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Investigations into the Cellular Sources of Type 2 Cytokines and Interleukin-17A using Cytokine Reporter Mice

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Investigations into the Cellular Sources of Type 2 Cytokines and Interleukin-17A using Cytokine Reporter Mice

by

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CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

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Investigations into the Cellular Sources of Type 2 Cytokines and Interleukin-17A using Cytokine Reporter Mice

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ABSTRACT

Cytokines play a pivotal role in the maintenance of homeostasis and in the coordination of immune responses. Our lab has designed a variety of cytokine reporter mice, which allow for the determination of cellular sources of cytokines directly *in vivo*. In this dissertation, reporter mice designed to mark cells expressing IL-4, IL-5, IL-13, IFN-γ, and IL-17A are described.

First, we focused on type 2 immunity, an immune program that is induced in response to allergens and parasitic helminths. We used IL-4 and IL-13 reporter mice to identify and characterize a subset of lineage-negative cells called Ih2 cells that participate in type 2 immune responses *in vivo*. We demonstrated that Ih2 cells were widely distributed at rest. We further showed that these cells expanded robustly in response to the epithelial cytokines IL-25 or IL-33 and after infection with the helminth *Nippostrongylus brasiliensis*. Finally, we demonstrated that activation of Ih2 cells with IL-25 is sufficient to mediate worm clearance, even in the absence of adaptive immunity.

Next, we used an IL-17A reporter mouse to identify cells that participate in IL-17-associated immune responses, which are involved in host defense against extracellular bacteria and fungi and also in the development of certain autoimmune disorders. We

demonstrated that during *Klebsiella pneumoniae* infection or experimental autoimmune encephalomyelitis, a substantial proportion of γδ T cells, invariant (i)NKT cells, and other $CD4^-$ $CD3\varepsilon^+$ cells and a smaller percentage of $CD4^+$ T cells were marked with the reporter *in vivo*. Administration of IL-1β and/or IL-23 elicited rapid expression of the reporter by resident γδ T cells, iNKT cells and other $CD4^-$ $CD3\varepsilon^+$ cells *in vivo*, demonstrating that these innate-like T cells are poised for rapid IL-17A production and likely comprise major sources of this cytokine during infection and autoimmunity.

Taken together, the results of these studies suggest that the cytokines associated with type 2 or IL-17-associated immune responses can be produced by a wide variety of cell types, including innate cells, innate-like T cells, and adaptive immune cells. Coordination of the effector functions of these various cell types is likely critical for the efficacy of these immune programs.

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CHAPTER I: INTRODUCTION

Part A: Cytokines in immunity

The evolution and function of cytokines

Cytokines are small, secreted proteins that have a specific effect on the interaction, communication, or behavior of cells. Although putative cytokines have been identified in many invertebrates species, including Spatzle and Unpaired in Drosophila (1, 2) and Insect chemotactic peptide in the moth (3), the majority of cytokines utilized by modern day vertebrates most likely evolved after the split from invertebrate lineages, coincident with the development of adaptive immunity. Proinflammatory cytokines, such as interleukin (IL)-17A and interferons, evolved early during the evolution of lower vertebrates, while regulatory and immunosuppressive cytokines, such as IL-4 and IL-10, are thought to have evolved more recently. In vertebrates, cytokines play a pivotal role in the maintenance of homeostasis and in the coordination of innate and adaptive immune responses.

Much of the work on cytokines has focused on the production of these proteins by specialized subsets of $CD4^+$ T cells that play a pivotal role in the coordination of immunity. These $CD4^+$ T cell subsets, called Th1, Th2, and Th17 cells, are defined by their production of unique combinations of cytokines and participation in distinct immunological programs (4). Specifically, Th1 cells produce the signature cytokine IFN-γ and are involved in immunity to intracellular pathogens, Th2 cells produce IL-4, IL-5 and IL-13 and participate in control of helminth infection, and Th17 cells produce IL-

17A and IL-17F and are important for immunity to extracellular pathogens such as bacteria and fungi. Dysregulation of either of these $CD4^+$ T cell subsets can be detrimental to the host, as Th1 cells can be associated with organ specific autoimmunity, Th2 cells are linked to the development of allergy and asthma, and Th17 cells appear to play critical roles in the development of multiple autoimmune diseases including inflammatory bowel disease, multiple sclerosis, and arthritis. Although much of the work aimed at elucidating the features of these three immune programs has focused the role of $CD4^+$ T cells, it is becoming apparent that cells of the innate immune system also play a crucial part in these responses and are important sources of the cytokines associated with each type of immunity. With our studies, we aimed to better identify and characterize important cellular sources of cytokines associated with Th2-associated type 2 immune responses and Th17-associated IL-17-mediated immune responses.

Reporter mice as tools for analyzing cytokine production

Cytokines are secreted proteins with a relatively short half-life, which makes tracking their production *in vivo* more challenging. Multiple experimental approaches have been developed to measure cytokine production and to identify cytokine-producing cells. The simplest method is enzyme linked immunosorbent assay (ELISA), in which the presence of a cytokine in a supernatant is measured by binding to specific antibodies followed by a detection step utilizing an enzyme/substrate reaction. A modification of the ELISA, called the ELISpot assay, allows for the enumeration of cytokine-secreting cells by the presence of localized spots of cytokine detection (5). Cytokine secretion assays allow for the detection of cytokine-secreting cells using flow cytometry by

tethering secreted cytokines to the cell surface using a heterodimeric antibody. The surface-captured antibody can then be detected using a second specific fluorochrome conjugated antibody (6). Intracellular cytokine staining protocols also allow for the detection of cytokine-secreting cells. With this method, cells are stimulated *ex vivo* with either phorbol myristate acetate (PMA) and ionomycin or specific antigens or stimuli to elicit cytokine production. The cytokines produced are then trapped within the cell by protein transport inhibitors such as brefeldin A or monensin and can be detected by flow cytometry after fixing and permeabilizing the cells and staining with a specific antibody. Although these methods have provided many valuable insights into the functions of cytokines and the nature of cytokine-secreting cells, they generally rely upon some form of stimulation to elicit *ex vivo* cytokine production that can then be quantified. Thus, an important caveat to these approaches is that they may induce cytokine production in cells that were not actively making the cytokine *in vivo* or in quantities that are not physiological.

To overcome these potential problems and more reliably assess *in vivo* cytokine production, multiple researchers have generated cytokine reporter mice (7). These mice allow for the tracking of cytokine gene expression by using the expression of a reporter gene, generally a fluorescence molecule such as GFP, YFP or RFP, a surface marker such as Thy1.1, or an enzyme such as β-galactosidase, as a surrogate for expression of the cytokine gene of interest. The first cytokine reporter mice were transgenic mice that were generated by introducing a human IL-2 promoter-driven β-galactosidase gene into the mouse germ-line (8). While multiple reporter mice have been created using a similar transgenic strategy, these mice can display variable efficacy depending on the site of

transgene insertion and the transgene dosage. Additionally, the transgenes introduced may not include important regulatory elements and, therefore, the expression of the reporter gene may not completely mimic endogenous gene expression. In an attempt to avoid these potential caveats, investigators have generated transgenic cytokine reporter mice utilizing bacterial artificial chromosomes (BACs). These constructs are much larger in size, and therefore presumably are much more likely to include all the relevant genetic regulatory elements surrounding the cytokine gene of interest. BAC transgenic mice also have less integration position effects and issues with dosage compared with traditional transgenic mice (9). However, BAC constructs often include additional unrelated genes that could potentially have unexpected effects when they introduced and subsequently expressed in the transgenic mouse.

