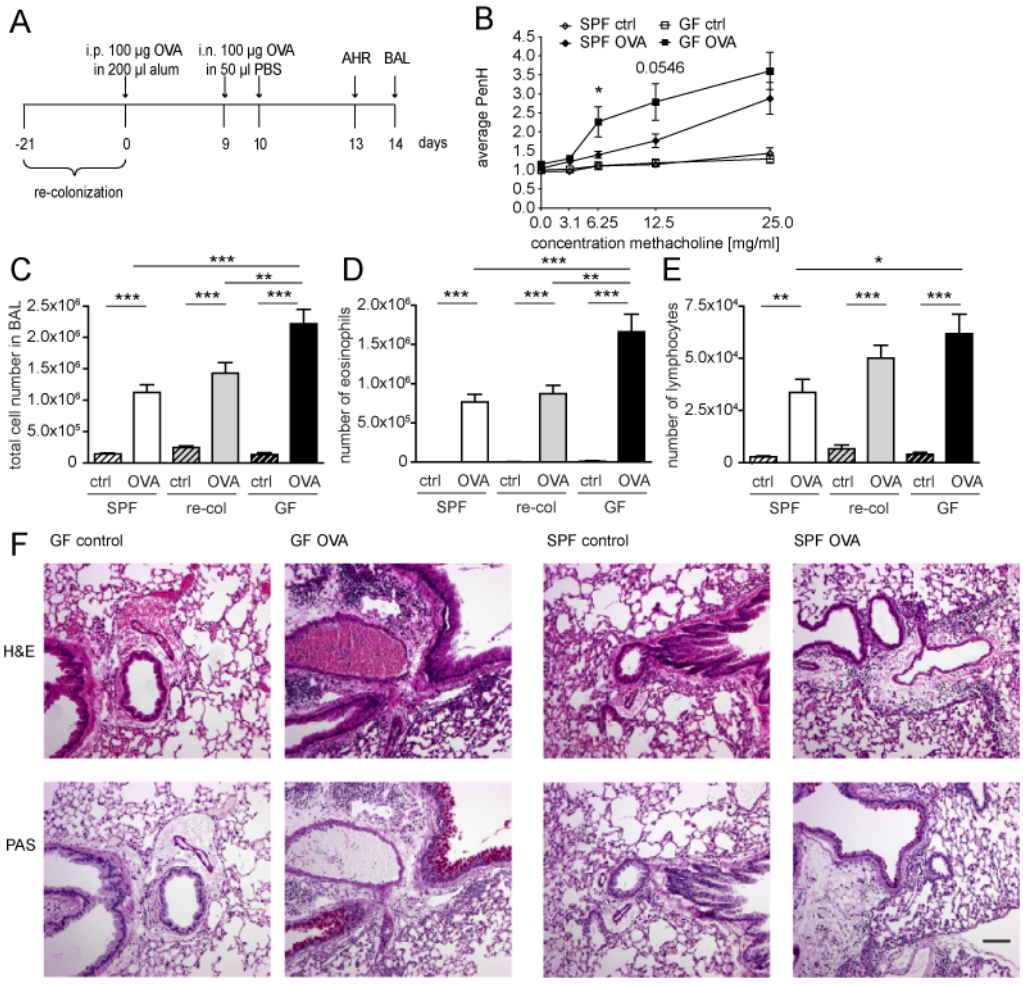


**Figure 1.** Commensal bacteria down-modulate exacerbated allergic airway inflammation. A) Experimental setup of OVA-induced allergic airway inflammation in C57BL/6 SPF, GF or re-colonized mice. B) Airway hyperresponsiveness as assessed in OVA (OVA/alum immunized, OVA challenged) or control (alum only immunized, OVA challenged) mice by whole body plethysmography (n=5-9) C-E) Numbers of C) total cells, D) eosinophils, E) lymphocytes in the broncho-alveolar lavage (BAL) as assessed by counting total BAL cells and performing differential cell counting of cytospins (controls: n=6, OVA: n=10-14). Results are pooled from three experiments and are representative of 5 independent experiments. Mean ± SEM is shown. Increased infiltration and mucus production in GF compared to SPF OVA

immunized and airway challenged mice. F) Histological sections of lungs from SPF and GF control or OVA mice were stained with H&E or PAS and analyzed by light microscopy (black bar = 100 μm). Pictures show representative samples of 4-6 mice per group from one experiment and are representative of two independent experiments.

To provide further evidence of the physiological relevance of this model, we applied an intranasal only sensitization and challenge model in germ-free compared to SPF mice. During this model the allergen OVA was administered via intranasal injections on three consecutive days, in the time course of three following weeks. We found an increase in eosinophils both in germ-free and SPF OVA treated groups compared to controls (Figure 2 A-B). Eosinophils seem to be increased in germ-free compared to SPF OVA treated mice, however these findings did not reach significant levels. Also the bacterial controls showed a contamination of the germ-free group. Because the procedure acquired more manipulations of the mice and carries therefore increased risk of contamination, we decided to proceed with the *intra peritoneal* immunization protocol.

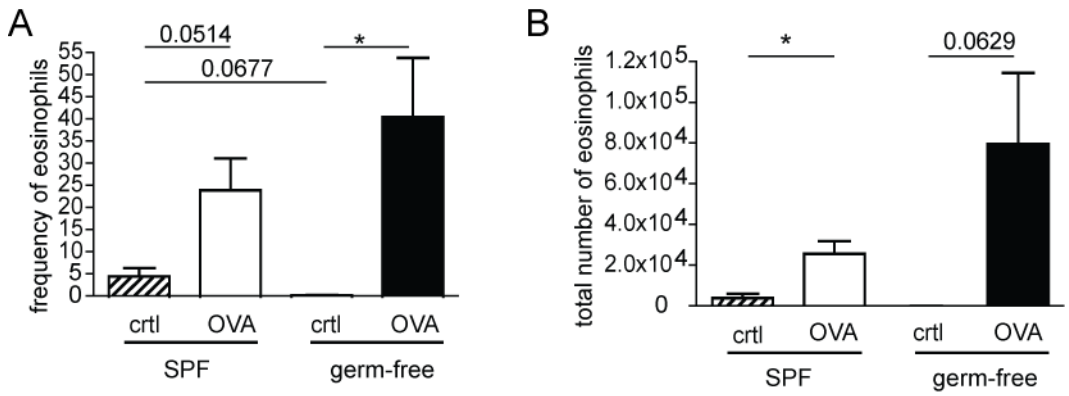


**Figure 2.** Commensal bacteria down-modulate exacerbated allergic airway inflammation. For immunization and challenge of SPF and germ-free mice an OVA intranasal only model was applied. Frequency of A) and total number of B) of eosinophils in the broncho-alveolar lavage (BAL) as assessed by counting total BAL cells and performing differential cell counting of cytospins (controls: n=4, OVA: n=5). Results are representative of one experiment. Mean ± SEM is shown.

To investigate the activation state and function of Th2 cells, $CD4^+$ T cells isolated from the broncho-alveolar lavage (BAL) were stimulated *ex vivo* with PMA and ionomycin and the production of IL-4, IL-5, IL-10, and IFN-γ determined by intracellular cytokine staining. In keeping with the increased allergic infiltrate, the percentage of $CD4^+$ T cells producing IL-4 and IL-5 were increased in GF mice as compared to SPF or re-colonized groups (Figure 3 A-B). By contrast, the percentage of cells producing the regulatory cytokine IL-10 (Figure 3 C) or the prototypic Th1-type cytokine IFN-γ (Figure 3 D) were similar for all groups.

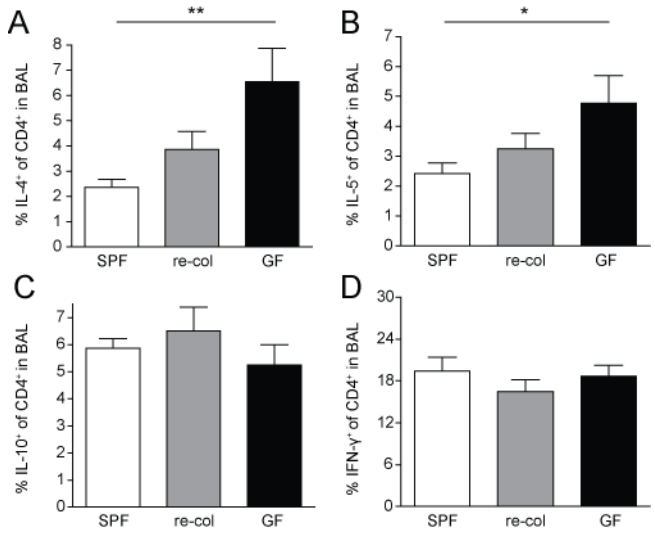


**Figure 3.** The absence of commensal bacteria results in enhanced Th2 cytokine production by BAL T cells. C57BL/6 SPF, GF, and re-colonized mice were subjected to OVA immunization and challenge (OVA) or OVA challenge only (control) as depicted in Figure. 1. A-D) Percentage of airway $CD4^+$ T cells producing A) IL-4, B) IL-5, C) IL-10 or D) IFN-γ following *ex vivo* re-stimulation with PMA/ionomycin/monensin were determined by intracellular staining and flow cytometry. Results are pooled from two experiments (n=7-10) and are representative of 4 independent experiments. Mean ± SEM is shown.

Additional multiplex or ELISA analysis of BAL fluid confirmed the increase of IL-4 *in situ* in inflammatory GF samples whilst no significant differences between GF and SPF mice were noted for IL-5, IL-13, IL-10, IFN-γ or TGF-β (Figure 4).

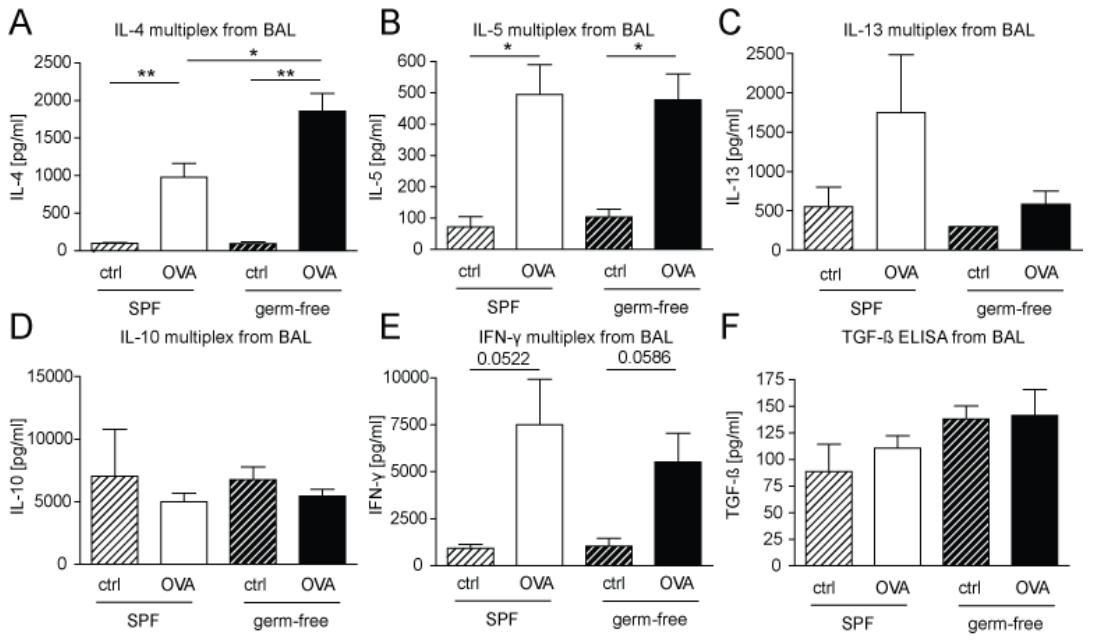


**Figure 4.** The absence of commensal bacteria results in increased levels of IL-4 in the BAL fluid of allergic mice. C57BL/6 SPF or GF mice were subjected to OVA immunization and intranasal challenge (OVA) as depicted in Figure 1. 1 ml of BAL fluid was collected, immediately placed on ice and then stored at -20°C. Cytokine concentrations were determined by Multiplex according to the protocol of BioLegend Bioplex assay. Levels of A) IL-4, B) IL-5, C) IL-13, D) IL-10, and E) IFN-γ. Data from 3-4 mice per group in one experiment are shown and represent mean ± SEM. Levels of TGF-β in BAL supernatant are presented in F) and were assessed using the eBioscience ELISA Ready-Set-Go! assay. Results are pooled data of three independent experiments and represented as mean ± SEM.

These results provide evidence for a selective increase in the production of IL-4 within the airways of GF mice following OVA immunization and intranasal challenge, which does not appear to be related to a dysregulated development of type 1 immunity or altered production of regulatory cytokines.

**Altered antibody production in the absence of commensal bacteria is not responsible for enhanced allergic airway inflammation.**

IgE production has previously been reported to be increased in GF mice (14-17). We therefore investigated the production of antibodies following OVA immunization and challenge. Total IgE levels were non-significantly elevated in the serum of GF mice prior to OVA immunization and challenge (Figure 5 A-B, control groups). As

expected, levels of total IgE in the serum increased following OVA immunization in all groups (Figure 5 A). However, GF mice exhibited an augmented IgE response following OVA intranasal challenge as compared to the SPF or re-colonized groups (Figure 5 B). This correlated with enhanced production of IL-4 in the airways and indicated that the most striking impact of commensal bacteria on this Th2-mediated response occurred following local exposure to OVA within the airways and lung. Consistent with these findings total IgE levels in the BAL and the production of OVA-specific IgE could only be detected in significant amounts in the serum of GF mice subjected to OVA sensitization and challenge (Figure 5 C-D). No significant differences were noted for OVA-specific IgG1 in the serum of any group (Figure 5 G).

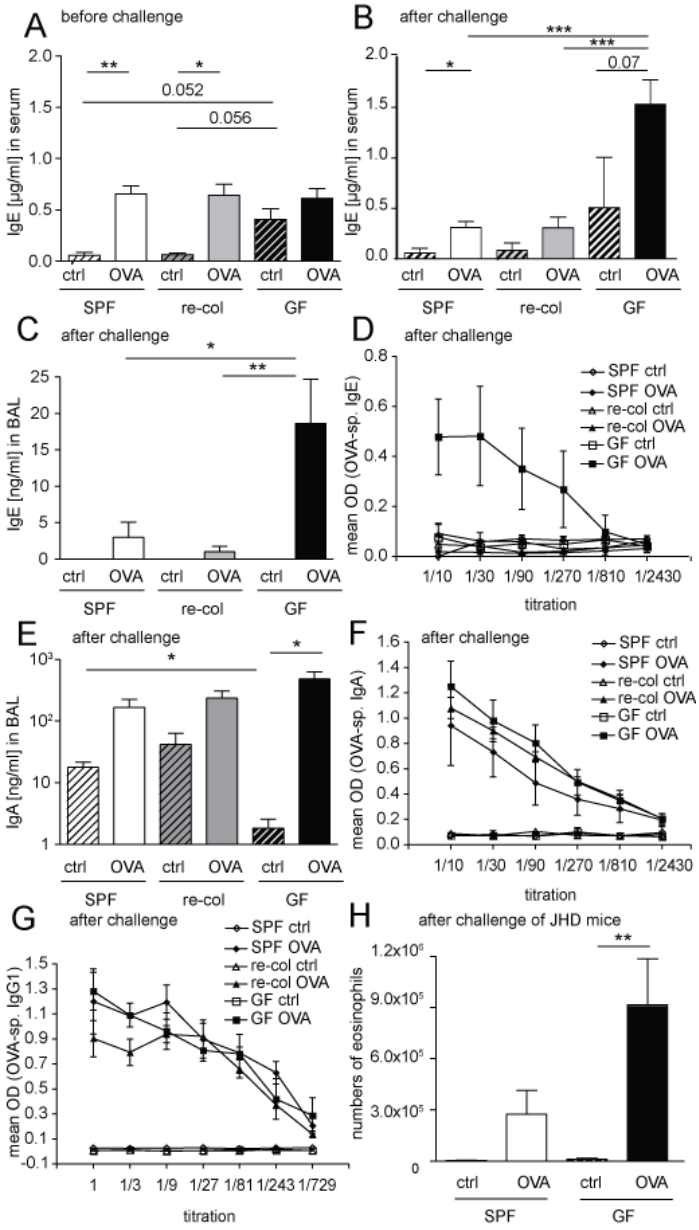


**Figure 5.** Elevated antibody production in the absence of commensal bacteria is not responsible for increased allergic airway inflammation. A-G) Antibody titers in C57BL/6 SPF, GF, and re-colonized mice subjected to OVA immunization and challenge (OVA) or OVA challenge only (control) as depicted in Figure. 1. A-C) Total levels of IgE are shown for A) serum before intranasal OVA challenge or B) serum after OVA challenge (controls: n=4-9, OVA: n=12-20) and C) BAL fluid after OVA challenge (controls: n=4, OVA: n=6-10). D) OVA-specific IgE in serum after intranasal OVA challenge. E) Total and F) OVA-specific IgA in BAL after intranasal OVA challenge (n=3-6). G) OVA-specific IgG1 levels in serum after intranasal OVA challenge (n=3-6). Results A-C) are pooled data from three experiments and are representative of 5 independent experiments. Results D-G) are data from one experiment and are representative of 3 independent experiments. H) Total numbers of eosinophils in BAL of JHD GF or SPF mice following OVA immunization and intranasal challenge. Results are pooled from two independent experiments (n=3-9). Mean ± SEM is shown.