Cytokine reporter mice have also been generated using a knock-in strategy, whereby the endogenous cytokine locus is modified to include a reporter gene. The reporter gene can be engineered to replace the endogenous gene, thus creating a knock-out allele for the cytokine gene. Alternatively, the reporter gene can be introduced into the 3' untranslated region (UTR) of the endogenous gene following an internal ribosome entry site (IRES), which allows for the generation of a bicistronic transcript and the concurrent translation of the cytokine gene and the reporter gene. Although knock-in reporter mice avoid the integration-site artifacts and gene dosage effects of transgenic mice, use of replacement knock-ins creates a null-allele and bicistronic knock-ins potentially alter the stability of the endogenous cytokine mRNA which can alter cytokine expression patterns. Knock-in strategies are also more time-consuming than BAC transgenesis. Another potential complication associated with all reporter mice is that the

reporter used may have enhanced or reduced stability and half-life when compared to the endogenous cytokine, consequently creating a reporter system that either over- or underestimates actual cytokine gene expression. Thus, although cytokine reporter mouse provide obvious advantages over previous *ex vivo* cytokine detection methods, each reporter mouse engineering strategy is associated with potential pitfalls. However, we feel that the specificity gained by targeting the endogenous locus with knock-in constructs makes this a superior strategy, and all the reporter mice described in these studies were generated using this targeting method.

A variation on standard reporter mice utilizes Cre recombinase technology (10). In these systems, mice are engineered to express Cre recombinase instead of or in addition to a reporter gene. Cytokine-specific Cre-expressing mice can then be crossed to mice that contain loxP recombination sites. In the resulting mice, activation of the cytokine gene drives expression of Cre, which moves into the nucleus and mediates a recombination event at the loxP-containing sites. For example, cytokine-Cre mice can be crossed to mice that are engineered to express a floxed Stop cassette followed by a reporter gene such as GFP or YFP driven by the ubiquitously expressed Rosa promoter (11). This allows for fate-tracking experiments, as all cells that have ever expressed the cytokine will be permanently marked by the reporter gene. Along similar lines, cytokine-Cre mice can be crossed to mice that contain a Rosa-driven floxed stop cassette followed by the gene for diphtheria toxin or the diphtheria toxin receptor, which allows for deletion of cytokine-expressing cells upon expression of the cytokine either immediately or conditionally after addition of diphtheria toxin, respectively (12). Although Cre-mediated recombination is a powerful approach for marking or deleting cytokine-

expressing cells, there are challenges associated with this technology. Major caveats include the fact that expression of Cre does not always accurately mirror endogenous cytokine gene expression, recombination efficiency is variable between different Cre constructs, and in certain cases Cre can be associated with cellular toxicity (13).

Part B: Cytokine production during Type 2 immune responses

Evolution of type 2 immune responses

Chronic infection with helminths and other parasites was a defining feature of vertebrate evolution. Infection with parasites has been documented in almost all vertebrate species, including ancient humans. The Tyroleon Iceman, a frozen corpse found in the European Alps and projected to have died around 3300 B.C., was infected with whipworm at the time of his death (14). The first written description of parasitic infection can be traced back to 1500 B.C. from Egyptian papyrus, and calcified helminth eggs were found in mummies dating from 1200 B.C. (15). Humans have been reported to host nearly 300 species of parasitic worms and over 70 species of protozoa. Some of these species can be traced back to primate ancestors, while others were acquired by surrounding animal species (15). Helminth infection is still extremely prevalent among modern humans, with billions of humans in the developing world harboring chronic parasitic infections. These chronic helminth infections were likely major forces driving the evolution of vertebrate immunity, and the components of type 2 immunity are believed to have evolved to specifically control and combat these infections (16).

Helminth infections have many unique features compared to infections with bacteria, viruses or fungi. Helminths are multicellular and do not replicate in the mammalian host. Once they enter the host and establish infection, the infective stages of the worm develop into adulthood and then produce eggs or live offspring for transmission to the next host. The migration of these macroparasites through the host can cause significant tissue damage, and the components of type 2 immunity may have evolved

from pathways involved in wound healing and repair (16). Many cells, such as mast cells and eosinophils, and processes, such as goblet cell hyperplasia and matrix remodeling, are shared between wound healing and type 2 immune programs. Unlike many single-celled pathogens, helminths can persist in their hosts for decades, and it has been hypothesized type 2 immunity evolved not to facilitate elimination of helminths, but rather to control and coexist with these parasites while minimizing consequent tissue damage (17). This "Old Friends" hypothesis is supported by the fact that, unlike the effector mechanisms utilized during type 1 or IL-17-associated immunity, the pathways involved in type 2 immune responses are largely regulatory or immunosuppressive.

Intriguingly, the same immune pathways that are involved in the control of helminths are associated with the development of atopic disease, such as allergy and asthma. The pathogenesis of these diseases is associated with aberrant type 2 responses after exposure to allergens such as pollen or house dust mite antigens. The link between helminth infection and atopic disease is supported by recent evidence in humans showing that some polymorphisms in type 2 associated loci can both enhance resistance to helminth infection and also predispose to the development of asthma. Specifically, polymorphisms in the 3' UTR of STAT-6 are associated with differential resistance to *Ascaris* infection in a Chinese cohort and asthma among a British population (18, 19). Similarly, a polymorphism in the IL-13 promoter leading to increased transcription of the gene is associated with enhanced susceptibility to asthma and also enhanced resistance to schistosome infection in Mali (20, 21). In the developing world, where helminth infection is widespread, the establishment of these type 2 genes with enhanced functionality would be largely beneficial for the society. However, in the developed

world, where chronic helminth infections have largely been eliminated, enhanced type 2 immune responses may predispose individuals towards the development of allergic inflammation and immunopathology. Thus, the genes and pathways involved in promotion of type 2 immune responses might be predominately maladaptive within modern societies.

In broad terms, the type 2 immune response to helminth infection or allergen challenge can be referred to as a "weep and sweep" response, where the host attempts to dislodge and flush the parasite or allergen from the tissue through which it is migrating or anchored, usually the intestine or lungs (22). The defining features of this response include elevated IgE and IgG1 antibody titers, goblet cell metaplasia with enhanced mucus production, smooth muscle hyperreactivity, and recruitment of innate cells such as eosinophils and basophils to the site of inflammation. Type 2 responses require the coordinated actions of many cell types and the production of a variety of mediators including a specialized subset of cytokines.

Cytokines in type 2 immunity

Although multiple other cytokines can participate in type 2 immune responses, the signature cytokines associated with this immune program are IL-4, IL-5, IL-9 and IL-13. The genes for all four of these cytokines are found on the same chromosome in humans, and IL-4, IL-5 and IL-13 are found on the same chromosome in mice. The degree of homology between IL-4 and IL-13 suggests that they originated through a gene duplication event (23). Research from multiple groups has revealed that, while some of the functions of these cytokines can overlap, they each play important and nonredundant

roles in type 2 immune responses. Transgenic overexpression or exogenous administration of each of these cytokines can lead to the development of pulmonary inflammation (24-26). The coordinated actions of these cytokines mediate the anti-helminth immune response and can promote the development of allergic lung inflammation.

IL-4 is often considered the hallmark cytokine of type 2 immunity. IL-4 can bind both the type I IL-4 receptor, which consists of the IL-4Rα chain paired with the common γ chain, or the type II IL-4/IL-13 receptor, which consists of the IL-4Rα chain paired with the IL-13Rα1 chain (27). In contrast, IL-13 does not bind the type I IL-4 receptor, but can bind the type II IL-4R or the decoy receptor IL-13Rα2, which is membrane-bound in humans and both membrane-bound and soluble in mice. Type I IL-4 receptor is mainly expressed on hematopoietic cells, while type II IL-4 receptor is expressed ubiquitously, including on nonhematopoietic cells. Binding of IL-4 to IL-4Rα leads to sequential activation of JAK-1 and STAT-6, which leads to the downstream effector functions of this cytokine (28). Binding of IL-4 to type I IL-4R is associated with promotion of Th2 differentiation and also the switching to IgE and IgG1 antibody isotypes by B cells (29). Binding of IL-4 or IL-13 to type II IL-4R mediates the tissue manifestations of allergic immunity including goblet cell hyperplasia and mucus secretion, smooth muscle contraction and the development of fibrosis. The actions of IL-4 and IL-13 and type II IL-4R signaling have also been associated with the promotion of macrophage alternative activation (30, 31). However, it is important to note that differential affinities and binding patterns of IL-4 and IL-13 to the type II IL-4R are such that IL-13 is likely a more potent driver of type II IL-4R signaling (27). Indeed, despite utilization of the same

receptor, multiple groups have demonstrated that IL-13 has multiple nonredundant functions during helminth infection and the development of allergic inflammation (24, 32-34).