Sudo *et al.* (16) also demonstrated increased basal and OVA-induced IgE in GF mice and reported that mono-colonization with *Bifidobacterium infantis* could reverse elevated IgE production but only if the bacterium was present from birth. In this report we show that exposure of GF mice to a complex commensal flora as adults is also effective at reversing elevated IgE production (Figure 5 A-D). These data indicate that exposure to a mixture of bacterial species may be more potent at attenuating IgE production as compared to a single species. Accordingly, the findings by Sudo *et al.* (16) that bacterial exposure must occur at birth may simply reflect a difference in the total length of time required for a single bacterium, or bacterial species, as compared to a complex mixture of bacteria to modulate IgE production.

Smits *et al.* (18) recently reported that the delivery of cholera-toxin pulsed DC to the airways of experimental mice promotes the production of local IgA and provides protection against allergic airway inflammation. We therefore investigated the secretion of IgA into the BAL of OVA immunized and intranasally challenged GF mice. In keeping with previous reports of attenuated IgA in GF mice (7), significantly decreased amounts of total IgA were detected in the BAL of control GF mice compared to control SPF mice (Figure 5 E). However, total and OVA-specific IgA levels in the BAL were similar for all groups having received both OVA immunization and intranasal challenges (Figure 5 E-F).

Taken together, these data indicate that the absence of commensal bacteria leads to an attenuated production of IgA in the airways prior to allergen sensitization, and to exaggerated total and OVA-specific IgE responses after intranasal challenge. To determine whether the observed alterations in antibody production contributed to the enhanced airway inflammation observed in GF mice, we investigated OVA-mediated allergic eosinophilia in GF JHD mice, which carry a targeted mutation in the $J_H$

region of the IgM locus and cannot generate mature B cells (19). As observed for C57BL/6 mice, the absence of commensal bacteria in JHD mice resulted in enhanced allergic airway eosinophilia (Figure 5 H), although this did not reach statistical significance. These data indicate that although commensal bacteria modulate antibody production, this process is independent of their impact on allergic airway inflammation.

## 2.5 Discussion

Our findings of exaggerated OVA-induced airway inflammation in GF mice provide the first experimental evidence for a functional impact of commensal bacteria on allergic inflammation in the lung. Re-colonization of GF mice with a complex SPF microbiota for 3 to 4 weeks prior to OVA sensitization was sufficient to protect against the increased allergic airway inflammation. Such re-colonization presumably models the process of bacterial colonization that occurs in every infant following its birth. Our findings may therefore offer an explanation as to why environmental factors experienced during early childhood, when commensal bacteria are first encountered, exert a strong impact upon the development of allergic diseases; in addition, our data provides promise for the 'reconfiguring' of susceptibility to allergy by reconstitution or alteration of the commensal flora.

Our observations that exaggerated allergic airway inflammation can be overcome by the re-colonization of GF mice provides a novel model by which the impact of commensal bacteria on, and their possible therapeutic capacity for, allergic airway inflammation can be further examined. Many interesting questions remain to be addressed, particularly given the progressive characterization of the microbiota in the lung itself (11); are respiratory immune responses impacted by commensal bacteria

residing in the intestine, airways, skin or all of the above? Do certain species of commensal bacteria direct the immune system down distinct paths? Providing answers to such questions will be of great importance for our understanding of basic immunological mechanisms and for the development of novel strategies aimed at preventing or treating allergic inflammation.

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# 3 Proposed mechanisms of dysregulation of allergic airway inflammation in the absence of microbial colonization

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This manuscript presents data that is part of a published study in the American Journal for Respiratory and Critical Care Medicine in 2011 (1).

## 3.1 Abstract

As previously shown by our group, the absence of commensal bacteria leads to increased allergic airway inflammation with eosinophilic infiltration, elevated levels of IL-4 and IL-5, as well as increased IgE. To address the mechanisms by which commensal bacteria impact on the development of allergic asthma, we utilized a model of allergic airway inflammation in germ-free (GF) mice that lack any exposure to pathogenic or non-pathogenic microorganisms. Allergic airway inflammation was induced in GF, specific pathogen free (SPF) or recolonized mice by sensitization and challenge with ovalbumin (OVA). Our results showed exaggerated airway eosinophilia, which correlated with increased local production of Th2 associated cytokines, elevated IgE production and an altered number and phenotype of conventional dendritic cells (cDC). Regulatory T cell populations and regulatory cytokine levels were unaltered but GF mice exhibited an increased number of basophils and decreased numbers of alveolar macrophages (AM) and plasmacytoid dendritic cells (pDC). These data demonstrate that the presence of commensal bacteria is critical for ensuring normal cellular maturation, recruitment, and control of allergic airway inflammation.

## 3.2 Introduction

We have previously shown that commensal bacteria normally function to prevent exaggerated allergic airway responses using a model of OVA sensitization and airway challenge in GF mice (Chapter 2.1). These studies also indicated that the major effect of commensal bacteria on the allergic response occurred following airway challenge with OVA indicating that bacteria and/or their products may alter the local environment within the lung and airways. Previous studies by Hammad et al. (2) and

Phythian-Adams et al. (3) have shown that lung resident dendritic cells are essential for both the induction and effector phases of allergic Th2 responses (3, 4). Basophils have also been shown to be capable of amplifying allergic airway responses (2), whilst pDC have been shown to promote anti-inflammatory properties and provoke a tolerogenic response by inducing regulatory T cells (4-7). Indeed, during the past decade there has been a growing body of evidence highlighting the important role or T regulatory (Treg) cells in the cause of asthma and allergic inflammatory diseases (8-10). Depletion as well as adoptive transfer studies confirmed the suppressive capacity of Tregs both on the induction and control on already ongoing allergic airway responses (11-13). In addition, lung resident myeloid cells such as interstitial and alveolar macrophages (AM) are thought to play a suppressive role by dampening the inflammation to control tissue injury and releasing IL-10 (14, 15). Lastly, immuno-modulatory components altering allergic airway inflammation have been proposed to be cytokines such as IL-10 and tumor growth factor (TGF) beta which may be produced by a variety of other cell types including epithelium, macrophages or regulatory T cells (16, 17).

To address the question of how commensal bacteria alter these cellular components during allergic airway responses we used in-bred GF mice subjected to a model of OVA sensitization and airway challenge as previously described (chapter 2). Using this model we demonstrate, for the first time, that commensal bacteria play a fundamental role in shaping the type and extent of Th2 inflammatory responses in the lung. The absence of a commensal microbiota leads to dysregulated maturation and recruitment of dendritic cell subpopulations and altered macrophage number and function, and an increased basophil response. By contrast no impact of the commensal

microflora on the number of lung or airway regulatory T cells or on IL-10 and TGF-β production could be determined.

## 3.3 Materials and Methods

**Mice**

Germ-free C57BL/6 mice (originally obtained from Harlan) were kindly provided by the Institute of Laboratory Animal Science, University of Zurich or from the Clean Animal Facility, University of Bern, Switzerland. Germ-free mice were housed in flexible isolators until the day of sacrifice. Fecal samples of the germ-free mice and swabs of the inner wall of the isolator were cultured under aerobic and anaerobic conditions. Additionally, gram stains and DNA stains were performed of fecal samples collected immediately before export from the isolators. SPF C57BL/6 mice (originally obtained from The Jackson Laboratory) were maintained in a pathogen-free animal facility and were given the same food as germ-free mice and water *ad libitum*. Studies were carried out with mice aged 8-12 weeks. Animal experiments were performed according to institutional guidelines and to Swiss federal and cantonal laws on animal protection.

**Protocol for experimental allergic airway inflammation**

Mice were immunized with 100 μg OVA (Sigma-Aldrich, Steinheim, Germany) and 200 μl 2% aluminum hydroxide (SERVA Electrophoresis GmbH, Heidelberg, Germany) via the intraperitoneal route. On day 9 and 10 post immunization, mice were challenged intranasally with 100 μg OVA suspended in 50 μl sterile PBS. Control mice either received immunization with aluminum hydroxide only and were challenged with OVA in sterile PBS, according to the protocol used for allergic mice, or were untreated. Both control groups showed comparable results. All mice were

sacrificed on day 4 after last intranasal challenge, the day after airway hyperresponsiveness test. To analyze the cellular compartment of the airway lumen broncho-alveolar lavage (BAL) was performed. For this purpose a small incision on the trachea was made, through which a catheter was introduced. The lungs were flushed three times with 1 ml PBS. BAL cells were harvested by centrifugation and used for flow cytometry. Lungs were digested with collagenase IV for 40 min at 37°C. Total cell numbers per BAL were determined by using Coulter Counter (IG Instrumenten Gesellschaft). Differential cell counts were performed on cytospins stained with QuickDiff (Dade Behring, Siemens Healthcare Diagnostics, Deerfield, USA) and the percentage of eosinophils, neutrophils, macrophages and lymphocytes within a total population of 200 cells was determined.

**Flow Cytometry**

For analysis of APC subsets, lung tissue was digested using collagenase IV (BioConcept, Worthington, Lakewood, New York) in IMDM medium for 45 min at 37°C. Samples were then filtered through a 70 μm cell strainer (MILIAN), washed with 0.2% BSA/PBS and counted with help of a Coulter Counter. APC were stained with antibodies against CD11c APC-Cy7 (N418, BioLegend, San Diego, USA), CD11b PerCP/Cy5.5 (M1/70, BioLegend, San Diego, USA), F4/80 APC (BMB, eBioscience, San Diego, USA), I-A/I-E Alexa700 (M5/114.15.2, BioLegend), PDCA-1 Alexa647 (927, BioLegend, San Diego, USA), CD45R/B220 FITC (RA3-6B2, BioLegend), and CD40 PE (1C10, BioLegend, San Diego, USA), CD80 PE (16-10A1, BioLegend, San Diego, USA), Ox40L PE (RM134L, BioLegend, San Diego, USA), CD86 Biotin (GL-1, BioLegend, San Diego, USA), CD137 Biotin (17B5, BioLegend), and ICOSL Biotin (HK5.3, BioLegend, San Diego, USA) for activation markers using Streptavidin PE-Cy7 as detection antibody (Streptavidin, BioLegend,

San Diego, USA). The percentage of T regulatory cells of the whole $CD4^+$ T cell compartment of lung and BAL was estimated by staining with antibodies against CD4 PerCP (GK1.5, BioLegend, San Diego, USA), CD25 PE (PC61.5, eBioscience, San Diego, USA), and Foxp3 APC (FJK-16s, eBioscience, San Diego, USA). Basophils were identified using a staining including CD49b PE (HMα2, BD Pharmingen, New Jersey, USA) and IgE FITC (RME-1, BioLegend, San Diego, USA). All cells were recorded using BD FACSCalibur or BD LSR II. Samples were analyzed using FlowJo 8.8.6 software (Tree Star Inc., Ashland, USA).

**Transfer of alveolar macrophages**

Alveolar macrophages (AM) of 40 naïve SPF donor mice were harvested using BAL. Cells were centrifuged and re-suspended in pure RPMI 1640 (LONZA CAMBREX-Bio Whittaker, Switzerland). A total number of $1.0\text{-}1.5 \times 10^5$ AM in a total volume of 50 µl of medium were transferred to OVA immunized GF and SPF mice intranasally on day 8 post immunization, the day before first OVA challenge.

**Statistical analysis**

Student's t test (unpaired, two-tailed) was used to calculate significance levels between treatment groups. *P* values of less than 0.05 were considered significant and are depicted as *p<0.05; **p<0.01; ***p<0.001. Graph generation and statistical analysis was performed using Prism version 4.0c software (GraphPad, La Jolla, USA).

## 3.4 Results

**Commensal bacteria alter the number and activation status of lung APCs.** Numerous APC populations have been described in the lung and it is clear that they play a fundamental role in shaping the polarization and effector function of T cells

either locally or following migration to the draining lymph nodes. We hypothesized that the APCs in the lung might be directly influenced by the host's microbiota. As such, we investigated the number and functional status of the major APC populations in the lung as described by Plantinga et al. (18). Dendritic cells from the lung were first distinguished by their expression of CD11c but lack of the macrophage marker F4/80 (Figure 1).

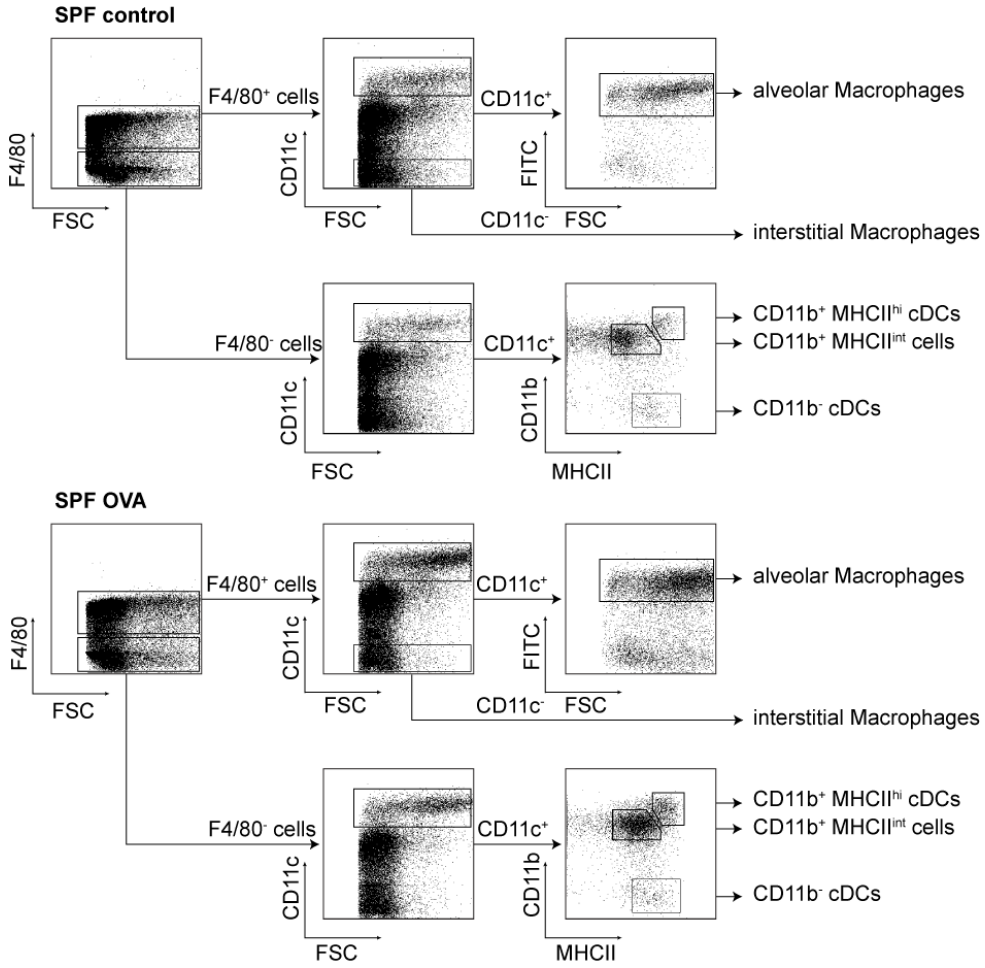


**Figure 1.** Identification of conventional DC subsets and macrophages in lung tissue by flow cytometry. To differentiate between macrophages and dendritic cells, the expression of the macrophage marker F4/80 was determined on live cells. $F4/80^{+}$ cells were further subdivided into $CD11c^{+}$ autofluorescent ($AF^{+}$) alveolar macrophages and $CD11c^{-}$ interstitial macrophages in accordance with Bedoret et al. (14). From the F4/80 negative cell population, $CD11c^{hi}$ cells were identified and further subdivided into CD11b- cDCs, $CD11b^{+}MHCII^{hi}$ cDCs, and $CD11b^{+}MHCII^{int}$ cells. (19).