IL-5 is another cytokine associated with type 2 immune responses. IL-5 signals through a receptor comprised of IL-5Rα and common β chain subunits (35). Signaling through the IL-5Rα chain is associated with activation of JAK2, STAT-5 and multiple additional downstream signaling pathways (36). IL-5 is a growth factor for a specific subset of B cells called B1 cells, which have limited B cell receptor diversity and are major sources of IgM (36, 37). IL-5 transgenic mice display a remarkable increase in the number of B1 cells, resulting in increased serum IgM, IgA and IgE, and IL-$5^{-/-}$, and IL-$5R\alpha^{-/-}$ mice have reduced numbers of B1 cells and half as many IgA-secreting cells in the intraepithelial lymphoid tissue of the intestine (38, 39). IL-5 is also has multiple effects on eosinophils. IL-5 prolongs eosinophil survival by preventing eosinophil apoptosis, possesses eosinophil chemotactic activity, increases eosinophil adhesion to endothelial cells promoting migration, and also enhances eosinophil effector function (40). Consequently, IL-$5^{-/-}$ and IL-$5R\alpha^{-/-}$ also have reduced baseline eosinophil numbers, and the resulting eosinophils appear to be functionally impaired (39).

IL-9 production is also associated with type 2 immunity. IL-9 signals through a receptor composed of IL-9Rα and common γ chains, and signaling involves activation of JAK-1 and STAT-1, STAT-3 and STAT-5 (41). IL-9 was first characterized as a T cell and mast cell growth factor (42, 43). However, the role of this cytokine in promotion of T cell growth and function is controversial (44). IL-$9^{-/-}$ mice have reduced goblet cell hyperplasia and defects in mast cell recruitment in a pulmonary granuloma model (44). In

contrast, IL-9$^{-/-}$ mice display normal levels of lung inflammation and airway hyperreactivity in a standard sensitization model (45), and have no observable defects in immunity to the helminth *Nippostrongylus brasiliensis* (44). Further studies are necessary to fully elucidate the role of IL-9 in type 2 immunity.

Potential sources of type 2 cytokines

While the production of type 2 cytokines is generally associated with CD4$^+$ T cells, there is increasing evidence that cells of the innate immune system might also be important sources of these cytokines. Parasite infection or allergic lung inflammation leads to the accumulation and activation of unique populations of innate immune cells, including eosinophils, basophils, mast cells, NKT cells, and newly defined type 2-associated innate lymphoid cells. These innate cells cooperate with CD4$^+$ T cells, and are involved in both the initiation and effector phases of type 2 immune response.

CD4$^+$ T cells CD4$^+$ T cells have been shown to produce IL-4, IL-5, IL-9 and IL-13 in a variety of different models. Most of this production is thought to derive from Th2 cells, naïve CD4$^+$ T cells that have been differentiated under specific conditions leading to a series of epigenetic modifications and transcriptional changes that define the Th2 lineage. IL-4 potently induces Th2 differentiation *in vitro*, but the early sources of IL-4 during inflammation remain undefined. Additionally, it is unclear whether IL-4 is even required for the differentiation of Th2 cells *in vivo*, and alternative pathways such as Notch signaling might be more relevant in certain contexts (46). In most cases, adoption of a Th2 fate has been shown to require activation of STAT-6, which operates together with AP-1, NF-κB, and other signals to promote expression of IL-4 and the transcription

factor GATA-3. These events mediate an autocrine feedback loop that further potentiates Th2 differentiation. Unlike IL-4, IL-5, and IL-13, IL-9 can be produced by multiple $CD4^+$ T cell lineages. Production of IL-9 was first documented by Th2 cells (47), but has more recently been observed to be produced by Th17 cells (48-50) and possibly by regulatory T cells (48, 51). It has been proposed that IL-9-producing $CD4^+$ T cells may constitute a separate lineage of "Th9 cells" that are induced by the combination of TGF-β and IL-4 (52-54). Differentiation of these putative Th9 cells has been shown to require the actions of the type 2-associated transcription factors STAT-6 and GATA-3 and also the additional transcription factors PU.1 and IRF-4 (55, 56). However, it remains to be determined whether Th9 cells represent a truly distinct and stable lineage or if IL-9 production is indicative of plasticity among other $CD4^+$ T cell subsets (57).

Eosinophils Eosinophils are multifunctional leukocytes that participate in type 2 immune responses (58). Eosinophils develop in the bone marrow and require the coordinated actions of at least three transcription factors - GATA-1 (a zinc family finger member), PU.1 (an ETS family member), and C/EBP members (CCAAT/enhancer-binding protein family) (59, 60). At rest, a majority of eosinophils traffic to the gastrointestinal tract, in a process that has been demonstrated to be independent of lymphocytes or the presence of commensal flora (61). Eosinophils can also be found preferentially in the thymus, mammary gland, and uterus under homeostatic conditions, and baseline eosinophil trafficking is thought to depend on the chemokine eotaxin-1 (also known as CCL11). During inflammation, eosinophils migrate from the peripheral blood into tissues in a process that has been suggested to involve multiple mediators such as cytokines (including IL-4, IL-5 and IL-13), adhesion molecules (including β1, β2 and β7

integrins), and chemokines (including RANTES/CCL5 and the eotaxins) (58). Eosinophil granules can contain a wide variety of mediators, including the cytotoxic cationic proteins major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin (62). Eosinophils have also been documented to secrete a plethora of additional molecules including proinflammatory cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and TGF-α/β), chemokines (RANTES/CCL5 and eotaxin-1), and lipid mediators (platelet-activating factor and leukotriene C4) after *ex vivo* stimulation (63), although it remains to be determined whether these factors are actually secreted during inflammation *in vivo*.

Basophils Basophils are rare granular leukocytes that are present in all vertebrate species. They comprise less than 1% of the circulating leukocytes in the blood, but are rapidly recruited to inflamed tissues during helminth infection or the establishment of allergic lung inflammation (64). Recruitment and accumulation of basophils into inflammatory tissues has been shown to be dependent on $CD4^+$ T cells (65, 66), and is therefore believed to occur during late-phase inflammation after antigen exposure (67). Basophil migration is suggested to involve a multitude of factors, including monocyte chemotactic proteins, eotaxins, RANTES/CCL5 and monocyte inhibitory protein-1α/CCL3 (68). However the precise mechanisms of basophil trafficking have not been fully elucidated. Basophils express the high-affinity IgE receptor FcεRI, and cross-linking of this receptor with IgE triggers a rapid signaling cascade that culminates in the release of allergic mediators, including eicosanoids, vasoactive amines and peptides, and proteolytic enzymes such as granzyme B (69, 70). Basophil effector function is also thought to include the secretion of type-2 associated cytokines. Murine and human

basophils have been shown to produce IL-4 under multiple conditions *in vitro* (71, 72), and murine basophils have been shown to produce IL-4 *in vivo* during helminth infection (65, 66) and after challenge with the cysteine protease papain (73). Mouse and human basophils have also been shown to produce IL-13 *in vitro* after culture with multiple stimuli, including anti-FcεRI, IL-3, IL-18 and IL-33 (74-76).

Mast cells Mast cells enter the circulation as immature precursors, and do not fully mature until they migrate into tissues. The differentiation and survival of mast cells is highly dependent on stem-cell factor (also known as KIT ligand), and the number and phenotype of mast cells can also be influenced by a variety of additional factors including growth factors, cytokines and chemokines (77). Mast cells are preferentially found in vascularized tissues that are exposed to the environment, such as the skin, gastrointestinal tract and airways. Like basophils, mast cells express FcεRI and can become activated by cross-linking with IgE. IgE or antigen-mediated activation can lead to mast cell degranulation, resulting in the release of a plethora of immunomodulatory compounds such as histamine, proteases, lipid mediators, and cytokines (including IL-4, IL-5, IL-9 and IL-13 among numerous others) (77). Studies utilizing mast cell deficient mice have revealed a crucial role for mast cells in multiple IgE-dependent processes, including tissue swelling (78), fibrin deposition (79), enhanced airway hyperreactivity (80) and recruitment of innate immune cells to the airways or intestine (81). Mast cell derived mediators such as TNF have been shown to contribute to airway hyperreactivity and inflammation during an asthma model (82). Although accumulation of mucosal mast cells is commonly observed during helminth infection, the precise role of these cells in parasite immunity is not well defined (83).

NKT cells Natural helper T (NKT) cells are unique lymphocytes that share features common to adaptive T cells and also to innate immune cells (84). NKT cells comprise less than 1% of total lymphocytes in the thymus, bone marrow, spleen, blood and lymph nodes, but are enriched in the liver (>10%). A majority of NKT cells recognize antigens that are presented by the MHC molecule CD1d, and are referred to as invariant (i)NKT cells. Like $CD4^+$ and $CD8^+$ T cells, iNKT cells undergo selection in the thymus and express an αβ TCR, although the receptor diversity is much more limited. Most iNKT cells have an invariant α chain encoded by a Vα14–Jα18 rearrangement. iNKT cells can also express markers found on innate NK cells, such as NK1.1, NKG2D and members of the Ly-49 family. The first foreign antigen shown to activate NKT cells was α-galactosyl ceramide (αGalCer), a compound isolated from a marine sponge. Subsequently, iNKT cells have been shown to respond to other hexose sugars α-linked to lipids including glycosphingolipids from *Sphingomonas* bacteria (85), and diacylglycerol antigens from *Borrelia burgdorferi* (86). It is likely that iNKT cells can also recognize specific self-antigens. One of the defining features of iNKT cells is their ability to rapidly produce cytokines, including IFN-γ and IL-4, upon stimulation with αGalCer (87, 88). Although less established, activated iNKT cells have also been shown to secrete other cytokines such as TNF, IL-5, and IL-13 (89). The mechanisms contributing to differential production of cytokines by iNKT cells are not well elucidated.

Type-2 associated innate lymphoid cells In the past ten years, a series of papers has revealed the existence of an additional cell type capable of secreting type 2-associated cytokines and participating in immunity to helminth infection and the establishment of allergic lung inflammation. The first descriptions of these cells came

from DNAX Research Inc. (now Schering-Plough Biopharma), where investigators described a novel lineage of "non-B/non-T" cells that dramatically expanded and expressed IL-4, IL-5 and IL-13 in response to stimulation with the cytokine IL-25 or IL-33 (90-92). The accumulation of these non-B/non-T cells was also observed during helminth infection, and the absence of these cells in IL-25$^{-/-}$ mice was associated with defects in worm expulsion (93). More recently, similar innate lymphoid type 2 cytokine producing cells have been described to reside in specialized structures called fat-associated lymphoid clusters and to play a role in the self-renewal of and IgA production by mucosal B1 cells (94). In this same study, these innate cells were shown to produce IL-13, which could contribute to goblet cell hyperplasia in a mouse model of helminth infection. The generation of a novel IL-13 reporter mouse by Neill et al. allowed for the characterization of similar cells in the mesenteric lymph nodes of mice (95). This group revealed that these IL-13-producing cells expand in response to IL-25 or IL-33 and during infection with *Nippostrongylus brasiliensis*, and that they fail to expand or become activated in mice doubly deficient in IL-25 and IL-33 receptors. Cells with a similar phenotype have been associated with influenza-induced airway hyperreactivity (96) and mouse models of asthma (97, 98). A subsequent paper has demonstrated that these cells are induced during papain-induced lung inflammation and are capable of secreting IL-9 (99). Although it appears likely that the innate type 2-associated cells described by multiple researchers are in fact cells of the same lineage, these cells have been given multiple names including natural helper cells (94), nuocytes (95), innate lymphoid cells (96, 99), and Ih2 cells (our work). Although individual reports identify some distinguishing characteristics of each subset, there appears to be a consensus that

these cells are negative for lineage markers and positive for IL-7R, c-kit, CD45, Thy1, ICOS and CD25 (100). However, it still remains possible that the cells described by various researchers are indeed distinct populations, representing phenotypically different effector cells derived from similar precursors. To simplify discussion, we will assume that these innate type 2-associated cells are a single lineage and will use the nomenclature we proposed on our manuscript and refer them as Ih2 cells.

Insights from cytokine reporter mice Reporter mice generated to report the expression of type 2-associated cytokines have provided important insights into the cellular sources of these cytokines *in vivo*. Multiple IL-4 reporter mice have been generated, including 4get mice (101) and KN2 mice (102) from our group and G4 knock-in GFP mice from William Paul's laboratory (103). The 4get reporter was generated such that GFP is produced when the IL-4 locus is active, and thus effectively marks cells that are competent or poised to secrete IL-4. Cell types that are either constitutively GFP^+ or can be induced to express GFP under certain condition in these mice include Th2 cells, eosinophils, basophils, mast cells, NKT cells, and Ih2 cells (66, 88, 93, 104). Although these 4get reporter-positive cells are competent to produce IL-4, it does not necessarily mean that they are actively producing IL-4 *in vivo*. In contrast, KN2 reporter mice accurately identify cells that are producing IL-4 protein. These reporter mice have been used to demonstrate that Th2 cells and basophils actively produce IL-4 during parasitic infection (102, 105). The G4 mice have confirmed that Th2 and basophils can produce IL-4 during infection (65). Although there is evidence using the KN2 mice that a very small percentage of eosinophils may produce IL-4 (106), it remains unclear whether eosinophils, NKT cells or Ih2 cells actively make IL-4 during infection or

allergic lung inflammation. IL-13 GFP knock-in reporter mice were generated by Andrew McKenzie's group and were used to demonstrate that Ih2 cells were major innate sources of IL-13 (95). This group further demonstrated that small numbers of iNKT cells, mast cells, basophils, and γδ T cells were IL-13 reporter-positive during *N. brasiliensis* infection. IL-9-Cre reporter mice have also recently been generated and used to fate map IL-9 producing cells (99). Activation of the IL-9 reporter was observed by $CD4^+$ T cells cultured with TGF-β and IL-4, conditions associated with polarization of the "Th9" lineage, and also by Ih2 cells during the establishment of papain-induced lung inflammation. Although the results have not yet been published, IL-5 reporter mice were generated by our group, and we have verified that Th2 cells and Ih2 cells express IL-5 at rest, after administration of IL-25 or IL-33, and during *N. brasiliensis* infection (Jesse Nussbaum – personal communication).

Initiation of type 2 immune responses

A multitude of recent studies have revealed new and important roles for the cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) in the initiation of type 2 immune responses. Although multiple cellular sources for each of these cytokines have been reported (107), the majority of studies have focused on their expression by epithelial cells. While epithelial cells were originally viewed as merely constituting a passive barrier to infection or injury, it is becoming increasingly apparent that epithelial cells are crucial and active participants in pathways that maintain mucosal homeostasis and also in innate and adaptive immune responses. Epithelial cells express many genes that allow them to participate in these responses, including major histocompatibility

complex (MHC) class I and II, innate immune receptors such as toll-like receptors (TLRs), costimulatory molecules, chemokines, cytokines, and prostaglandins. IL-25, IL-33 and TSLP are expressed during helminth infection and allergen challenge and overexpression of either of these cytokines can lead to the downstream features of a type 2 immune responses, including the production of IL-4, IL-5 and IL-13, increased serum IgE, IgA and IgG1, and tissue manifestations such as goblet cell hyperplasia and mucus production and smooth muscle hyperreactivity (90, 92, 108).

IL-25 (also known as IL-17E) is a member of the IL-17 family (discussed in detail in Part C). IL-25 binds to a receptor complex comprised of IL-17RA and IL-17RB (109, 110). IL-25 is preferentially expressed in mucosal tissues such as the lung, stomach, small intestine, colon and kidney. IL-25 mRNA has been detected in epithelial cells, Th2 cells, mast cells, and alveolar macrophages, although the production of IL-25 protein has not been confirmed in most of these cell types (100). IL-25$^{-/-}$ mice fail to expel *N. brasiliensis* efficiently, as worm clearance was observed on day 17 instead of the normal day 10. Conversely, administration of exogenous IL-25 can mediate rapid worm clearance, even in Rag$^{-/-}$ mice lacking B and T cells (93). Blocking antibodies to IL-25 lead to reduced airway hyperreactivity and lung inflammation in a model of allergic airway disease (111). These effects were associated with reduced type 2 cytokine production, and consequent reductions in eosinophil infiltration, goblet cell hyperplasia and IgE production. Although IL-25 likely operates through multiple mechanisms, one of the notable downstream effects of this cytokine is promotion of the expansion and effector function of Ih2 cells (90, 91, 93, 95).