These $CD11c^{+}$ $F4/80^{-}$ cells were further separated based upon their expression of CD11b and MHC class II, yielding three distinct subpopulations of cells (Figure 1). The number of $CD11b^{+}MHCII^{hi}$ conventional DCs (cDC) as defined in figure 1 showed a reduced trend in total numbers in the lung of GF mice, however this did not reach statistical significance (Figure 2 A). These $CD11b^{+}$ $MHCII^{hi}$ cDCs did however exhibit overall lower levels of the activation markers CD40, CD80, CD86, CD137, and OX40L in GF mice as compared to SPF controls (Figure 2 A). The two other cDC populations assessed, $CD11b^{+}MHCII^{int}$ cells (Figure 2 B), and $CD11b^{-}$ cDCs (Figure 2 C) exhibited a striking statistically significant reduction in total numbers. Similar to the CD11b+$MHC^{hi}$ cDCs, these cells also had reduced surface expression of CD40, CD80 and CD86 (Figure 2 C, D). Allergen challenge increased the numbers of $CD11b^{+}MHCII^{hi}$ (Figure 2 A), $CD11b^{+}MHCII^{int}$ (Figure 2 B) and $CD11b^{-}$ (Figure 2 C) cDCs in SPF mice, whilst only $CD11b^{+}MHCII^{hi}$ cells were significantly increased following allergen challenge of GF mice. Overall, it can be speculated that these differences in DC subpopulations and activation states may shape the lung environment such that it is more prone to the development of Th2 immune responses. Although the exact role of these distinct populations, or the implications of their activation status, during allergic asthma remains unclear, it is clear that the commensal microbiota can impact on both the number and phenotype of these cells. Of particular interest is the increased ICOSL expression on $CD11b^{-}$ cDCs (Figure 2 C). Although still controversial, ICOS-ICOSL interactions have been reported to play a role in promoting the expansion and effector function of differentiated Th2 cells within the lung (20).

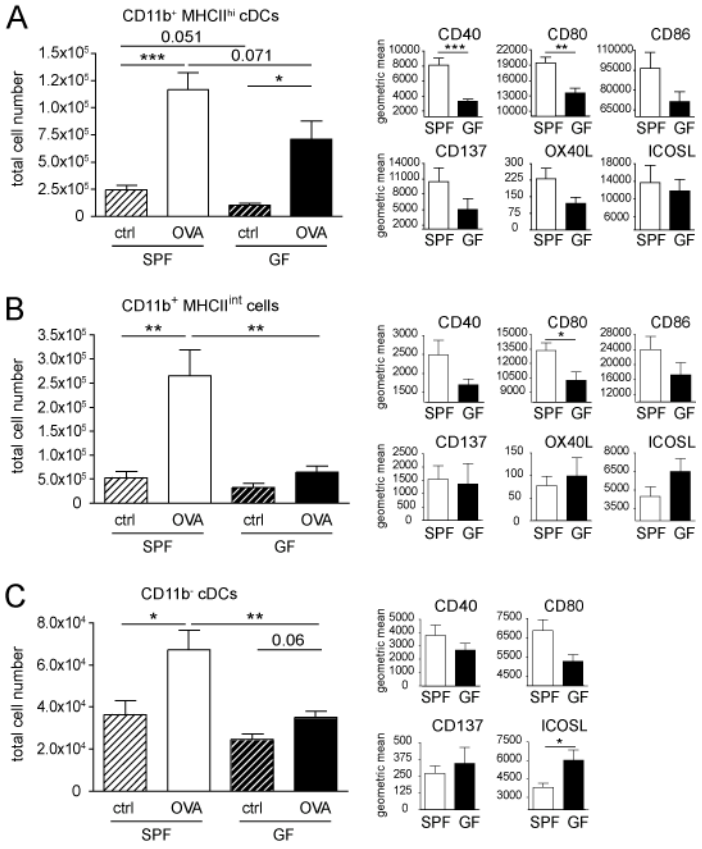


**Figure 2.** Commensal bacteria impact on the number and activation of lung cDCs in allergic airway inflammation. cD11c$^{+}$ cells in the lungs of OVA or control C57BL/6 SPF and GF mice were analyzed by flow cytometry as depicted in Figure 1A. The following cell subsets of CD11b$^{+}$classII$^{hi}$ cDCs, CD11b$^{+}$classII$^{int}$ cells, and CD11b$^{-}$ cDCs were defined as shown in Figure 1 and as indicated in representative FACS plots of SPF and GF OVA mice. A-C) The total cell number and activation state of each population was determined by flow cytometry. OX40L was not detected on CD11b$^{-}$ cDCs. Activation markers are shown as geometric mean of fluorescence intensity ± SEM and are normalized to isotype control antibodies or fluorescence minus one controls. Total numbers are shown as mean ± SEM. Results shown are from one experiment (controls: n=3-5, OVA: n=6) and are representative of two independent experiments.

**Decreased number of suppressive alveolar macrophages in absence of commensal bacteria contributes to exaggerated allergic response.**

We were particularly interested in the potential impact of commensal bacteria on interstitial and alveolar macrophages because they have been proposed to control local inflammatory settings in the lung. The number of interstitial and alveolar macrophages after induction of allergic airway inflammation in GF and SPF mice was determined by FACS analysis according to the gating strategies, which are depicted in

Figure 1. We found no differences in numbers of interstitial macrophages between the different groups (Figure 3 A). However, the frequencies of alveolar macrophages were decreased in naïve GF compared to SPF lung tissue and the same trend was obtained for OVA-sensitized and –challenged subjects (Figure 3 B).

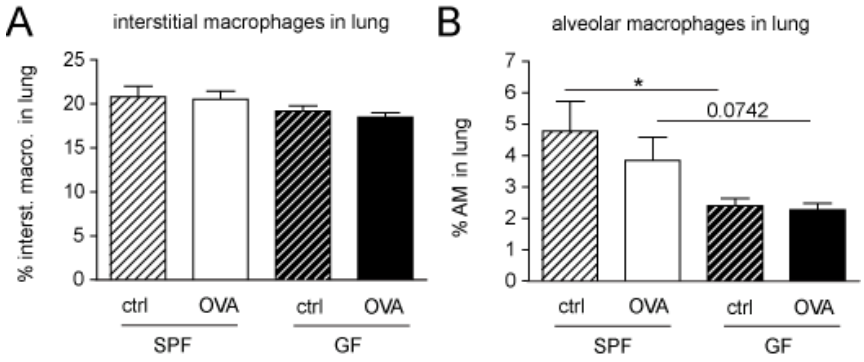


**Figure 3:** Interstitial and alveolar macrophages. The frequency of A) interstitial macrophages and A) alveolar macrophages in the lung (controls: n=5, OVA: n=5) were determined by flow cytometry.

We therefore decided to test whether there were functional differences in alveolar macrophages in the presence or absence of commensal bacteria. To address this question, adaptive transfer experiments were performed. Hence, AM from naïve SPF were isolated and transferred to the airways of SPF or GF mice prior to OVA intranasal challenge (see also experimental scheme Figure 4 A). The transfer of $1.0x10^5$ AMs cells from naïve SPF donor mice into OVA immunized GF and SPF mice suppressed airway eosinophilia in GF but not SPF recipients (Figure 4 B).

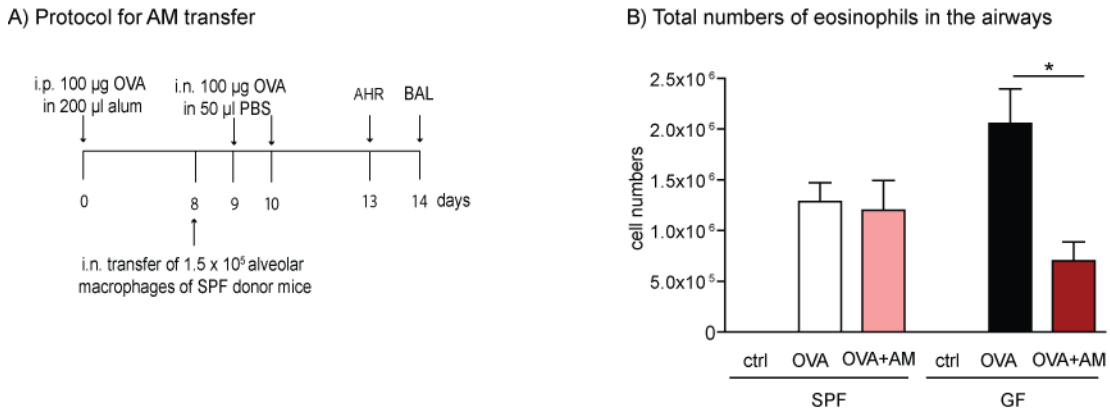


**Figure 4.** Commensal bacteria impact on the number and function of alveolar macrophages in allergic mice. C57BL/6 SPF, and GF mice were immunized and challenged with OVA. Airway eosinophilia could be reversed upon transfer of alveolar macrophage (AM) transfer from naïve SPF donor mice. A) Scheme of AM transfer and OVA-induced allergic airway inflammation in SPF and GF mice. B) Airway eosinophilia in SPF and GF with or without prior AM transfer. Results are pooled and representative of two independent experiments (n=5-7). Mean ± SEM is shown.

These data indicate that macrophages from SPF but not GF mice can modulate allergic responses. To confirm this hypothesis we would need to directly compare the suppressive ability of GF and SPF AMs in an adoptive transfer experiment. However these experiments would have acquired a large number of GF mice to simply serve as donors of naïve GF AM, which is for one extremely costly and for terms of feasibility would acquire a large GF facility to provide space and maintenance of these special care animals. Instead, we decided to compare the functional status of these cells using an *in vitro* suppressor assay. Thus, we isolated AM from naïve GF and SPF mice and incubated them together with CFSE-labeled OT-II T cells and splenic DC. According to previous publications, a mixture of $1x10^5$ T cells together with $2x10^4$ of with OVA-peptide pulsed DCs was used, while the number of AMs was titrated from $2x10^3$ to 20. After 72 hours of incubation, the proliferation of T cells was analyzed by FACS. To compare the suppressive activity of AM to that of other macrophage populations, peritoneal macrophages (PM) were also isolated and used for the co-culture. In spite of several attempts no proliferative difference could be obtained with or without AM

or PM incubation (Figure 5). We suppose the method of macrophage handling did activated or altered the maturation state so that they no longer possessed a suppressive phenotype.

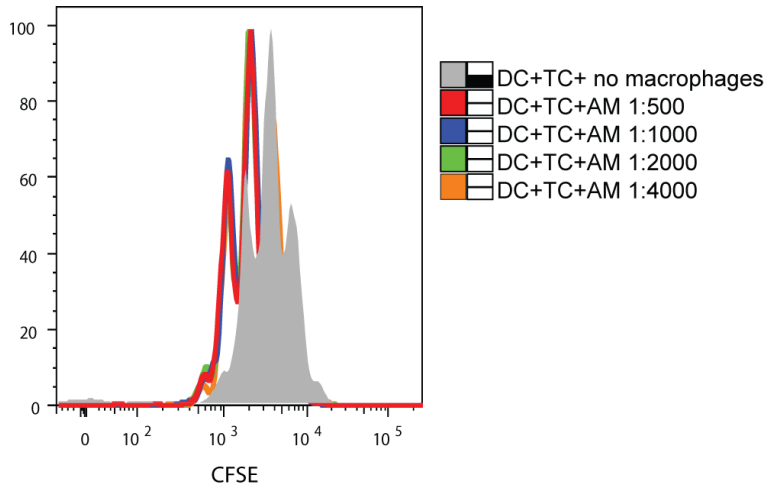


**Figure 5:** *In vitro* suppression assay comparing different dilutions of alveolar macrophages (AM). In brief, OVA specific T cells (TC) isolated from OT-II mice were labeled with CFSE, incubated with OVA pulsed dendritic cells (DC) in the present or absence of AM.

**Dysregulated numbers of regulatory immune cell types in the lungs in the absence of commensal bacteria.**

Numerous other cell types have been described to contribute to, or regulate Th2 mediated allergic asthma. Basophils are potent producers of IL-4 and have been proposed to contribute to the allergic response (2). By contrast, regulatory T cells (8), pDCs (6), and lung macrophages (14) have all been attributed with a regulatory function during allergic asthma.

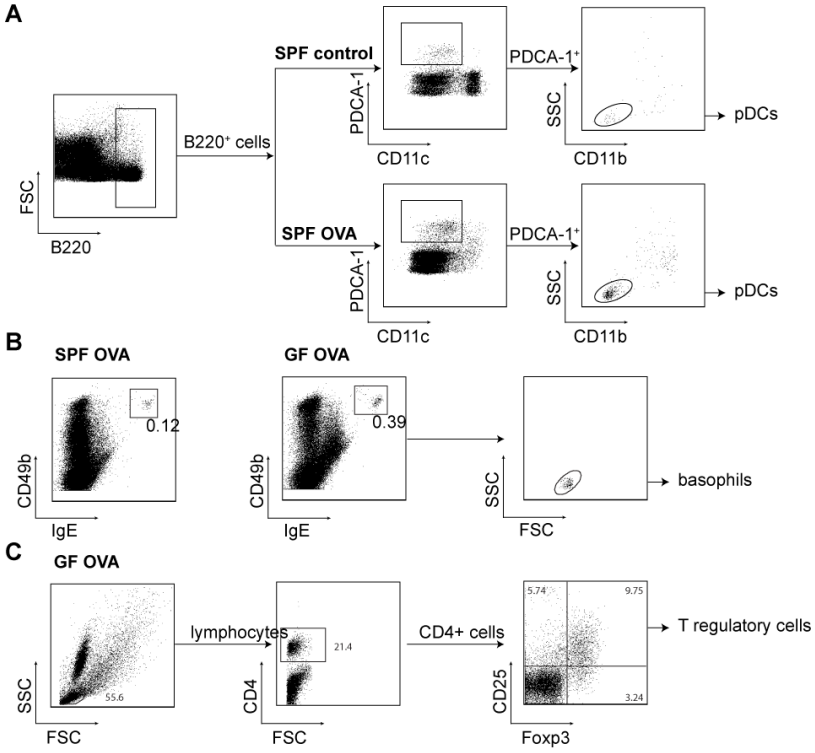


**Figure 6:** Gating strategy of plasmacytoid DCs, basophils and regulatory T cells. A) pDCs were defined by assessing PDCA-1 expression of $B220^+$ live cells, then gating on $CD11b^-$ cells of the $B220^+PDCA\text{-}1^+$ population. pDCs showed low expression of MHCII and were of small size (data not shown) as described previously (5, 21). B) Representative plots for the gating of GF and SPF OVA $CD49b^+IgE^+$ basophils. C) Depicted is the strategy to gate for $CD4^+CD25^+Foxp3^+$ T regulatory cells. Cells were identified as lymphocytes according to their forward and side scatter properties. $CD4^+$ cells of all lymphocytes were then differentiated according to their expression of CD25 and Foxp3.