IL-33 is a member of the IL-1 cytokine family, and signals through a receptor composed of T1/ST2 (also known as just ST2 or IL1lR1) and IL-1R accessory protein (abbreviated as IL-1RAcP). Early descriptions of ST2 described its expression by polarized Th2 cells and mast cells (112, 113). $ST2^{-/-}$ mice do not form normal granulomas in response to *Schistosoma mansoni* eggs (114) and have diminished inflammation in models of OVA-induced asthma (115). Intranasal administration of IL-33 contributes to the development of airway inflammation, characterized by epithelial hypertrophy and mucus production (92). Incubating Th2 cells with IL-33 *in vitro* results in enhanced production of IL-5 and IL-13 (92), suggesting that T cells can directly respond to IL-33. Subsequent reports demonstrated IL-33-mediated effects on innate cell populations *in vitro*, such as increased survival and cytokine production by human and murine mast cells (116-118), MyD88-dependent production of IL-4, IL-6 and IL-13 by basophils (119), and enhanced degranulation, IL-8 production, and survival of eosinophils (120). Like IL-25, IL-33 has been demonstrated to potently induce the accumulation of Ih2 cells, and to promote the production of IL-5 and IL-13 by these cells (92, 94, 95).

TSLP is structurally similar to IL-7, and signals through a receptor comprised of the IL-7Rα chain and a unique TSLPR chain. Basal expression of TSLP has been observed in the epithelial cells of the skin, lung and intestine (107). *In vitro* experiments demonstrated a role for TSLP in conditioning $CD11c^{+}$ dendritic cells (DC) to more effectively promote Th2 differentiation while suppressing Th1 differentiation (108, 121, 122). This conditioning has been shown to involve the promotion of dendritic cell secretion of Th2 chemoattractants such as CCL17 and CCL22 (108) and the expression

of OX40 ligand, which is a member of the TNF cytokine family that has been implicated in interactions between B cells, T cells and DCs and in the promotion of Th2 differentiation (122). Mice deficient in the TSLPR have normal Th1 responses, but fail to develop lung inflammation in a murine asthma model (108, 121).

IL-25, IL-33 and TSLP are upregulated during helminth infection or after challenge with allergens (93, 123-125), although the identity of the pathogen or allergen-specific motifs that are being sensed are not well-defined (126). It has been suggested that type 2 immune reactions can be initiated by recognition of low levels of lipopolysaccharide (LPS) (127), glycans on schistosome eggs (128), schistosome phosphatidylserine (129), and a variety of proteases, including the cysteine protease papain (73, 130), the protease allergen Der p1 from house dust mites (131, 132), and undefined protease components in the secretions of the *Necator americanus* hookworm (131). More recently, it was demonstrated by our group that chitin, a ubiquitous polymer that is a major component of fungal cell walls and the exoskeletons of arthropods, could function as a type 2 pathogen-associated molecular pattern (PAMP) (133). Administration of chitin, either into the lung or peritoneal space, led to leukotriene-mediated infiltration of eosinophils and basophils and the alternative activation of macrophages. Induction of epithelial cytokine production could also be induced indirectly, by exposure to proinflammatory or type-2-associated cytokines or other mediators. Indeed, enhanced expression of TSLP by epithelial cells was observed after exposure to IL-1β and TNF-α or to IL-4 and IL-13 (134-136). Administration of either IL-25 (123) or IL-33 (124) can induce the production of TSLP, suggesting the existence of cross-regulation between these epithelial cytokines. Epithelial injury or trauma

resulting from migrating helminths or exposure to allergens might be another trigger for release of epithelial cytokines. Epithelial damage associated with procuring biopsy samples was associated with production of TSLP (125). Intriguingly, IL-33 appears to be sequestered within the nucleus of intact cells and to be specifically released during necrosis, which is associated with tissue injury or infection (137). During non-inflammatory apoptotic death, IL-33 is cleaved by the apoptotic caspases 3 and 7 into a functionally inactive form (138). The release of IL-33 during necrosis but not apoptosis, led to its characterization as an "alarmin", as its release and subsequent signaling alerts the immune system to the presence of trauma or infection.

Type 2 cytokines in helminth infection

Nippostrongylus brasiliensis is a naturally occurring nematode parasite of rats. Experimental infection of mice with this pathogen is a widely used experimental model of helminth infection, and studies using this pathogen have been instrumental in elucidating the mechanisms of type 2 immunity. In this model, infective larvae are introduced into mice subcutaneously. The injected worms then migrate through the skin and enter venules, which deliver them to the lung. The larvae then migrate from the alveoli into the trachea, where they are coughed up, swallowed, and enter the digestive tract. Once anchored in the small intestine, the worms mature into adults and lay eggs that are then secreted in the feces. *N. brasiliensis* infection is generally cleared within 6-10 days by immunocompetent mice, and expulsion of worms is dependent upon the generation of a productive type 2 immune response. Infection of mice deficient in type 2-

associated cells, cytokines and transcription factors has allowed for the elucidation of the components of type 2 immunity that are crucial for immunity to this helminth.

The epithelial cytokines IL-25 and IL-33 are induced during *N. brasiliensis* infection and have been suggested to play a crucial role in the initiation of immunity to this pathogen (91-93). Supporting this model, IL-25$^{-/-}$ mice and mice deficient in IL-25 or IL-33 receptor chains have delayed worm clearance (95). These cytokines are likely able to partially compensate for each other during infection, as deficiency in both receptors leads to a much more severe defect in worm expulsion (95). One of the major effects of IL-25 and IL-33 during infection is the promotion of the expansion Ih2 cells in the lung and mesenteric lymph node (MLN) and the activation of these cells to produce cytokines such as IL-5 and IL-13 (91, 93). TSLP has also been proposed to play a role in the early phases of infection, as its expression is upregulated and TSLPR$^{-/-}$ mice have a slight delay in worm clearance. However, TSLPR$^{-/-}$ mice were shown to have no defect in the features of the *N. brasiliensis*-induced immune response, including normal differentiation of Th2 cells, type 2 cytokine production, and *N. brasiliensis*-specific IgE and IgG1 antibody levels (139).

T cells are crucial for the resolution of infection with *N. brasiliensis*, as SCID and Rag$^{-/-}$ mice lacking T and B cells or TCR-C$\alpha^{-/-}$ mice lacking T cells do not effectively expel the parasite (34, 140, 141). By day 9 after infection, approximately half of all CD4^{+} T cells in the lung have acquired a Th2 phenotype, as assessed by activation of the IL-4 locus (66). Accumulation of innate immune cells, such as basophils, eosinophils, and Ih2 cells are also a defining feature of *N. brasiliensis* infection, and these processes have been shown to depend on CD4^{+} T cells (66, 95). During infection, IL-4 is produced

by follicular helper T cells in the B cell follicle and promotes B cell class switching to IgE and IgG1 isotypes (142). However, despite being essential for the generation of parasite-specific IgE and IgG1 antibodies, IL-4 is not essential for worm clearance, as IL-$4^{-/-}$ mice resolve primary infection normally (143). Additionally, mice deficient in IL-5 and IL-9 do not have obvious defects in immunity to *N. brasiliensis* (44, 144). In contrast, mice deficient in IL-13 or elements downstream of IL-13 signaling, such as IL-4Rα and STAT-6 have severe defects in worm expulsion and the other features of the anti-helminth immune responses (33, 145). IL-13 has multiple downstream functions that contribute to worm clearance, including the promotion of goblet cell hyperplasia and mucus secretion, smooth muscle contraction, and the alternative activation of resident and inflammatory macrophages (30, 31).

Although $CD4^+$ T cells produce IL-4 and IL-13 during *N. brasiliensis* infection, there is evidence to suggest that innate cells might be more important sources of these cytokines. In an experiment by Voehringer et al. (141), it was demonstrated that the transfer of IL-$4/13^{-/-}$ T cells to TCR-$C\alpha^{-/-}$ mice could rescue the defect in worm clearance seen in TCR-$C\alpha^{-/-}$ mice, while the transfer of wild-type $CD4^+$ T cells to IL-$4/13^{-/-}$ TCR-$C\alpha^{-/-}$ mice did not rescue this response. This suggests that IL-4 and IL-13 production from innate cells might be necessary for the resolution of this infection. Basophils have been shown to be the major innate source of IL-4 during *N. brasiliensis* infection (65, 141), while Ih2 cells are the major innate source of IL-13 (95). Deficiency in either basophils or eosinophils does not impair the ability to clear worms (105, 141), suggesting that Ih2 cells might be the most important innate source of type 2 cytokines. Indeed, transfer of wild-type Ih2 cells into an IL-$4/13^{-/-}$ mouse after infection was enough to

almost completely rescue worm clearance in the donor mice (95). During the course of *N. brasiliensis* infection in an intact mouse, it is likely that type 2 cytokine production is at least somewhat redundant, such that the same cytokines are produced coordinately by cells of the innate and adaptive immune system and each compartment plays an important role in promoting anti-helminth immunity.

Other commonly used helminth models include infection with the intestinal nematodes *Trichuris muris* and *Heligmosomoides polygyrus*. *T. muris* is a whipworm that resides in the mouse cecum and colon, where it anchors itself within the intestinal epithelium to promote feeding. Interestingly, *T. muris* causes chronic infection in certain susceptible inbred mouse strains (such as AKR mice), but is efficiently expelled from other strains (such as C57BL/6) (146). *H. polygyrus* is a natural parasite of mice that establishes a chronic infection in the small intestine that lasts for a period of weeks to months (147). Although *N. brasiliensis*, *T. muris*, and *H. polygyrus* each have a unique natural history, including parasite life cycles and the route by which they establish and maintain intestinal infection, infection with either of these three parasitic helminths induces potent type 2 immune responses, including the hallmark features of IgE and IgG1 antibody production, goblet cell hyperplasia and mucus production, and smooth muscle hyperreactivity. However, the immune response to these different parasites is not identical. Focusing on the unique features of each pathogen and elucidating the subtle differences in immune mechanisms employed during infections with parasites that establish a chronic infection and those that are ultimately expelled should provide further insight into type 2 immunity.

Type 2 cytokines in allergic lung inflammation

Atopic diseases are becoming increasingly prevalent in the modern world. The Centers for Disease Control estimate that 54% of the population of the United States suffers from at least one allergy and that approximately 20 million people are affected by asthma alone (148). Allergic lung inflammation or asthma is a disease that is characterized by excessive inflammation in the lung and respiratory passages that is generally the result of an aberrant type 2 immune response. This disease can be initiated by chronic exposure to environmental allergens such as pollutants, pollens, grasses, animal dander, molds, or antigens from cockroaches or dust mites (149). The pathologic features of asthma include epithelial mucus cell hyperplasia, smooth muscle hypertrophy and deposition of matrix proteins in the airways. This process is referred to as airway remodeling and can contribute to the airway hyperresponsiveness that is a characteristic symptom of the disease. In many ways the features of allergic lung inflammation resemble those observed during parasitic helminth infection, including the involvement of the same cell types and the reliance on inflammatory mediators such as type 2-associated cytokines.

Although multiple mouse models are used to study the events involved in the initiation and propagation of human allergic asthma, no single model completely recapitulates all of the features of human disease (150). The most common method of initiating allergic lung inflammation is a sensitization/re-challenge model, whereby mice are first sensitized to a foreign protein by intraperitoneal injection(s) along with an adjuvant (typically aluminum hydroxide). After the cessation of the resulting immune response, the animal is rechallenged with the foreign antigen either intranasally or in the

form of an aerosol that can be inhaled. This protocol results in a marked pulmonary inflammation characterized by an influx of eosinophils, airway remodeling including epithelial thickening, and the development of airway hyperreactivity (150). The most common foreign antigen used is ovalbumin (OVA), a protein found in egg whites, although researchers have also utilized a variety of antigens alone or in combination with OVA including the pollutant nitric dioxide (151) and extracts from house dust mites (152) or the fungus *Aspergillus fumigatus* (153). The adjuvants used and dosing regimens have also been modified in multiple studies, and recent efforts have attempted to a develop a model for longer-term antigen exposure that more closely mirrors the chronicity of antigen exposure by asthmatic humans (150).

The initiation of allergic lung inflammation has recently been suggested to involve the cytokines IL-25, IL-33, and/or TSLP (107). Administration of either of these cytokines directly to the lungs of mice can lead to the development of pulmonary inflammation that results in elaboration of the hallmark features of allergic lung inflammation, including mucus production, airway remodeling and the induction of airway hyperresponsiveness (90, 92, 108). Additionally, IL-25, TSLP, and the IL-33 receptor component are found in the serum of asthmatic or allergic patients (154-156). Similar to the pathways involved in anti-helminth immunity, Th2 cells are thought to play a pivotal role in the coordination of the inflammatory response during the development of allergic lung inflammation. Th2 cells are required for the generation of allergen-specific IgE and IgG1. Allergic lung inflammation is also characterized by the recruitment and accumulation of a variety of innate immune cells including eosinophils, basophils, mast cells and Ih2 cells (97, 157). The innate and adaptive immune cells involved in the

initiation and propagation of allergic immunity exhibit a variety of effector functions, including release of inflammatory mediators such as cytokines. Expression of the type 2-associated cytokines IL-4, IL-5, IL-13 and IL-9 is induced during the establishment of inflammation, although the greatest contributions to pathogenesis appear to derive from IL-4 and IL-13 (158, 159). The important role of these cytokines in animal models has led to the establishment of IL-4, IL-13, and IL-4R antagonists as potential therapeutics for patients with asthma or allergic disease (160). While human clinical trials have so far failed to reproduce the positive results observed with animal studies, this method could eventually result in efficacious treatments for diseases that are becoming increasingly prevalent among the human population.

Part C: Cytokine production during IL-17-associated immune responses

Evolution of IL-17-associated immune responses

Although the IL-17 cytokine family is the most recent cytokine family to be discovered, this family appears to be one of the earliest to have evolved. Homologues of IL-17 family members have been found in multiple vertebrate species including mouse, rat, cow, chicken, zebrafish and rainbow trout. More recently, a homologue of IL-17 was identified in $VLRA^+$ T cell-like cells in sea lampreys, jawless vertebrates that diverged from jawed vertebrates approximately 500 million years ago (161, 162). It is likely that IL-17 family members evolved in order to provide protection against mucosal exposure or infection with extracellular bacteria or fungi. The sea lamprey IL-17 homologue *Lamp*IL-17 is expressed preferentially in the mucosal tissues of the skin, intestine and gill, and expression of *Lamp*IL-17 by skin epithelial cells was induced within 6 hours of exposure to lipopolysaccharide, a conserved feature of gram-negative bacteria (161). Further evidence for a conserved role for IL-17A in antibacterial and antifungal immunity is found in human genetic studies. Genetic deficiencies in IL-17 receptor A or IL-17F are associated with susceptibility to mucocutaneous infection with *Candida albicans* and, to a lesser extent, *Staphylococcus aureus* (163). Similarly, patients with hyper-IgE syndrome associated with mutations in Stat3 have deficits in the induction of Th17 cells that correlate with recurrent bacterial and fungal infections (164). The role of IL-17 family members in rapidly promoting proinflammatory responses, including the recruitment of neutrophils, likely played a role in the evolution and retention of this cytokine family.