Therefore, we next investigated the impact of commensal bacteria on other cell populations including pDC, regulatory T cells and basophils. The representative gating strategy of these cell populations is described in Figure 6. We found that the recruitment of basophils into the airways and lungs of OVA immunized and airway challenged mice was greater in GF compared to SPF mice (Figure 7 A-B). No differences were noted for airway or lung $CD4^+CD25^+Foxp3^+$ T cells (Figure 7 C-D) in GF or SPF mice. However, reduced frequencies of pDCs (Figure 7 E) were evident in OVA sensitized and challenged GF mice compared to SPF mice.

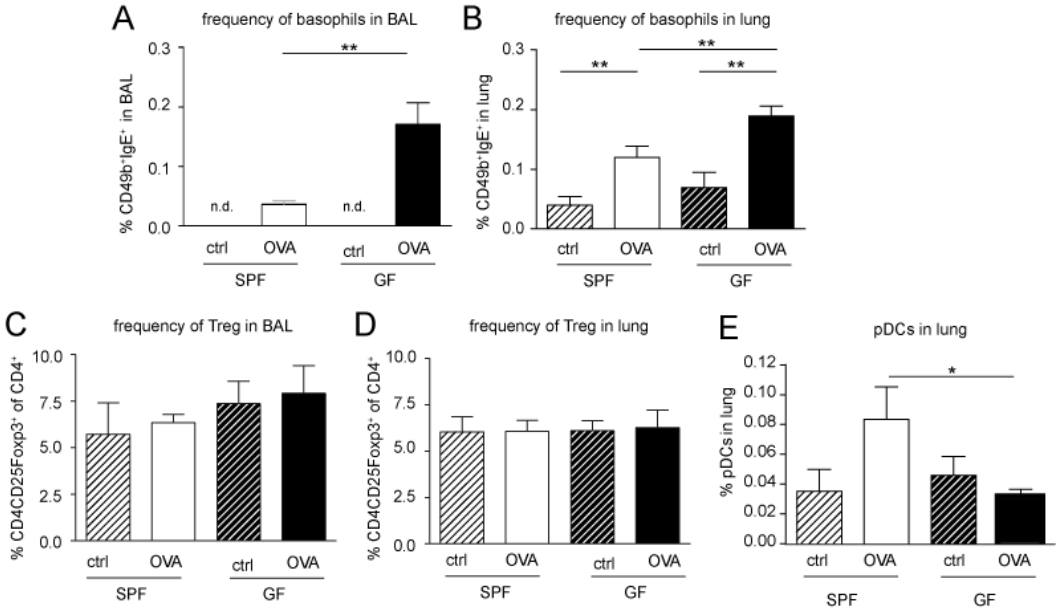


**Figure 7.** Commensal bacteria impact on the number and function of basophils, alveolar macrophages, and pDCs in allergic mice. Cell types in the lungs of OVA or control C57BL/6 SPF and GF mice were analyzed by flow cytometry as depicted in Figure 5. The frequency of $CD49b^+IgE^+$ basophils in A) airways (controls: n.d.= not detected, OVA: n=13-16) and B) lung (controls: n=5-8, OVA: n=14-17) are shown. Results are pooled data of three experiments and are representative of three independent experiments. Mean ± SEM is shown. C-D) The frequency of $CD4^+CD25^+Foxp3^+$ T cells was determined for the C) airways and D) lung (controls: n=4-6, OVA: n=9-10). Results are pooled data of two experiments and are representative of three independent experiments. E) The frequency of pDCs in lung (controls: n=5, OVA: n=5) is shown. Results are data of one experiment and representative of two independent experiments. Mean ± SEM is shown.

Taken together, our data suggest that GF mice exhibit decreased populations of regulatory AMs and pDCs and increased frequencies of pro-allergic basophils. Overall these findings correlate with, and are likely to contribute to, the exaggerated numbers of $CD4^+$ T cells producing Th2 cytokines and heightened IgE levels observed in GF mice following OVA airway challenge.

## 3.5 Discussion

Our findings of exaggerated OVA-induced airway inflammation in GF mice provide the first experimental evidence for a functional impact of commensal bacteria on allergic inflammation in the lung. Re-colonization of GF mice with a complex SPF

microbiota for 3 to 4 weeks prior to OVA sensitization was sufficient to protect against the increased allergic airway inflammation. Such re-colonization presumably models the process of bacterial colonization that occurs in every infant following its birth. Our findings may therefore offer an explanation as to why environmental factors experienced during early childhood, when commensal bacteria are first encountered, exert a strong impact upon the development of allergic diseases; in addition, our data provide promise for the 'reconfiguring' of susceptibility to allergy by reconstitution or alteration of the commensal flora.

The respiratory mucosa is constantly challenged by both pathogenic and non-pathogenic microorganisms in addition to environmental toxins and innocuous protein antigens. Thus, as for the intestine, immune homeostasis must be maintained such that exaggerated or inappropriate immune responses are avoided. It is perhaps not surprising therefore that the absence of commensal bacteria results in widespread changes to myeloid cell populations including altered numbers and functionally of lung antigen presenting cells and decreased numbers of regulatory AM and pDC. Pro-inflammatory basophils were also found to be altered and increased frequencies of basophils in the BAL and lung of GF mice may contribute to the increased *in situ* IL-4 production observed in chapter (2.1) and likely act to amplify the disease.

$CD4^+CD25^+Foxp3^+$ T regulatory cells have also been shown to control AHR and allergic airway inflammation in mice (8) and might be expected to be altered by the presence or absence of commensal bacteria (22). In our experiments normal infiltration of $Foxp3^+$ T cells into the airways of allergic GF mice indicates that defective expansion and/or recruitment of these cells is not likely to be responsible for the enhanced allergic airway disease observed. However, more subtle differences in the functional capacity of regulatory T cells cannot be ruled out by our experiments.

Until recently no phenotypic marker could distinguish natural and induced Tregs, until Thornton et al., described Helios as a natural Treg maker (23). In this instance, it would be interesting to repeat our experiments and also access the development of Tregs in order to identify natural versus induced Tregs.

In summary our data shows that the absence of commensal bacteria leads to dysregulated cDC activation status, elevated basophilia and a decreased presence of regulatory pDCs and AM cell populations in the lungs of allergic mice. Although any one of these cellular changes alone could impact on allergic asthma, we believe that the all of the observed changes likely act in concert to account for the increased allergic airway inflammation observed in GF mice. Preliminary transfer experiments with AM from SPF donors into GF recipients, revealed that AM are sufficient to reverse the increased eosinophil numbers, thus supporting this concept. Importantly our data indicates that the commensal flora 'educates' immune cells in the lung and redirects them from a Th2 prone activation state; such a process might be a fundamental step in the maturation of the immune cells responsible for controlling the balance between protective immunity and pathology in the lung.

## 3.6 References


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# 4 Antibodies support helminth-induced basophil expansion by eliciting autocrine IL-3 production

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## 4.1 Abstract

Basophils are powerful mediators of Th2 immunity and are present in increased numbers during allergic inflammation and helminth infection. Despite their ability to potentiate Th2 immunity the mechanisms regulating basophil development remain unknown. We have found a crucial role for affinity-matured and isotype-switched antibodies in mediating helminth-induced basophil production. Though the production of *Heligmosomoides polygyrus bakeri*-specific antibodies required Th2 cells, a passive transfer of helminth-specific antibodies was sufficient to promote basophil production even in the absence of Th2 immunity. The ability of *H. polygyrus bakeri* infection to increase numbers of basophils occurred independently of T cell-derived IL-3 but was, nevertheless, IL-3-dependent. Cross-linkage of IgG and IgE antibody receptors on the surface of basophils was found to promote autocrine IL-3 production. The depletion of basophil containing CD49b positive cells from the bone marrow and spleen in C57BL/6 and from the bone marrow but not from the spleen in BALB/c mice, significantly reduced IL-3 production. These data reveal a novel and essential role for the Th2 dependent antibody isotypes in promoting basophil production following *H. polygyrus bakeri* infection in C57BL/6 mice.

## 4.2 Introduction

Basophils are rare granulocytes representing less than 0.3% of peripheral blood leukocytes (1). They develop from hematopoietic stem cells and typically complete their maturation in the bone marrow, before entering the circulation as fully matured cells (2). Mature basophils express high levels of FcεR1 and FcRII/III, the activation of which results in degranulation and the release of vasoactive substances, including histamine. Basophil activation also results in the production of proinflammatory cytokines (IL-4, IL-5 and IL-13) and lipid mediators resulting in enhanced Th2 immunity and inflammation (2). Although cross-linking of antibody receptors elicits the most powerful response, basophils can also be activated by antibody-independent mechanisms including C5a, toll-like receptor (TLR) ligands and helminth antigens (reviewed in (2)). Historically, studies of basophils in mice have been hampered by their rarity and extremely short half-life (approximately 60 hrs) (1). However, recent developments have allowed the routine and precise identification of rare mouse basophils based on their unique combination of surface expressed FcεR and CD49b (3). Basophils additionally express CD200R3, FcRII/III and Thy1 but are negative for c-kit (2).

Murine basophils are continuously present within the circulation, but their number increases dramatically following helminth infection or during allergic inflammation. They have been proposed to be potent modulators of Th2 immunity as they represent a significant source of IL-4 *in vivo*. They have also been reported to act as antigen presenting cells (APC) and to promote Th2 cell differentiation from naïve cells *in vitro* (4-6). However, their exact function during the development of Th2 immune responses remains unclear. Several studies have shown an essential role of basophils

as APCs (4-7), whilst others favor a model whereby dendritic cells (DC) initiate Th2 immune responses (8-12) and basophils simply amplify the responses (8, 12). As effector cells, basophils are known to contribute to allergic inflammation and play a crucial role during IgG-mediated anaphylaxis (13), chronic allergic dermatitis (10, 14), and allergic airway inflammation (8). Basophils have also been reported to play a non-redundant role in acquired immunity against helminths (10, 15) and ticks (16).

IL-3 plays an essential role in basophil biology and can 'prime' enhanced IL-4 production following antibody-mediated activation (17, 18). Moreover, IL-3 selectively increases the number of mature basophils from bone marrow cells *in vitro* (19) and *in vivo* (20), indicating its important function as a basophil hematopoietic growth factor. In support of this, IL-3 deficient mice fail to exhibit increased basophil numbers following infection with the helminths *Strongylodes venezuelensis* or *Nippostrongylus brasiliensis* (21). However, IL-3 does not appear to be required for basophil production during steady state conditions as baseline levels of circulating basophils are normal in IL-$3^{-/-}$ mice (21). Optimal production of basophils following helminth infection requires $CD4^+$ T cells (3) and IL-3 production by T cells has been demonstrated to promote basophil production following infection with the rodent helminth *N. brasiliensis* (22). Yet, essentially all activated T cells, including $CD8^+$ T cells, are able to secrete IL-3 (2). Thus, it has been difficult to reconcile an exclusive role for T cell-derived IL-3 in promoting increased basophil production during Th2 immune responses. Instead, additional factor(s) that are associated with Th2 immunity, and capable of promoting basophil production, must exist. Indeed, recently TSLP, but not IL-3, has been reported to be required for *Trichuris muris*-induced basophilia (23).

In the current study we identify a novel role for IgG1 and IgE antibodies in regulating basophil hematopoiesis and thus reveal important means by which antibodies may amplify Th2 immune responses.

## 4.3 Material and Methods

### Mice and parasites

C57BL/6, BALB/c, AID$^{-/-}$ (24), IL-4$^{-/-}$ (25), TCRβδ$^{-/-}$ (26), CD4$^{-/-}$ (27), C57BL/6 FcR$^{-/-}$ (28), FcRIII$^{-/-}$ (29), IgE$^{-/-}$(30), and C57BL/6 or BALB/c IL-4R$^{-/-}$ (31) mice were bred and maintained under specific pathogen-free (SPF) conditions at Ecole Polytechnique Fédérale de Lausanne (EPFL). BALB/c IL-3$^{-/-}$ mice (32) were provided by Dr Chris Lantz (James Madison University, Harrisonburg, VA) were bred and maintained at the Biomedical Research Unit, Malaghan Institute, NZ. All animal experiments were performed according to guidelines set by the State Veterinary Office of Vaud, Switzerland or Victoria University Animal Ethics Committee, New Zealand. Where indicated mice were infected orally with 200 L3 *H. polygyrus bakeri* or subcutaneously with 500 live L3 *N. brasiliensis*. For experiments using pooled immune serum, serum was collected from naïve mice or at day 13-20 post secondary or tertiary infection for *H. polygyrus bakeri* and post challenge infection for *N. brasiliensis*.

### Analysis of helminth-specific cytokine production

Mesenteric lymph node cells were collected from *H. polygyrus bakeri* infected mice at the indicated times and cultured in medium (IMDM, Lonza, Walkersville, USA) plus 7% FCS (Lonza, Walkersville, USA) and 5 μg/ml HES (excretory/secretary products collected from adult L5 *H. polygyrus bakeri*) for 72 hrs. Afterwards, cells were re-stimulated with phorbol-12-myristat-13-acetat (PMA) (Sigma-Aldrich,

Missouri, USA) and ionomycin (Sigma-Aldrich, Missouri, USA) for 4 h at 37°C in IMDM medium plus 5% FCS with Brefeldin A (10 μg/ml) added to the cultures for the final 2 hours. Permeabilized cells were stained with anti-IL-4 APC or PE conjugated (11B11, BioLegend, San Diego, USA), anti-IFN-γ-FITC (XMG1.2, Biolegend, San Diego, USA) or anti-IL-3-PE (M12-SF8, Biolegend, San Diego, USA).

**Analysis of basophils, basophil progenitors and eosinophils.**

Blood smears were stained with Diff-Quik (Dade Behring, Siemens Healthcare, Deerfield, Illinois) and differential cell counts performed using standard criteria. For the analysis of basophils by flow cytometry cell suspensions were stained with anti-IgE-FITC (r35-72, BD Pharmingen, New Jersey, USA or RME-1 Biolegend, San Diego, USA) or anti-FcεR-FITC (Mar-1, eBioscience, San Diego, USA), anti-CD49b-PE (HMα2, BD Pharmingen, New Jersey, USA) and anti-Thy1.2-APC (53-2.1, BD Pharmingen, New Jersey, USA). FcR expression on bone marrow derived basophils was determined by anti-FcRII/III-FITC (2.4G2, BD Pharmingen, New Jersey, USA) or rat anti-mouse FcRI (290322, R&D systems, Minneapolis, USA) and goat anti-rat IgG2a-FITC (Southern Biotech, Birmingham, USA). For analysis of IgG binding to basophils isolated from the peripheral blood anti-mouse IgG1-FITC (goat-anti mouse F(ab')2, Southern Biotech, Birmimgham, USA), anti-mouse IgG2a-FITC (polyclonal, Southern Biotech, Birmingham, USA) or a FITC conjugated isotype control antibody, (Southern Biotech, Birmingham, USA) were used. According to previous literature (35), basophil progenitors were defined by their expression of surface markers lineage (CD3, CD19, NK1.1, Ly6G) negative, Sca-1 (D7, BioLegend, San Diego, USA) negative, c-kit (2B8, BioLegend, San Diego, USA) negative $CD34^{+}$ (RAM34,

eBioscience, San Diego, USA), CD16/CD32$^{+}$ (93, BioLegend, San Diego, USA) and FcεRIhigh, stained with anti-IgE (RME-1, BioLegend, San Diego, USA).

To investigate IL-3 production by whole spleen or bone marrow cells, the cell suspensions were incubated with PMA/ionomycin for 24 hours at 37°C, thereafter the supernatant was collected and analyzed by ELISA. For depletion of CD49b positive cells prior to culture magnetic cell sorting (MACS) technique was applied using CD49b labeled magnetic beads (DX5, Miltenyi Biotec, Bergisch Gladbach, Germany).