The IL-17 cytokine family

The founding member of the IL-17 cytokine family, IL-17A (originally known as CTLA-8) was identified in 1993 as a cytokine secreted by activated T cell clones (165, 166). Subsequently, additional IL-17 family members were identified by sequence homology, and termed IL-17B-F. All members of this family include four conserved C-terminal cysteine residues. Among the IL-17 family members, IL-17A and IL-17F share the most homology (55%), are found on the same chromosome in both mice and humans, and appear to have similar proinflammatory effects when overexpressed (167, 168). IL-17A and IL-17F induce the secretion of a variety of cytokines (including IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, and TNF-α), chemokines (including CXCL-8, CXCL1, CXCL10, GRO-α, and MCP-1), and prostaglandins (such as PGE_2) from a variety of hematopoietic and stromal cells (169). One of the major downstream effects of IL-17A and IL-17F is rapid generation, mobilization and recruitment of neutrophils to sites of inflammation. IL-17A also appears to play a role in the maintenance of neutrophil homeostasis (170). Although IL-17A and IL-17F appear to be coordinately regulated and have similar functions in many cases, they have differential effects in certain models such as asthma and DSS-induced colitis (168). IL-17A and IL-17F can be secreted as homodimers or heterodimers, which bind to a complex of the IL-17RA and IL-17RC receptor chains and signal through a pathway that involves the adaptors ACT-1 and TRAF-6.

Recently, multiple reports have ascribed functions to another IL-17 family member, IL-17C (171-173). In the first two reports, IL-17C was shown to be produced

by epithelial cells and to promote protective antibacterial immune responses at epithelial surfaces (171, 172). In a third report, IL-17C production was observed by $CD4^+$ Th17 cells during a mouse model of experimental autoimmune encephalomyelitis (EAE) and served to further potentiate IL-17A and IL-17F production by Th17 cells (173). IL-17C signals through complexes of IL-17RA and IL-17RE, and downstream signaling involves activation of NF-κB and mitogen-activated protein kinases. IL-17E, which is better known as IL-25, has the least sequence homology to IL-17A (17%) and appears to play a role in the initiation and propagation of type 2 immune responses (explained more fully in Part B) (169). IL-25 signals through IL-17RA/IL-17RB receptor complexes. A fifth IL-17 family member, IL-17D, has been identified based on sequence homology, but a function for this cytokine remains to be determined. Similarly, an additional putative IL-17 family member receptor, IL-17D, has been identified but the respective ligand(s) and downstream signaling pathways remain to be characterized (174).

Potential sources of IL-17A

For our studies, we decided to focus on the IL-17 family member IL-17A. As mentioned previously, IL-17A was the first member of the IL-17 family to be identified and characterized. Since its discovery, a plethora of studies have focused on the role of this cytokine in immune homeostasis, during infection, and during the initiation and propagation of autoimmune diseases in both human and mice. One of the major unresolved questions in IL-17A biology involves determining the *in vivo* cellular sources of this cytokine at rest and during inflammatory responses. Evidence from multiple groups suggests that IL-17A can be produced by adaptive Th17 and $CD8^+$ cells, innate-

like T cells including γδ T cells and iNKT cells which have a recombined TCR but recognize self and foreign antigens in a manner similar to the pattern recognition receptors of the innate immune system, and multiple populations of innate immune cells. However, it is important to bear in mind that most of these studies used *ex vivo* restimulation and subsequent intracellular cytokine staining to identify cytokine-producing cells, which may skew actual *in vivo* cytokine expression patterns.

Th17 cells IL-17A was initially discovered as a cytokine secreted by activated T cells (165). Subsequently, a series of pivotal studies demonstrated that IL-17A was secreted by a specialized subset of $CD4^+$ T cells, later termed Th17 cells (175-177). Th17 cells were shown to be developmentally and functionally unique from canonical Th1 and Th2 cells, and represented a major modification to the Th1/Th2 hypothesis originally proposed by Mosmann and Coffman (178, 179). Th17 cells in both humans and mice are characterized by expression of the chemokine receptor CCR6 (180, 181) and the transcription factor RORγt. Th17 cells have been shown to play an important role in host defense against a variety of infections and also to contribute to the pathogenesis of multiple autoimmune diseases.

$CD8^+$ T cells $CD8^+$ T cells have also been shown to secrete IL-17A in both mice and humans. Murine $CD8^+$ T cells produced IL-17A after stimulation with IL-1β and IL-23 or conditioned medium from DCs that were activated with the extracellular bacterium *Klebsiella pneumoniae* (182-184). Human memory $CD8^+$ T cells also secrete IL-17A after *in vitro* stimulation of peripheral blood cells with PMA and ionomycin (185).

γδ T cells γδ T cells have been shown to be potent sources of IL-17A in multiple models (186). γδ T cells have features of both innate and adaptive immune cells. These

cells express T cell receptors (TCR), although in both humans and mice they use a limited number of Vγ and Vδ genes. Like αβ T cells, γδ T cells develop in the thymus, although, unlike αβ T cells, a subset of γδ T cells can mature and exit the thymus without encountering their cognate antigen (187). In a study by Jensen et al. (188), it was demonstrated that these antigen-inexperienced γδ T cells are more likely to express IL-17A when activated, while antigen-experienced γδ T cells express IFN-γ. Identifying γδ T cell ligands has proven challenging, and it appears that γδ T cells can be activated through both TCR-independent and TCR-dependent mechanisms. Ligands that have been identified include a variety of nonpeptide antigens from pathogens (including lipids, phosphoantigens and alkyl amines) and self-derived molecules often associated with stress or tissue damage (including the murine MHC class Ib molecules T10 and T2, the human MHC class-I-like molecules MICA and MICB and ATP synthase F1–apolipoprotein A-I complex) (189). γδ T cells are generally thought to reside in mucosal tissues, where they play a role in immune surveillance. Despite their relatively low numbers (1-5% of total T cells), mice deficient in γδ T cells have deficits in host defense against a variety of pathogens and delayed onset of some autoimmune disorders, suggesting that γδ T cells may constitute an important component of inflammatory response (189, 190). γδ T cells capable of producing IL-17A upon restimulation have been identified in resting mice, during multiple infections, and during autoimmune diseases, and can be further characterized by expression of CD25, CCR6, IL-1R, IL-23R and the scavenger receptor SCART2 (191).

iNKT cells As mentioned previously, NKT cells are lymphocytes that have features common to both adaptive T cells and innate NK cells. iNKT cells are a subset of

NKT cells that possess an invariant a chain encoded by a Vα14–Jα18 rearrangement and utilize a limited number of β chains. iNKT cells are further defined by their ability to respond to αGalCer presented by the MHC molecule CD1d. iNKT cell subsets have been identified that produce either IL-4 or IFN-γ (192). More recently, a subset of iNKT cells that can produce IL-17A has been defined (193-197). These iNKT cells were isolated from the thymus based on their $CD44^{+}NK1.1^{-}CD4^{-}$ phenotype and produced IL-17A in a TCR-dependent fashion in response to *ex vivo* stimulation with αGalCer or the addition of the cytokines IL-1β and/or IL-23. Additionally, $CCR6^{+}$ iNKT cells isolated from the peripheral blood of rhesus macaques infected with simian immunodeficiency virus were capable of secreting IL-17A (198).

Innate lymphoid cells Multiple populations of $CD3^{-}$ lymphoid cells have been suggested to produce IL-17A. Indirect evidence for a non-T cell source for IL-17A was found in experiments demonstrating the presence of IL-17A in $Rag^{-/-}$ mice, which lack B and T cells, during a mouse model of inflammatory colitis induced by administration of a monoclonal antibody to CD40 (199). Cells resembling lymphoid tissue inducer (LTi) cells have been isolated from multiple organs of the mouse and can produce IL-17A after stimulation with IL-23 or the TLR-2 agonist zymosan (200). These "LTi-like" cells were defined as being negative for lineage markers (including CD3) and IL-7R positive, and these cells also constitutively expressed CD25, aryl hydrocarbon receptor, and CCR6. A subsequent study further characterized these cells as being positive for the surface markers Thy1, Sca-1 and IL-23R and the transcription factor RORγt and referred to this population as innate lymphoid cells (201). These authors further demonstrated that these innate lymphoid cells respond to IL-23 and play a pathogenic role in both bacterial innate

colitis and T cell-dependent colitis models. A subset of peritoneal NK1.1$^+$ cells was also demonstrated to express IL-17A during infection with *Toxoplasma gondii* (202). The localization of these cells make it possible that these cells were not conventional NK cells, but rather a subpopulation of a recently identified NKp46$^+$ population of mucosal NK-like cells that likely more closely resembles LTi-like/innate lymphoid cells (202, 203). However, these cells have mainly been noted to produce the IL-17-associated cytokine IL-22 rather than IL-17 (204-206). Further work is needed to characterize these innate lymphoid populations, to determine the relevant cytokines they produce, and to determine their role during inflammatory responses.