**ELISA**

ELISA assays were performed as previously described (33). Antigen-specific IgG1 and IgE were measured in a similar manner after coating with 1-5 μg/ml HES. Bound antibody was detected with secondary peroxidase-conjugated anti-IgG1 (Zymed, USA) or biotin-conjugated anti-IgE (RIE-4) followed by AP-conjugated streptavidin (Zymed, USA) and developed as described (33). To calculate concentrations an internal standard consisting of pooled serum from C57BL/6 mice after challenge infection with *H. polygyrus bakeri* was used. IL-3 ELISA was performed following the standard protocol of BioLegend, using 1 μg/ml LEAF IL-3 (MP2-8F8, BioLegend, San Diego, USA) capture antibody and 1 μg/ml biotinylated IL-3 (MP2-43D11, BioLegend, San Diego, USA) detection antibody.

**Purification of immune serum IgG**

Pooled serum of previously tertiarily infected C57BL/6 mice was incubated with Sepharose G beads over night at 4°C to allow binding of IgG. Beads were washed over a column and eluted with 0.1M glycine buffer, pH=2.75, followed by

neutralization with 1M Tris-HCl. 220 μg of purified IgG were injected intraperitoneal every other day.

**Detection of IL-3 mRNA expression by quantitative RT-PCR**

Total RNA was isolated from indicated cells using TRI Reagent (Molecular Research Center, Inc., Cincinnati, USA) and reverse transcribed using Superscript III RT kit (Invitrogen, California, USA) or using Fast Lane Kit (Qiagen, Hilden, Germany). Transcribed cDNA was used as a template for the PCR reaction. Real-time RT-PCR was performed using Brilliant SYBR Green (Stratagene, California, USA) and an iCycler (Bio-Rad Laboratories, California, USA). Expression was normalized according to the expression of the housekeeping gene β-Actin. Sequences of primers used were IL-3; 5'-TTA GCA CTG TCT CCA GAT C-3' and 5'-ACT GAT GAT GAA GGA CC-3', and β-Actin; 5'-CTT TTC ACG GTT GGC CTT AG-3 and 5'-CCC TGA AGT ACC CCA TTG AAC-3'.

**In vitro basophil culture and stimulation**

For basophil in vitro culture bone marrow from C57BL/6 mice was grown in IMDM containing 8% FCS and 5% IL-3 (supernatant of X63). Basophils were purified by FACS sorting of $FcεRI^{+}CD49b^{+}c\text{-}kit^{-}$ cells. $2x10^{5}$ cells were incubated with 1 μg/ml IgE (TIB141, in house) or 1 μg/ml IgG (24.G2, in house) for 60 min at 37°C. To crosslink bound antibodies cells were incubated with 1 μg/ml anti-IgE (6HD5, in house), or 1 μg/ml anti-IgG2a (2A 8F4, Southern Biotech, Birmingham, USA) for additional 60 min. Thereafter, total RNA was extracted and IL-3 RT-PCR was performed.

**Statistical analysis**

For all data significant differences were determined between gene-deficient or treatment groups and wildtype mice by a two-tailed, unpaired Student's t-test. Significant p-values are shown at $p<0.05$ (*), $p<0.01$ (**), or $p<0.001$ (***).

## 4.4 Results

**Helminth-induced basophil production is supported by affinity-matured and isotype-switched antibodies.**

We previously reported a central, non-redundant, role of antibodies in providing protective immunity against the enteric helminth *H. polygyrus bakeri* (33). In order to further assess the protective role of antibodies following *H. polygyrus bakeri* infection we utilized $AID^{-/-}$ mice. These mice exhibit normal numbers of B cells and IgM secreting plasma cells, but have lost the ability to undergo isotype class switching or somatic hyper-mutation (24). Although $AID^{-/-}$ mice are unable to develop protective immunity against challenge infections with *H. polygyrus bakeri* (33), they mounted normal helminth-specific $CD4^+$ T cell responses as evidenced by IL-4, IL-3 and IFN-γ production comparable to wildtype C57BL/6 mice (Figure 1 A-C). Differential cell counting of H&E stained blood smears from C57BL/6 and $AID^{-/-}$ mice revealed comparable increases in circulating eosinophils following helminth infection (Figure 1 D-E). A striking increase in the percentage of circulating basophils was also observed in blood smears from C57BL/6 mice (Figure 1 D, F). Surprisingly, however, no increase in the percentage of circulating basophils was observed in $AID^{-/-}$ mice (Figure 1 F).

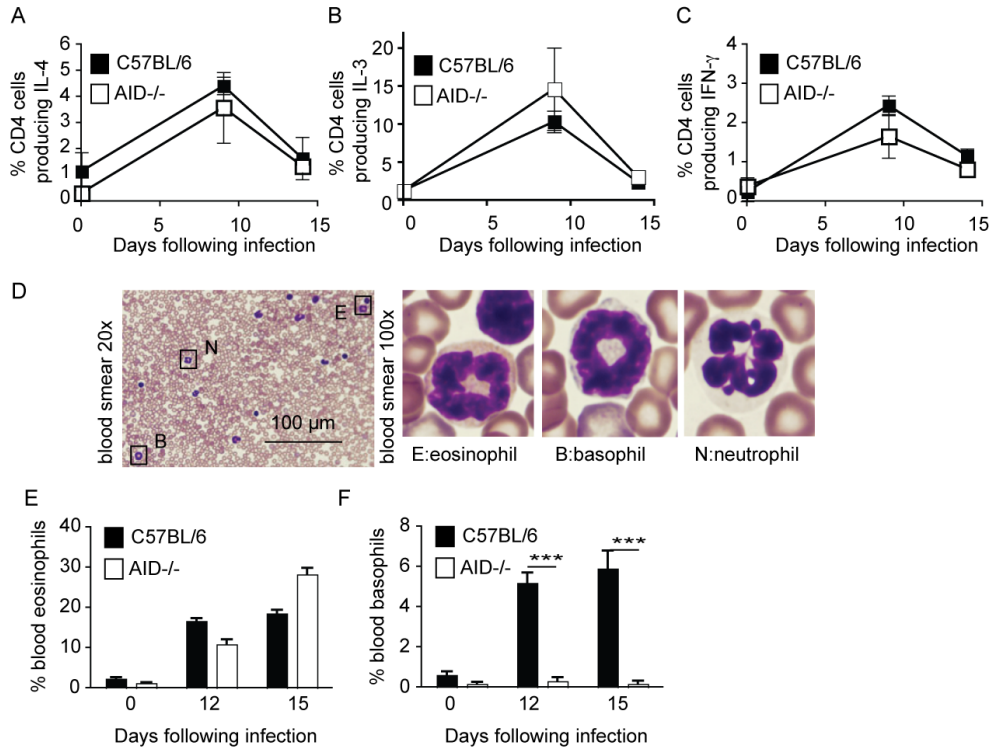


**Figure 1.** AID-/- mice fail to produce increased numbers of basophils following *H. polygyrus* infection**.** C57BL/6 or AID$^{-/-}$ mice, were infected with 200 L3 *H. polygyrus backeri*. (A-C) At the indicated time-points following infection mesenteric lymph node cells from C57BL/6 (filled squares) or AID$^{-/-}$ (empty squares) mice were cultured with L5 excretory/secretory antigens (34) and cytokine production was determined by intracellular staining. (D) A blood smear stained with Diff-Quik®, showing representative eosinophils, basophils and neutrophils. The percentage of white blood cells represented by (E) eosinophils or (F) basophils was determined by differential cell counting of blood smears from infected C57BL/6 (filled bars) or C57BL/6 AID$^{-/-}$ (empty bars) mice. All data is shown as the combined mean ± S.E.M. of individual mice (n=3-6 mice per group) from one experiment and are representative of at least two independent experiments.

To confirm this finding we analyzed the numbers of circulating basophils in C57BL/6 or AID$^{-/-}$ mice using flow cytometry. Basophils from C57BL/6 mice were identified as IgE$^{hi}$CD49b$^{hi}$, whereas basophils from AID$^{-/-}$ mice were identified as FcεR1$^{hi}$CD49b$^{hi}$ (Figure 2 A). All basophils in C57BL/6 mice were additionally stained for FcεR1. However as a high degree of IgE binding in infected mice was observed to interfere with the efficiency of anti-FcεR1 staining, surface IgE was determined as a more reliable marker of FcεR1 expression in these mice. Analysis of basophils by flow cytometry confirmed that helminth-induced increases in circulating basophils were

abrogated in AID$^{-/-}$ mice (Figure 2 B). Increased numbers of circulating basophils in C57BL/6 mice were also observed following secondary infection with *H. polygyrus bakeri*, but again this response could not be observed in AID$^{-/-}$ mice (Figure 2 B). Taken together these data demonstrate a non-redundant role for affinity-matured and/or isotype-switched antibodies in regulating *H. polygyrus bakeri*-induced increases in circulating basophils.

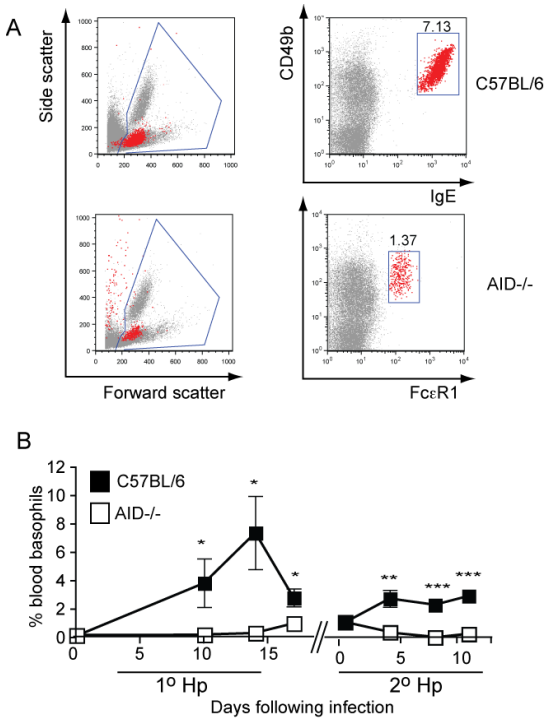


**Figure 2.** (A) Representative FACS plots of basophil stains for C57BL/6 or AID$^{-/-}$ mice using CD49b and IgE or FcεR1 as markers. Backgated basophils are shown in red to indicate their forward versus side scatter properties. (B) The percentage of total white blood cells that are basophils in primary and secondary infected C57BL/6 (closed squares) or AID$^{-/-}$ (open squares) mice were determined by FACS. All data are shown as the combined mean ± S.E.M. of individual mice (n=3-6 mice per group) from one experiment and are representative of at least two independent experiments.

**Antibodies promote *H. polygyrus bakeri*-induced basophil generation independently of Th2 cells.**

A requirement of Th2 cells for eliciting increased numbers of circulating basophils following *H. polygyrus bakeri* infection was found using mice deficient for either

$CD4^+$ T cells (Figure 3 A) or the IL-4 receptor (IL-4R) (Figure 3 B). Th2 cells were also required for the production of polyclonal IgG (Figure 3 C) and for helminth-specific (Figure 3 D) IgG1 and IgE following *H. polygyrus bakeri* infection. Thus we reasoned that Th2 cells may not directly regulate basophil numbers, but may rather act in an indirect manner by promoting the production of helminth-specific antibodies.

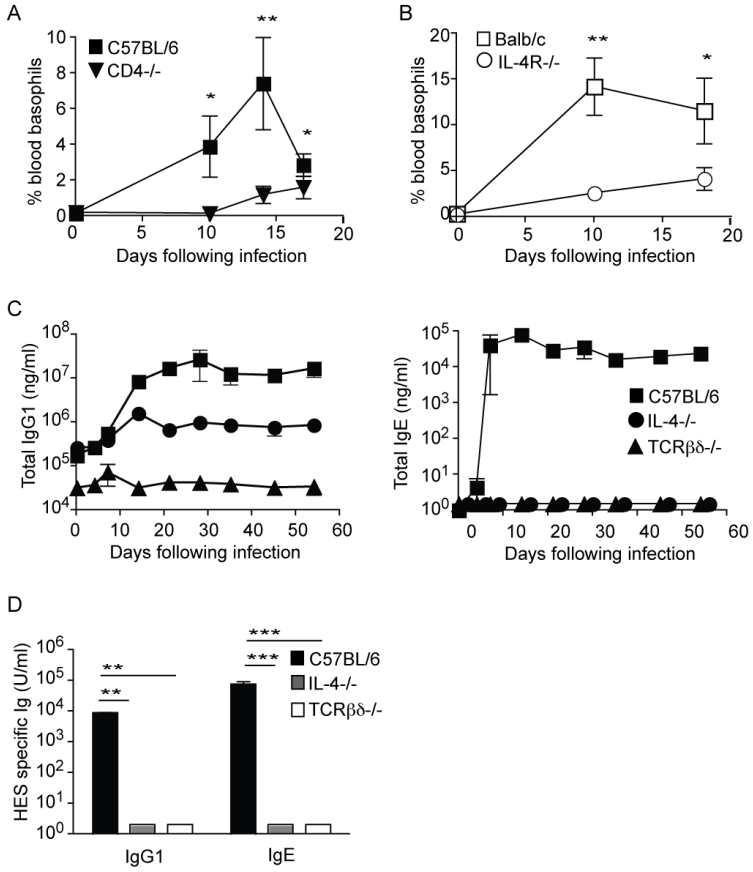


**Figure 3.** Helminth-specific antibodies promote basophil production independently of Th2 cells. C57BL/6 (closed squares) and $CD4^{-/-}$ (closed triangles) mice or (B) BALB/c (open squares) and IL-$4R^{-/-}$ (open circles) mice were infected with 200 L3 *H. polygyrus bakeri* and the percentage of total white blood cells that are basophils determined by FACS. (C) Total IgG1 and IgE levels present in the serum of C57BL/6 (closed squares), C57BL/6 IL-$4^{-/-}$ (closed circles) or C57BL/6 TCRβ$\delta^{-/-}$ (closed triangles) mice infected with *H. polygyrus backeri* were determined by ELISA. (D) IgG1 or IgE antibodies exhibiting specificity for L5 HES products were determined by ELISA using serum from mice C57BL/6 (black bars), C57BL/6 IL-$4^{-/-}$ (grey bars) or C57BL/6 TCRβ$\delta^{-/-}$ (open bars) mice after a secondary parasite infection.

To formally address this hypothesis, we supplemented IL-$4R^{-/-}$ mice with naïve or immune serum purified from wildtype *H. polygyrus bakeri* infected mice. As

expected, IL-4R$^{-/-}$ mice receiving naïve serum did not exhibit increased numbers of circulating basophils (Figure 4 A). By contrast, IL-4R$^{-/-}$ mice receiving immune serum exhibited a robust increase in blood basophils, comparable to that seen in infected C57BL/6 mice (Figure 4 A). Interestingly, the kinetics of helminth-induced blood basophilia were accelerated by the presence of immune serum, suggesting that the production of helminth-specific antibodies represents the main limiting factor in this process. To confirm that the antibodies and not circulating cytokines, such as IL-3, were responsible for the ability of immune serum to promote basophil production in IL-4R deficient mice we next dialyzed immune serum with a 20 kilo Dalton MW cut off membrane that should remove all small molecules but retain soluble antibodies (MW = 150 kilo Dalton). Dialyzed secondary immune serum (Figure 4 B) did induce increased numbers of basophils in IL-4R$^{-/-}$ mice on day 14 post infection. However, basophil numbers did not reach a level comparable to wildtype animals following helminth infection. The transfer of IgG purified from tertiary immune serum (Figure 4 C) did yield significantly more basophils at day 10 post infection than treatment with IgG purified from naïve serum. We also took advantage of another helminth model, *Nippostrongylus brasiliensis*, for which it had been reported by Min et al. that CD4 cells can restore basophil production without the need of antibodies (3). We infected C57BL/6 and AID-/- mice with *Nippostrongylus brasiliensis* and followed the blood basophilia after transfer of naïve or immune serum (Figure 4D). In keeping with the literature, antibodies were not necessary but could elevate the levels of basophils in AID$^{-/-}$ mice. Taken together, these data demonstrate that soluble antibodies can elicit increased numbers of circulating basophils even in the absence of Th2 cells.

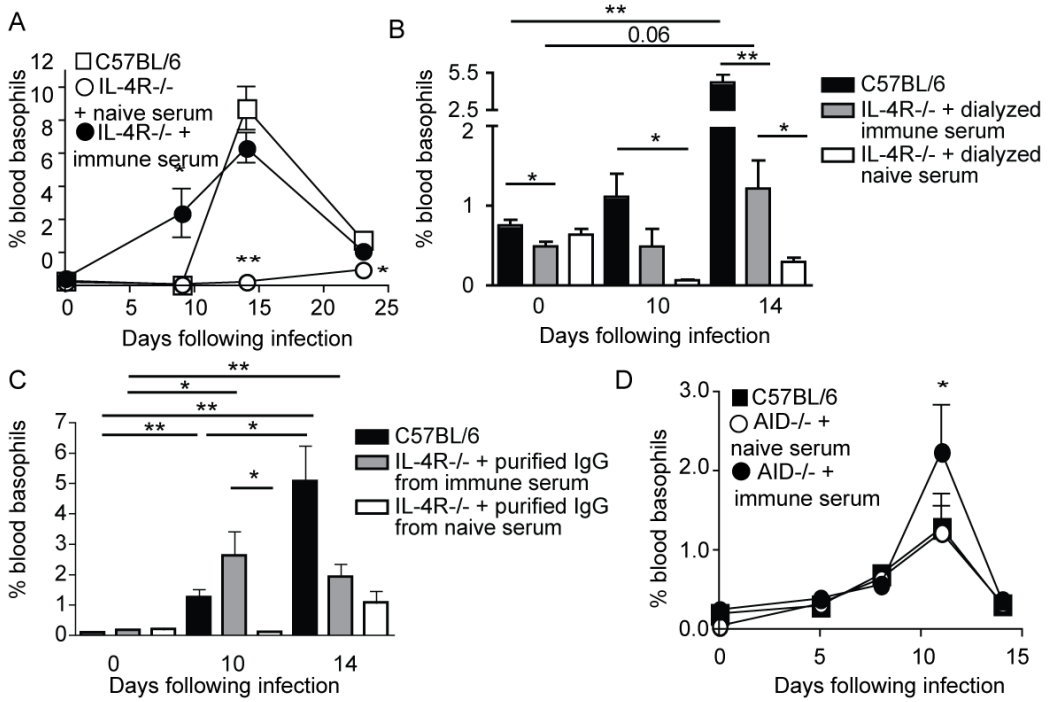


**Figure 4.** Helminth-specific antibodies support basophil production in IL-4R$^{-/-}$ and AID$^{-/-}$ mice. C57BL/6 (closed squares) and IL-4R$^{-/-}$ (open circles) mice were infected with 200 L3 *H. polygyrus bakeri* and the percentage of total white blood cells that are basophils determined by FACS. (A) Naïve IL-4R$^{-/-}$ mice were treated with intraperitoneal injections every other day from the time of infection of 100 μl of pooled serum from naïve (open circles) or immune C57BL/6 mice (closed circles) then infected with *H. polygyrus bakeri* and the percentage of total white blood cells that are basophils determined by FACS. *H. polygyrus bakeri* infected C57BL/6 mice (open squares) were included as a positive control. B) Naïve IL-4R$^{-/-}$ mice were treated with dialyzed immune or naïve serum every other day. C) Naïve IL-4R$^{-/-}$ mice were treated with 220 μg IgG purified from immune or naïve serum every other day. D) AID$^{-/-}$ mice received intraperitoneal injections of 100 μl of pooled serum from naïve (open circles) or immune C57BL/6 mice (closed circles) every other day from the time of infection with *Nippostrongylus brasiliensis.* The percentage of total white blood cells that are basophils determined by FACS. *Nippostrongylus brasiliensis* infected C57BL/6 mice (closed squares) were included as a positive control. All data are shown as the combined mean ± S.E.M. of individual mice (n=4-5 mice per group) from one experiment and are representative of at least two independent experiments.

**Antibodies promote the production of basophils within the bone marrow and spleen.**

We next set out to determine whether antibodies function to promote basophil hematopoiesis, or whether their impact on basophil numbers reflected changes in

basophil survival and/or trafficking. For this purpose we performed a kinetic analysis of basophil numbers in various tissues following *H. polygyrus bakeri* infection. Helminth infection resulted in an increased percentage of basophils in the blood, spleen, mesenteric lymph node (MLN) and liver of C57BL/6-, but not $AID^{-/-}$ mice (Figure 5 A-D). For the blood, spleen and MLN increased basophil numbers were detectable at day 10, and peaked at day 14, following infection (Figure 5 C). For the liver an increased percentage of basophils were only apparent at day 14 post-infection and only in C57BL/6 mice (Figure 5 D). Interestingly, the percentage of basophils present in the peritoneal cavity of naïve mice was higher than the one found in the circulation indicating that basophils traffic to this compartment during steady-state conditions (Figure 5 E). *H. polygyrus bakeri* infection resulted in a dramatic decrease in the proportion of basophils found at this site with no apparent difference between C57BL/6 and $AID^{-/-}$ mice (Figure 5 E). In the bone marrow, basophil numbers peaked earlier than in the periphery (Figure 5 F), and again helminth-induced increases in basophil numbers were absent in $AID^{-/-}$ mice (Figure 5 F). These data suggest that the bone marrow is an important site of basophil expansion following *H. polygyrus bakeri* infection, and that helminth-specific and isotype-switched antibodies are important mediators of this process.

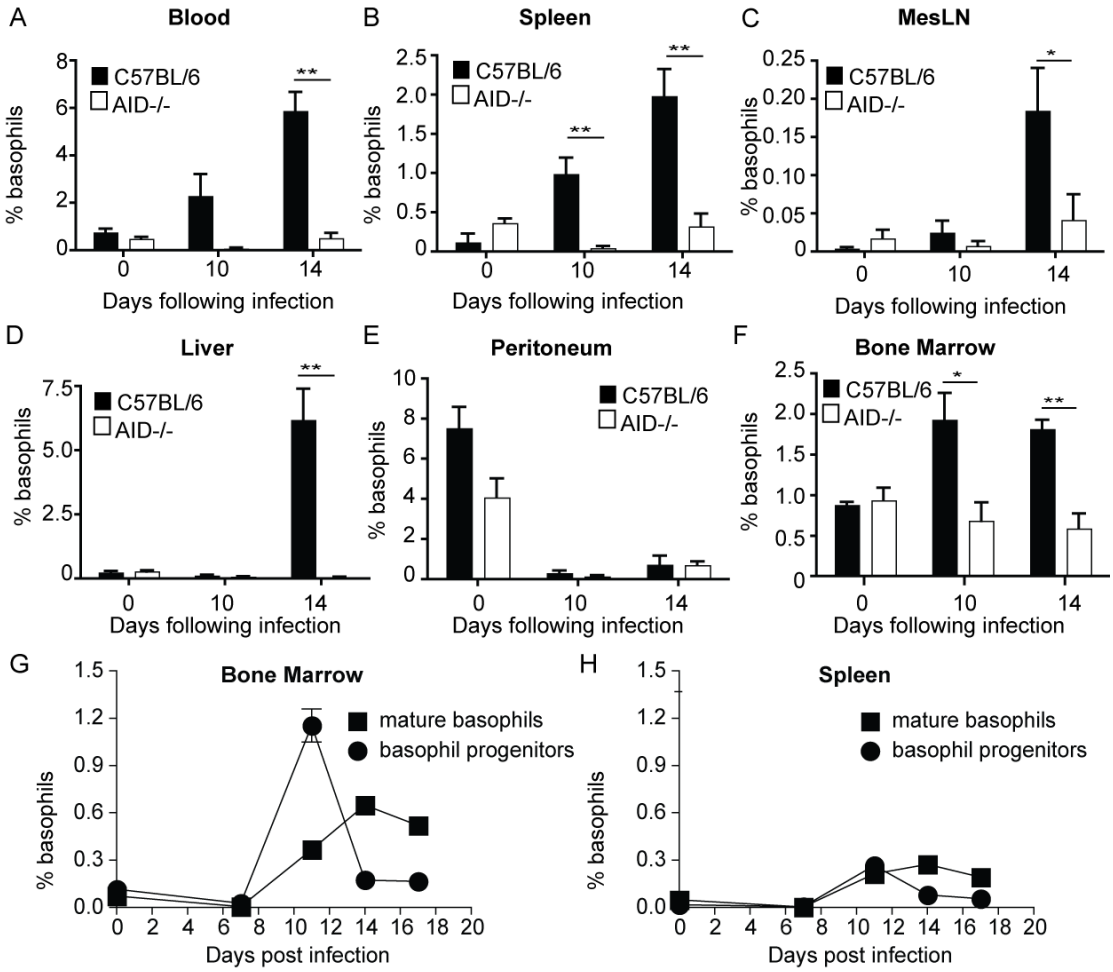


**Figure 5.** Antibodies function to promote basophil production and release from the bone marrow. C57BL/6 or AID$^{-/-}$ mice were infected with 200 L3 *H. polygyrus bakeri*. At the indicated time-points following infection the percentage of live cells that are basophils in (A) blood, (B) spleen, (C), mesenteric lymph node, (D) Liver, (E) Peritoneum and (F), Bone marrow of C57BL/6 (closed bars) or AID$^{-/-}$ (open bars) mice were determined by FACS. Basophil progenitors (closed circles) and mature basophils (closed squares) in (G), bone marrow, and (H) spleen were analyzed by FACS. All data are shown as the combined mean ± S.E.M. of individual mice (n=4-5 mice per group) from one experiment and are representative of two independent experiments.

To determine when and where the defect of basophil expansion occurred after infection we analyzed the relative proportion of basophil progenitors versus mature basophils in these organs based on their expression of CD34 (35) as depicted in the representative gating strategy (Figure 6).

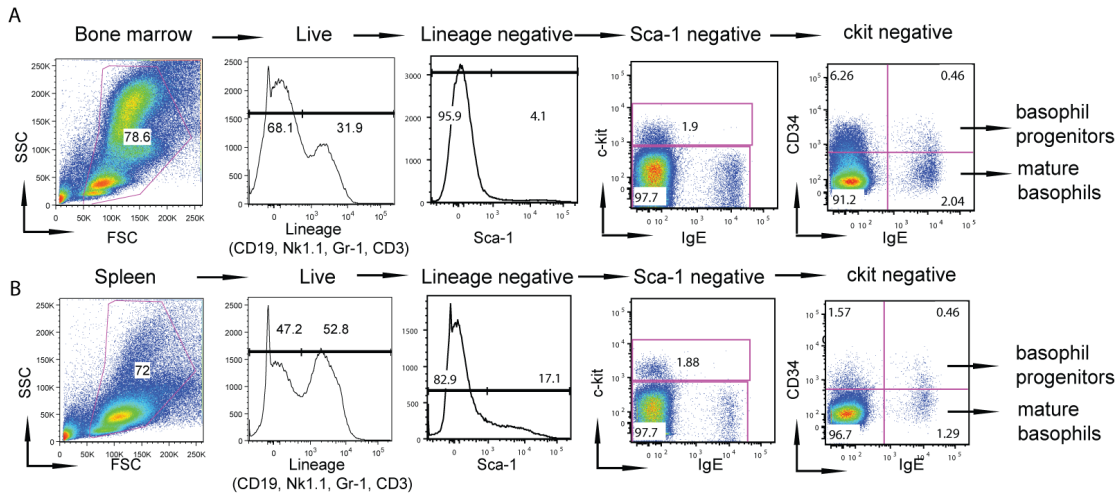


**Figure 6:** Gating strategy for basophil progenitors and mature basophils. Whole organ single cell suspensions of A) bone marrow and B) spleen were stained for linage markers, including CD19, NK1.1, Gr-1 and CD3. Lineage negative, Sca-1 negative and c-kit negative cells were identified as basophil progenitors or mature basophils according to their expression of CD34 and FcεR, stained with anti-IgE.

The number of basophil progenitors and mature basophils in the bone marrow and spleen following helminth infection were increased and peaked at day 11. Thereafter, basophil progenitor frequencies returned to baseline levels on day 14 (Figure 5 G-H), whereas the mature basophils reached a maximum on day 14 both in the bone marrow and spleen.

**Antibodies promote basophil production by eliciting autocrine IL-3 production.**

Given the essential role of IL-3 in promoting helminth-induced basophil production following infection with *S. venezuelensis* and *N. brasiliensis* (21) we investigated whether IL-3 was also required for increased basophil production following infection with *H. polygyrus bakeri.* IL-3$^{-/-}$ mice failed to exhibit increased numbers of blood basophils following *H. polygyrus bakeri* infection (Figure 7 A), indicating a crucial role of this cytokine. This finding contrasted with our earlier observation that *H. polygyrus bakeri*-specific T cells produced normal levels of IL-3 in AID$^{-/-}$ mice (Figure 1 B), and indicated that other sources of IL-3 must be present. As basophil

production was found to be driven by IgG1 or IgE in a redundant manner, and since basophils are the major bone marrow resident cell type expressing receptors for both of these antibody isotypes we reasoned that basophils themselves may represent the major source of antibody elicited IL-3 following helminth infection.

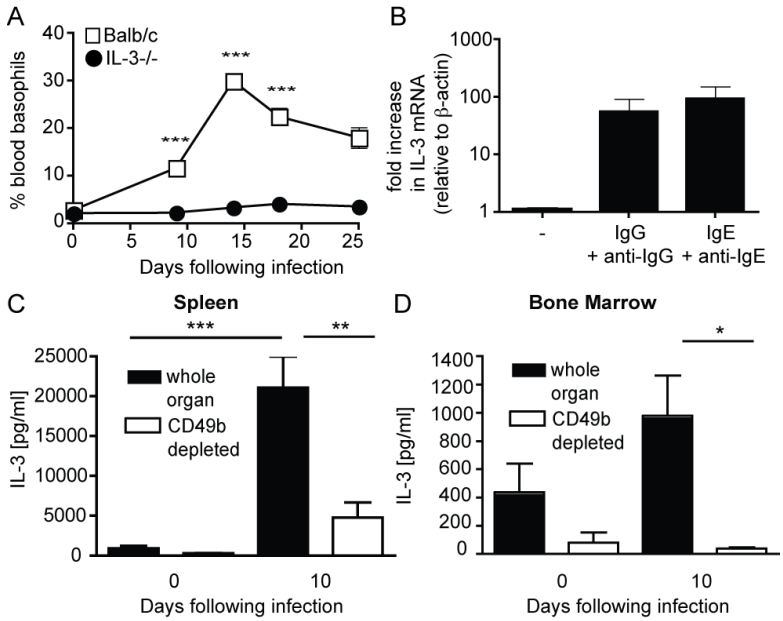


**Figure 7.** Antibodies support the production of basophils by eliciting the production of autocrine IL-3. (A) BALB/c (open squares) or IL-3$^{-/-}$ (closed circles) mice were infected with 200 L3 *H. polygyrus bakeri* and the percentage of white blood cells that are basophils determined by FACS. (B) Relative mRNA expression of IL-3 was determined by quantitative real-time RT-PCR using in vitro grown bone marrow derived basophils from C57BL/6 mice, incubated for one hour with monoclonal IgE (TIB141) or anti-mouse FcRII/III rat IgG2b mAb (24.G2) followed by 2 hrs of crosslinking with 1 μg/ml anti-IgE (6HD5) or 1 μg/ml anti-rat IgG2b (2B 10A8). Data are expressed as fold change of FcR cross-linked cells as compared to basophils incubated in media alone. Data show mean ± S.E.M. of 3 wells of stimulated cells per condition and were repeated in two independent experiments. (C) spleen and (D) bone marrow cells were isolated from infected C57BL/6 mice and restimulated with PMA/Ionomycin for 24h. Levels of IL-3 in supernatant of the whole organ culture or the CD49b depleted cell fractions were measured by ELISA. Data are shown as mean ± S.E.M. of 3 samples of stimulated cells per condition and were C) pooled from two experiments or D) representative of one experiment and repeated in at least two independent experiments.

In support of this hypothesis Schroeder *et al.* (34) recently reported that human basophils generate IL-3 following cross-linking of surface bound IgE and that these cells then rapidly bind to and utilize this IL-3 *in vitro*. Further, we could show that

either IgG- or IgE-mediated FcR cross-linking could elicit IL-3 production by murine bone marrow-derived basophils (Figure 7 B). To test whether basophils were also able to produce IL-3 *ex vivo* after helminth infection we collected spleen or bone marrow cells from mice previously infected with *H. polygyrus bakeri* infection and lyzed whole organs to detect IL-3 in the supernatant of this cell lysis. Since IL-3 was below detection, we next restimulated the isolated spleen and bone marrow cells with PMA/ionomycin for 24 hours (Figure 7 C-D). We observed increased IL-3 production in both the spleen and bone marrow following *H. polygyrus bakeri* infection, but this reach significance only in spleen samples. To address the contribution of basophils to splenic or bone marrow IL-3 production we additionally determined IL-3 production by cultures previously depleted of CD49b positive cells. The depletion of basophil within the CD49b positive cells from the bone marrow and the spleen, reduced IL-3 production levels significantly (Figure 7 C-D).

**IgG and IgE antibodies contribute to antibody-mediated basophil production.** We next set out to determine how isotype switched affinity matured antibodies could promote basophil hematopoiesis in the bone marrow and subsequent entry of mature basophils into the circulation. We first reasoned that soluble antibodies may reach the bone marrow and promote basophil production via direct intereaction with this cell type and/or basophil progenitors.

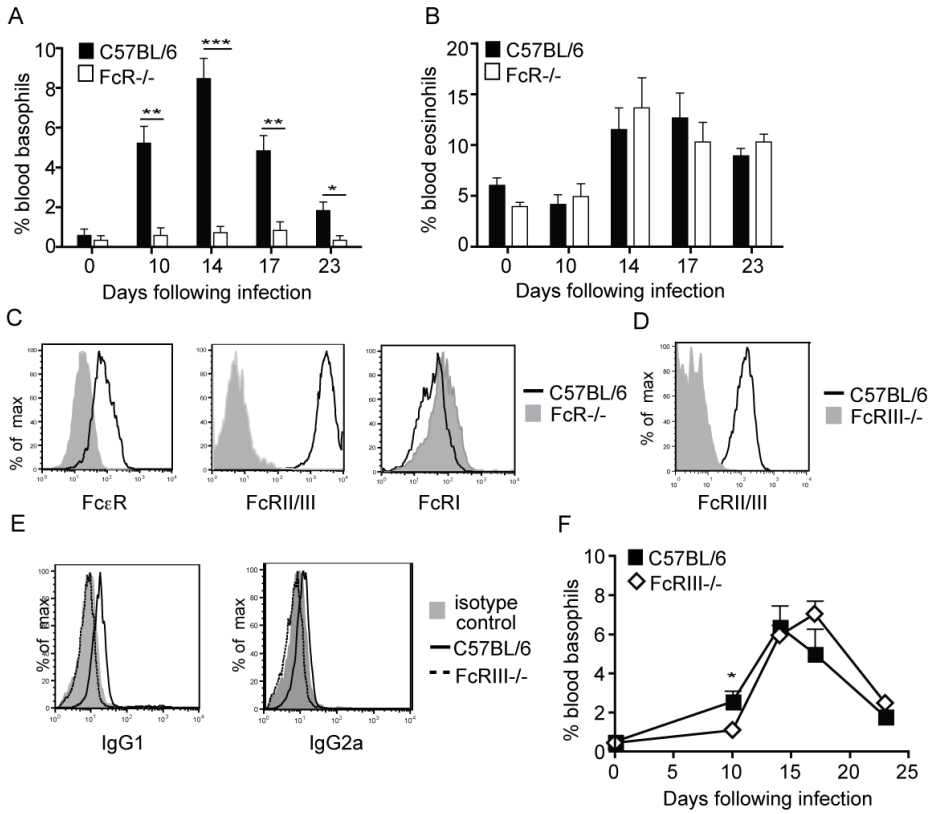


**Figure 8.** Antibody-mediated increases in basophil production require IgG1 and IgE binding to Fc receptors. (A-B) C57BL/6 (closed bars) or $FcR^{-/-}$ (open bars) mice were infected with 200 L3 *H. polygyrus bakeri*. At the indicated time-points following infection blood smears were stained with Diff-Quik® and the percentage of white blood cells represented by (A) basophils or (B) eosinophils was determined by differential cell counting. (C) Bone marrow derived basophils from C57BL/6 (solid line) or $FcR^{-/-}$ (shaded histogram) ($CD49b^{hi}Fc\varepsilon R^{pos}$) were stained for FcRII/III (CD32/16) or FcR1 (CD64). (D-E) Basophils were identified in the blood of C57BL/6 (solid line) or C57BL/6 $FcRIII^{-/-}$ (shaded histogram) mice infected 15 days previously with *H. polygyrus bakeri* as $CD49b^{+}IgE^{+}$ and their surface expression of (D) FcRII/III or determined by FACS. (E) Basophils were identified in the blood of C57BL/6 (solid line) or (E) IgG1 and IgG2a determined by FACS. (F) C57BL/6 (closed squares) or $FcRIII^{-/-}$ (open diamonds) mice. Data for A, B, F, G and H are shown as the combined mean ± S.E.M. of individual mice (n=4-6 mice per group) from one experiment and are representative at least two independent experiments. For C, D and E, FACS histograms from the bone marrow or blood of one mouse are shown but are representative of all animals analyzed and of at least two independent experiments.

To verify this hypothesis we utilized mice lacking the Fc gamma-chain receptor ($FcR^{-/-}$) which no longer express activating receptors (FcεR1, FcRI, FcRIII and FcRIV) for the isotpye-switched antibodies IgE and IgG (28). $FcR^{-/-}$ mice exhibited reduced numbers of circulating basophils following *H. polygyrus bakeri* infection (Figure 8 A), but demonstrated a normal increase in circulating eosinophils (Figure 8 B),

mimicking the effect of AID deficiency. To determine which Fc receptors might be responsible for this effect we examined bone marrow-derived basophils for FcεR1, FcRI and FcRII/III using monoclonal antibodies (mAb). Basophils expressed the high affinity receptor for IgE (FcεR1), but did not express the high affinity IgG receptor (FcRI) (Figure 8 C). Bone-marrow derived basophils also exhibited a high level of reactivity to the 2.4G2 mAb which exhibits cross-reactivity for the low affinity IgG receptors FcRII and FcRIII.

As FcRIII, but not FcRII, is absent in $FcR^{-/-}$ mice we investigated a specific role for FcRIII during basophil production. Interestingly, basophils from $FcRIII^{-/-}$ mice were not reactive with antibodies directed against FcRII/III indicating that these cells only express FcRIII and not FcRII (Figure 8 D). To identify the IgG subclasses bound by FcRIII we investigated IgG binding in wildtype and $FcRIII^{-/-}$ mice *ex vivo*. Wildtype basophils from helminth-infected mice exhibited significant binding to IgG1 and low-level binding of IgG2a (Figure 8 E), but did not bind to IgG2b or IgG3. IgG1 and IgG2a binding was abrogated in $FcRIII^{-/-}$ mice suggesting that this receptor was responsible for all IgG binding to blood basophils. *H. polygyrus bakeri* infection of $FcRIII^{-/-}$ mice led to an increased proportion of circulating basophils that was comparable to that observed in C57BL/6 mice at peak time-points (Figure 8 F). However $FcRIII^{-/-}$ mice did exhibit a slight, but highly reproducible, delay in the production of basophils following *H. polygyrus bakeri* infection (Figure 8 F). These findings indicate that IgE, and to a lesser extent IgG1, isotypes are likely to be responsible for the ability of helminth-specific antibodies to promote basophil production.

**Alternative IL-3 source in spleen of *H. polygyrus bakeri* infected BALB/c mice**

To take advantage of different knockout animals, which have been created on BALB/c background we next investigated the basophil kinetics after *H. polygyrus bakeri* infection in Balb/c mice. We confirmed that helminth infection resulted in increased numbers of basophils with the earliest peak observed in the bone marrow following infection of BALB/c mice (Figure 9 A-D). While basophil numbers in BALB/c bone marrow (Figure 9 A), spleen (Figure 9 B, blood (Figure 9 C), and liver (Figure 9 D) overshoot the basophilia in C57BL/6 mice, kinectics are the same. Taken together besides the accerlatered kinetics in BALB/c mice, we conclude that basophil hematoposiesis was largely occurring in the bone marrow. However, the frequency of basophil populations over all was clearly higher in BALB/c than in C57BL/6, probably due to a stronger Th2 response in the fromer mouse strain.

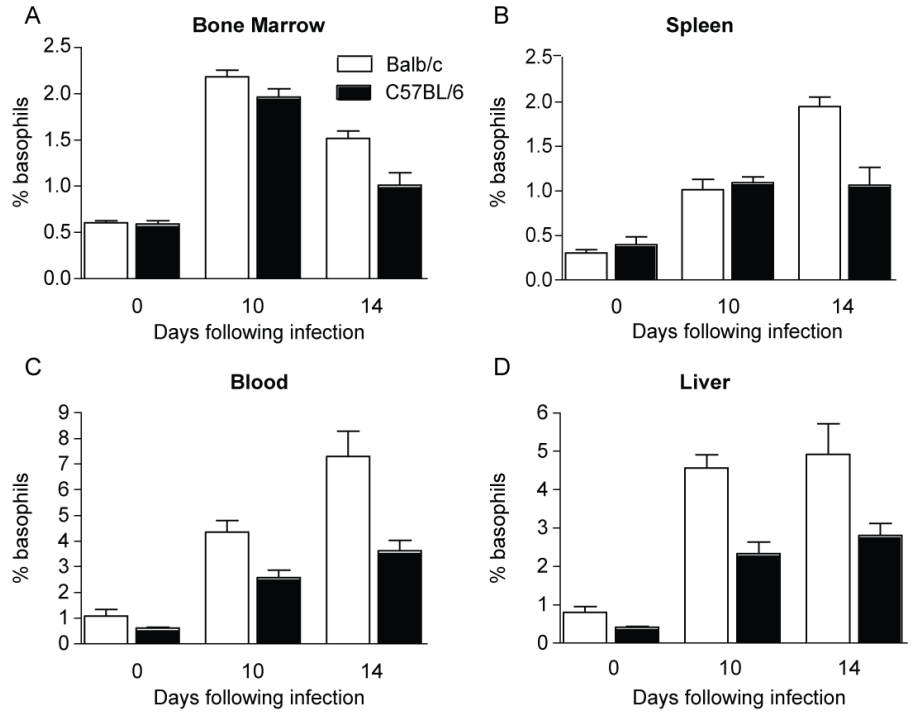


**Figure 9.** Comparison of basophil numbers analyzed by FACS stains for C57BL/6 or BALB/c mice using CD49b and IgE as markers. The percentage of total white blood cells that are basophils (A) bone marrow, (B) spleen, (C) blood, and (D) liver of C57BL/6 (closed bars) or BALB/c (open bars) mice were determined by FACS. All data are shown as the combined mean ± S.E.M. of individual mice (n=3-6 mice per

group) from one experiment and are representative of at least two independent experiments.

Next we checked the source of IL-3 production in the bone marrow and spleen in whole organ suspensions or previous CD49b MACS depleted samples. Depletion of basophil containing CD49b positive cells from the bone marrow but not from the spleen, reduced IL-3 production levels significantly (Figure 10 A-B). We even found an increased level of IL-3 after restimulation of CD49b depleted spleen cells 10 days post helminth infection. This might reflect that in BALB/c there are other mechanisms that regulate IL-3 production, T cells might contribute to a larger extend than basophils. However, further studies with AID$^{-/-}$ mice on BALB/c background would be desirable to elucidate the underlying mechanism.

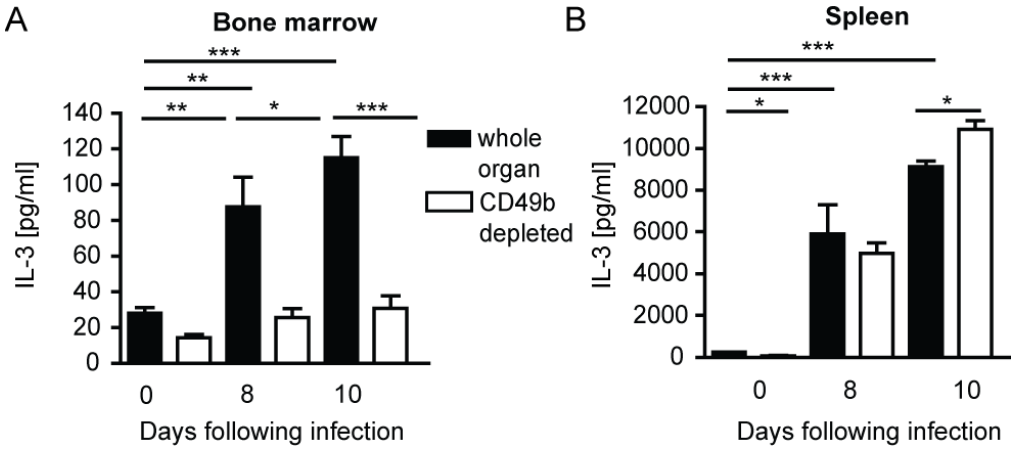


**Figure 10:** Autocrine IL-3 production in BALB/c mice coming mainly form CD49b positive basophils in the bone marrow but not in the spleen.

We utilized IgE$^{-/-}$ mice and found no significant differences in basophil numbers compared to wildtype control mice following helminth infection (Figure 11 A). These findings indicate that the Th2 dependent isotypes IgE and IgG1 act in a redundant manner to promote basophil expansion following helminth infection. However, we have to keep in mind that concerning the BALB/c background of these mice there might be other cells producing IL-3, which may act on basophil expansion.

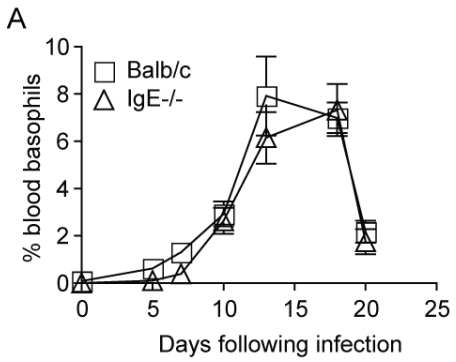


**Figure 11.** Mice deficient in IgE display increased basophils after helminth infection. (A) BALB/c (open squares) and $IgE^{-/-}$ (open triangles) mice were infected with *H. polygyrus bakeri* and the percentage of white blood cells that are basophils determined by FACS.

## 4.5 Discussion

Our data reveals a novel role for antigen-specific antibodies in promoting basophil development following helminth infection. We further show that this promotion is IL-3-dependent, but can occur independently of Th2 cells and T cell derived IL-3 production. Instead IgE and IgG antibodies can bind to FcRs expressed by basophils and elicit autocrine IL-3 production and promote the further expansion of this rare cell type.

Importantly, increased basophil production following *H. polygyrus bakeri* infection was observed to peak at day 15 but decline thereafter (Figure 1). Yet we know that helminth-specific antibodies are present for long period after this time and that titers even increase over time (33). Our *in vivo* data therefore indicate that antigen-antibody complexes, but not antibody alone, are necessary for basophil production. This conclusion is supported by our *in vitro* data showing a role of Fc receptor cross-linking in promoting autocrine IL-3 production by basophils. Helminth derived antigens are also necessary for promoting basophil production following *N. brasiliensis* infection (3). It has been further hypothesized that *N. brasiliensis* derived

antigens may bind directly to basophils resulting in their activation (22). This hypothesis is supported by findings that the protease allergen papain (36) and *Schistosoma* egg antigens (37) can activate basophils directly. We hypothesize, based on our own data, that helminth-induced basophil proliferation requires autocrine IL-3 elicited by helminth-derived products, binding directly or as antibody-antigen complexes, to bone marrow resident basophils and basoophil progenitors. A similar mechanism is likely to be responsible for increased basophil production during allergic diseases, and to be potentiated in the presence of pre-existing antibodies.

We have preliminary data suggesting that IL-3 production in the spleen of BALB/c mice is coming largely form another cell population than basophils. Further studies would be required to elucidate the underlying mechanisms, if maybe T cells in the systemic circulation are the main IL-3 producing populations as has been suggested for *Nippostrongylus brasiliensis* infections (22). However, we could confirm that the main population of IL-3 producing cells in the bone marrow comes from the pool of CD49b positive cells, which comprise basophils and NK cells.

In summary, we have demonstrated that IgE and IgG1 antibodies not only function to elicit basophil degranulation and IL-4 production, but can also promote basophil proliferation within the bone marrow. To the best of our knowledge, these are the first data indicating a role for antibody binding to activating FcRs in modulating myeloid cell hematopoiesis. Our observations also help to resolve the paradox of why basophils are typically only associated with Th2 immune responses and shed light on an important mechanism by which antibodies contribute to Th2-mediated immunity. Interestingly basophils themselves have been reported to promote IgE production by human B cells (17). Thus the ability of IgE to promote IL-3 production by basophils

may represent a novel pathway in which antibodies and basophils act in concert to positively regulate their own production and to amplify Th2 immunity.

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# 5 Discussion and conclusions

## 5.1 Commensal bacteria regulate allergic disease

Dysbiosis of commensal bacteria has been linked to pathological conditions other than allergy, including type 1 diabetes (1), obesity (2), rheumatoid arthritis (3), and experimental autoimmune encephalomyelitis (the animal model for multiple sclerosis) (4). Thus, indicating that changes in the timing of exposure and/or complexity of the commensal flora may impact on a multitude of chronic inflammatory diseases. Intriguingly, the prevalence of these autoimmune diseases has also increased selectively within the developed world over the past few decades (5), indicating a likely link between the environmental cues responsible for providing protection or susceptibility against allergy, inflammatory bowel disease and autoimmune conditions. Indeed, it was recently reported that dietary habits impact on intestinal bacterial composition (6). This could be shown by De Filippo et al., which associated different microbiota dominated by bacteroides species in fecal samples of children from a rural African village of Burkina Faso compared with more abundant firmicutes species found in fecal samples of European children (6).

Immune modulation by commensal bacteria is likely to be complex and their impact on different inflammatory disease may be mediated by distinct mechanisms. In this regard, a recent report by Maslowski *et al.* (7) has shown that germ-free (GF) mice exhibit exaggerated inflammation using models of experimental colitis and arthritis. Further these authors showed that colitis could be attenuated in these mice following treatment with acetate, a short chain fatty acid (SCFA) normally produced during fermentation of dietary fiber by intestinal microbiota. The ability of acetate to

attenuate inflammation was attributed to its ability to bind to the host G protein coupled receptor (GPR43). Maslowski et al. additionally showed that a lack GPR43 resulted in increased susceptibility to allergic airway inflammation (7). This raises the possibility that substituting mice with diets high in fiber would increase the availability of SCFA and could help to control inflammation. Lastly, although the vast majority of research on commensals has focused upon the intestine, it remains plausible that for lung responses the microbiota of the airways may also be a key player. Indeed, Hilty et al. recently reported that the microbial flora of the lung changes depending upon whether individuals have asthma or not (8). The airways of healthy patients were more frequently colonized with bacteroidetes (particularly *Prevotella spp*.), therefore it would be interesting to determine whether intratracheal application of these bacteria could attenuate inflammation in a mouse model of allergic airway inflammation (8). This also implies that therapies with inhaled corticosteroids might impact on the locally present microbiota as has been reported for neonatal intestinal microbiota already (9, 10). Whether the different microbiota observed in the human airways are causal of, or consequential to, the disease remains to be deciphered. In our results we show that the lack of commensal bacteria induces exaggerated allergic airway inflammation with increased levels of IgE, eosinophilia and elevated levels of IL-4 and IL-5. The lack of microbiota also altered the number of basophils, alveolar macrophages, and plasmacytoid dendritic cells. These data indicate that altered exposure to bacteria and/or their products can play a causative role in disease.

Mono-colonization with different strains of bacteroides or lactobacilli might induce a more differentiated manipulation of the distinct cell types, manipulating either basophils or different subsets of antigen-presenting cells (APCs). By performing a

gastric gavage, bacterial strains could be induced into the intestine, whereas intratracheal applications would lead to pulmonary colonization. However, since also bacteria from the airways could be coughed up and swallowed it would be difficult to control for distinct local colonization. It has been reported that monocolonization with *Bacteroides fragilis* and especially the polysaccharide A (PSA) produced by this species can correct the immune defects of GF mice (11). In this context, PSA expression displayed protective capacity in a model of *Heligobacter hepaticus* induced colitis (12). Other strains of bacteria such as *Lactobacillus reuteri* have shown beneficial effects on asthma outcome in a TLR-9 dependent manner (13). An easy way to address the maturation effects of these bacteria or their products could be to utilize *in vitro* cultures using freshly isolated tracheal explants as has been done by Perros et al. (14). Thus, one could isolate naïve lung tissue and compare it to with bacteria or bacterial product challenged lung samples. FACS analysis of activation markers could provide some suggestions which and how host cells can be modulated. This could then be further investigated during experimental asthma models *in vivo* as has been proposed for pulmonary application of *Escherichia coli*, which protected from airway inflammation (15). This also suggests that there might be a difference of colonization of lung and intestine. Therefore it would be interesting to analyze in more detail beneficial commensal strains of the lung and elucidate how commensal bacteria contribute to immune homeostasis locally.

Another influencing factor might be environmental danger signals introduced by the diet or inhaled aerosols. Even though the source of food and water was sterile and the same used for all groups, the ovalbumin (OVA) that was used has been reported to show high contamination with lipopolysaccharide (LPS) (16). Toll like receptor (TLR) 4 recognizes LPS and has been reported to be crucial for the initiation of Th2

immunity during allergic airway responses against inhaled antigens (17). Indeed, the expression of TLRs especially on pulmonary stromal cells has shown to drive immunity in the airways and fosters maturation of APCs (14). In a sterile environment these signaling pathways might not be fully activated leading to pre-mature phenotype of airway APCs. Endotoxin contamination of OVA was shown to dampen the allergic airway response, but this might be restricted to the SPF mice, which had been previously in contact with LPS from the environment and not account for GF mice (16). Pretreatment of GF mice with LPS could address this question in more detail. Recently, more physiological asthma models are being used more widely and it would be of great importance to confirm our findings in the context of e.g. house dust mite (HDM) induced allergic inflammation. By Hammad et al. was revealed that HDM driven allergic responses also depend on TLR4 signaling on airway structural cells (18). The use of HDM allergens (e.g. *Dermatophagoides pteronyssinus* (Der p)) is more clinically relevant than the use of the chicken egg protein ovalbumin and is mostly administered locally into the airways, which also reflects more physiological priming (19).

The remaining question is; what cells are driving the enhanced Th2 response in GF mice? Thus, it would also be of great interest to determine the underlying mechanism for the enhanced Th2 immunity in GF mice. In this regard, the increased numbers of basophils in the GF lung that were found and the fact that basophils have recently been described to promote Th2 priming during allergic asthma could provide a possible explanation how basophils add to the increased inflammatory phenotype (20). To further address the role of basophils in the GF setting one could specifically target these cells via depleting antibodies CD200R3. It is also well appreciated that basophils produce leukotriene C4, which causes bronchocontriction and increased

mucus production. Leukotriene receptor antagonists and synthetase inhibitiors are available or being developed for treatment of asthma. The impact of commensals on leukotriene pathways would be interesting to decipher since we found increased numbers of basophils in lungs of GF asthmatic mice. In the literature, the suppressive effect of both alveolar macrophages and plasmacytoid dendritic cells has been well characterized but the impact of commensal bacteria on these processes are a novel finding of the work presented here (21-23).

An alternative immuno-modulatory mechanism induced by enteric helminths and their excretory/secretory products has been proposed to be beneficial for asthma outcome, although the reports remain controversial depending on the strain of parasites used (24). There the impact of commensal bacteria has not been yet evaluated, but with the helo of the current model one could address these questions. As proposed by Walk et al. the protective effect of *H. polygyrus bakeri* infection upon inflammatory bowel disease in C57BL/6 mice could be consequential of altered microbiota in the ileum of these mice (25). Additionally it has been shown that infection with *H. polygyrus* bakeri can induce TLR4 expression on T cells upon LPS stimulation, therefore regulating host response towards commensals (26).

Finally, the model of allergic airway inflammation in GF mice, which was developed as part of this work provides us with a valuable tool to address further questions in more detail. Another advantage is that for further studies one can selectively introduce single or multiple bacterial strains into an “empty” host and then follow the impact this has on local allergic airway inflammation.

## 5.2 Antibodies promote helminth-induced basophilia

Basophils have recently been determined to play various roles during antibody mediated immune responses (27). For instance it was recently shown that basophils

acted as antigen-capturing cells following immunization with allophycocyanin together with *B. pertussis* as adjuvant, and that this process was dependent on IgE and the presence of the IgE receptor (28). In concert with our findings, which describe the supporting effect of isotype-switched and affinitiy-matured antibodies on the expansion of basophils following helminth infection, this might propose a novel link between basophils and humoral immunity. Intestringly, Gomez et al. were able to show that basophils could prolong plasma cell survival *in vitro* and *in vivo* (29). It has also been discovered that basophils are the main IL-4 producing cell population during a primary helminth infection (30). Therefore, basophil derived IL-4 could contribute to B cell activation during helminth infections. A valuable tool to further elucidate the role of basophils during helminth infection would be the use of neutralizing antibodies to deplete basophils. Previous studies often took advantage of anti-FcεR antibodies (clone MAR-1), which are also directed against mast cells. However, today also CD200R3 antibodies are available and have been suggested to specifically deplete basophils (31). Drawbacks might be that also CD200R3 is an activation marker, which sufficiently depletes basophils from the systemic circulation but might not impact on "resting/pre-mature" basophils in the bone marrow. Another tool has been introduced by Wada et al., which selectively depleted basophils in a diphtheria toxin dependent manner (32). To further assess the functional impact of basophils on protective Th2 immunity and worm rejection during challenge infection with *H. polygyrus bakeri* depletion experiments using depleting antibodies anti-FcεR, anti-CD200R3 or even the diphtheria toxin approach to deplete basophils could be useful.

Regarding the factors influencing basophil expansion it has been known for some time that IL-3 is a potent growth factor for basophils and that in vivo IL-3 required for

helminth-induced expansion of basophils, but does not affect baseline levels of basophils (33). Interestingly Siracusa et al. has shown recently that TSLP can also drive basophil expansion in a IL-3 independent manner (34). Previous work from our group revealed the need of TSLP in protective immunity against *Trichuris muris* but not against *H. polygyrus bakeri* or *N. brasiliensis* infection (35). Our data however shows an exclusive role for IL-3 for increased basophil numbers during *H. polygyrus bakeri* infection in chapter 4. We could also confirm that for the parasite *N. brasiliensis* the role of antibody-mediated increases in basophil numbers is not as prominent. It has been shown by Kim et al. that basophils are dispensable for the protective Th2 immunity during *N. brasiliensis* infection (36). These authors also reported that the lack of IL-3 receptor beta (IL-3Rβ) and not IL-3 did impair the development of specific Th2 immunity, which could be due to the shared common β chain of IL-3/IL-5/GM-CSF receptors (36). However, base line levels of basophils are maintained even in the absence of IL-3 (37). To address if baseline basophils originate from a TSLP dependent lineage one could use TSLP depleting antibodies in IL-3 deficient mice. Additionally the role of antibodies for TSLP elicited basophils has not been addressed so far, which could be addressed in helminths infected AID mice with or without treatment of TSLP depleting antibodies.

In this study, it was proposed that autocrine IL-3 production is important for helminth-induced basophilia, which is in keeping with the literature where Schroeder et al. propose autocrine priming of human basophils in the setting of allergic disease (38) However, the main production of IL-3 came from the CD49b positive cell population of the bone marrow, which also includes NK cells. To confirm that basophils act in an autocrine fashion and that is is not NK cells that are producing IL-3 following *H. polygyrus bakeri* infection further experiments utilizing NK1.1

depletion of NK cells from the bone marrow before re-stimulation are planned.

Overall our findings support the role of isotype-switched and affinity-matured antibodies on basophils during helminth infection. We could also show that for the parasite *N. brasiliensis* the role of antibody-mediated increases in basophil numbers is not as prominent. It has been shown by Kim et al. that basophils are dispensable for the protective Th2 immunity during *N. brasiliensis* infection (36). The activation of basophils by the cross-linking of bound antibodies, such as IgG1 and IgE would lead to further IL-4 secretion of basophils. Thus, our data may indicate a positive feedback loop in which antibodies promote basophil expansion and basophils in turn promote Th2 differentiation and advocate antibody production. It would be extremely interesting to investigate whether the ability of antibodies to promote basophilia is also apparent in other Th2 responses such as allergy.

After all, the relevance and novelty of antibodies impacting on hematopoesis and possible interest of basophils being the major IL-3 source in the bone marrow could impact on hematopoiesis of other cell types. It has long been appreciated that IL-3 is a hematopoietic growth factor which impacts on renewal of hematopoietic stem cells, granulocyte-macrophage progenitors as well as megakaryocytic and erythroid lineages and is being widely used for *in vitro* cultures. Depleting basophils (39), at the same time as IL-3 and TSLP could also deliver valuable insights into regulation of hematopoietic processes.

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# 6 Appendix

## 6.1 Abbreviations

| | |
|---|---|
| AAM | alternatively activated macrophages |
| ADCC | antibody-dependent cellular cytotoxicity |
| AHR | airway hyperresponsiveness |
| AID | activation-induced cytidine deaminase |
| AM | alveolar macrophages |
| APC | antigen presenting cell(s) |
| BAL | broncho alveolar lavage |
| BSA | bovine serum albumin |
| CD | cluster of differentiation |
| cDC | conventionell dendritic cell(s) |
| pDC | plasmacytoid dendritic cell(s) |
| DC | dendritic cell(s) |
| DNA | deoxyribonucleic acid |
| ELISA | Enzyme Linked Immuno Sorbent Assay |
| ELISPOT | Enzyme Linked Immuno Spot Technique |
| FACS | fluorescence associated cell sorting |
| FcR | Fc receptor |
| FCS | fetal calf serum |
| GALT | gut-associated lymphoid tissue |
| GATA-3 | GATA binding protein 3 |
| GF | germ-free |
| GM-CSF | granulocyte macrophage – colony stimulating factor |
| GMP | granulocyte monocyte progenitors |
| HDM | house dust mite |
| H&E | hematoxylin and eosin |
| IFN | interferon |
| Ig | immunoglobulin |
| IH2 | innate helper 2 (cells) |
| IL | interleukin |

| | |
|---|---|
| ILF | isolated lymphoid follicle |
| IMDM | Iscove's Modified Dulbecco's Media |
| LN | lymph node |
| LP | lamina propria |
| LPS | lipopolysaccharide |
| ITAM | immunoreceptor tyrosine based activating motif |
| ITIM | immunoreceptor tyrosine based inhibiting motif |
| MACS | magnetic activated cell sorting |
| MALT | mucosal associated lymphoid tissue |
| MetCh | methacholine-chloride |
| MHC | major histocompatibiliy complex |
| MMP | matrix metalloproteinase |
| $MPP^{type\ 2}$ | multi-potent progenitor type 2 |
| NHC | natural helper cell |
| OVA | ovalbumin |
| PAS | periodic acid Schiff reaction |
| PBS | phosphate buffered saline |
| pDC | plasmacytoid dendritic cells |
| pIgR | polymeric immunoglobulin receptor |
| PMA | Phorbol-12-Myristat-13-Acetat |
| PP | Peyer's patch |
| PSA | polysaccharide A |
| RPMI | Roswell Park Memorial Institute |
| SCF | stem cell factor |
| SIgA | secretory IgA |
| SPF | specific pathogen free |
| STAT | Signal Transducer and Activator of Transcription |
| Tfh | T follicular helper |
| Th | T helper |
| TLR | toll like receptor |
| TNF | tumor necrosis factor |
| Treg | T regulatory cell |
| TSLP | thymic stromal lymphopoietin |

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