Paneth cells Paneth cells are specialized epithelial cells that reside in the small intestine, just below intestinal stem cells. These cells play an important role in innate immunity in the gut by secreting antimicrobial peptides and inflammatory mediators from pre-formed granules upon stimulation with cytokines or through pattern recognition receptors (207). In a report by Takahashi et al. (208), immunohistochemistry was used to demonstrate that Paneth cells contain IL-17A under homeostatic conditions, and that this cytokine is rapidly secreted during a model of TNF-induced shock. IL-17A from Paneth cells was shown to induce the production of chemokines and downstream recruitment of neutrophils.

Myeloid cells As mentioned above, IL-17A plays an important role in neutrophil homeostasis (170). In addition, two reports have suggested that neutrophils can produce IL-17A directly. In a model of arthritis induced by administration of anti-neutrophil cytoplasmic antibodies, IL-17A mRNA was detected in purified neutrophils and restimulation of these neutrophils led to production of IL-17A (209). Neutrophils were

also shown produce IL-17A in response to acute kidney ischemia-reperfusion injury (IRI) (210). In this model $CD11b^{+}Gr1^{+}$ cells were shown to be the major source of IL-17A in the kidney, and transfer of $CD11b^{+}Gr1^{+}$ cells to IL-$17A^{-/-}$ mice after initiation of IRI promoted kidney inflammation. IL-17A has been suggested to be produced by human macrophages during prostate cancer (211) and by alveolar macrophages during allergic lung inflammation (212), although these reports have not been confirmed by other researchers.

Insights from IL-17A reporter mice IL-17A reporter mice were recently generated independently by two different groups (213, 214), which allowed for important validation and comparisons to previous studies, which relied almost completely upon *ex vivo* restimulation to assay cytokine production. The reporter mouse designed by Hirota et al. (213) is a knock-in, in which Cre recombinase was inserted into the *il17a* locus. These mice were crossed to mice that express YFP from the *Rosa26* promoter, in order to facilitate fate-tracking. Under homeostatic conditions, the authors identified $CD4^{+}$ Tcells, γδ T cells, $CD4^{-}CD8^{-}\alpha\beta TCR^{+}$ NKT cells, and lineage-negative innate lymphoid cells that were YFP^{+}, suggesting that these cells had expressed IL-17A at some point during their development. The authors further observed significant populations of YFP^{+} $CD4^{+}$ T cells and γδ T cells during the development of EAE and during cutaneous infection with *Candida albicans*. The IL-17A reporter mouse designed by Esplugues et al. (214) is also a knock-in, in which eGFP is inserted into the *il17a* locus which allows for the tracking of current or recent IL-17A production. The authors noted very small percentages of GFP^{+} $CD4^{+}$ T cells in naïve mice, suggesting that IL-17A is produced at low levels in a resting mouse. However, the percentage of $GFP^{+}CD4^{+}$ cells increased

dramatically during a model of tolerance induced by administration of a CD3ε$^+$ antibody. Numbers of GFP$^+$ CD4$^+$ T cells also increased during sepsis and influenza virus infection. Unfortunately, the authors did not show data for GFP expression in cells besides CD4$^+$ T cells. Additionally, IL-17F reporter mice were generated by three different groups (215-217). Although IL-17A and IL-17F are not functionally equivalent in certain models, they are co-expressed in many instances and the authors of all three papers used IL-17F expression as a surrogate for identification of IL-17A-producing CD4$^+$ Th17 cells (168). Although the groups used different targeting strategies and reporter molecules, they all identified reporter-positive CD4$^+$ T cells after Th17 polarization of naïve CD4$^+$ T cells *in vitro* and under various conditions *in vivo*. Croxford et al. (215) additionally identified reporter-positive CD8$^+$ T cells, but only after *in vitro* culture and not during development of EAE. The results from reporter mice provide a nice confirmation that IL-17A can be expressed by CD4$^+$ T cells, γδ T cells, NKT cells and innate lymphoid cells. It remains to be validated whether CD8$^+$ T cells, Paneth cells, or myeloid cells are true sources of IL-17A *in vivo*.

Initiation of IL-17-associated responses

As it has become increasingly apparent that IL-17A plays an important role in inflammation, researchers have become very interested in determining the mechanisms that lead to IL-17A secretion by both Th17 cells or IL-17A-producing innate or innate-like T cell populations. During an infection, recognition of pathogen associated molecular patterns (PAMPS) and nonspecific tissue damage lead to the release of a wide array of proinflammatory mediators. The unique composition of the inflammatory milieu

then promotes the differentiation of distinct $CD4^+$ T cell lineages. The differentiation of Th17 cells has been shown to require IL-6 and TGF-β (218-220). Signaling through IL-6 promotes the activation of the transcription factor STAT-3, which then leads to expression of a second transcription factor RORγt, which is critical for adoption of the Th17 program (220). Although IL-23 is dispensable for initial differentiation of Th17 cells, it appears to be important for the maintenance, stability and effector functions of this lineage (221, 222). IL-23 may also play a role in secretion of IL-17A by memory cells (175). The aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor that senses environmental toxins such as dioxin, also plays a role in the differentiation of and cytokine secretion by Th17 cells (223, 224). Other factors such as IL-1β, IL-21 and the transcription factor RORα have also been implicated in the differentiation or maintenance of Th17 cells (225-229).

Generation of antigen-specific Th17 cells presumably takes between 3-5 days, at which point Th17 cells can home to the inflamed tissue and participate in immune responses. In the interim, innate and innate-like T cells are likely the main sources of IL-17A. Interestingly, the requirements for innate cell or innate-like T cell production of IL-17A appear to differ slightly from those of adaptive Th17 cells. Unlike their Th17 counterparts, γδ T cells, iNKT cells and innate lymphoid cells do not require IL-6 to produce IL-17A (196, 230, 231), however the transcription factor STAT-3 is still required. It is possible that STAT-3 is being induced by IL-23 or IL-21 in these cells, although this remains to be formally proven. Secretion of IL-17 by innate cells and innate-like T cells requires RORγt and also expression of the aryl hydrocarbon receptor (191). IL-17A-producing innate cells and innate-like T cells constitutively express the

receptors for IL-23 and IL-1β, and are able to rapidly produce IL-17A after being exposed to these cytokines during inflammatory responses(196, 200, 232-234). Martin et al. (235) further demonstrated that IL-17A-producing γδ T cells express toll like receptor (TLR)1, TLR2, and dectin-1 and that engagement of these receptors or exposure to pathogen products can lead to IL-17A production from these cells that is enhanced by the presence of IL-23.

IL-17A in homeostasis

IL-17A and other cytokines associated with IL-17A-mediated responses contribute to the maintenance of homeostasis at mucosal barriers. IL-17A enhances the synthesis of claudins, which are tight junction proteins that are critical for epithelial integrity (236). Neutralizing IL-17A during a model of dextran sodium sulfate (DSS)-induced colitis exacerbated colonic injury (237). IL-17A and the associated cytokine IL-22 promote the production of antimicrobial peptides and compounds such as β-defensins, regenerating (REG) proteins, S100 proteins, lipocalins and lactoferrins by epithelial cells and Paneth cells in the intestine (191). IL-17A and IL-22 also cooperate to promote production of β-defensins and calgranulin by bronchial epithelial cells (238, 239). These antimicrobial compounds are important in preventing dissemination of commensal bacteria and also in defense against pathogenic species. Interestingly, colonization of mice with a single species, segmented filamentous bacteria (SFB), promotes an increase in differentiation of Th17 cells, which subsequently leads to production of antimicrobial compounds and enhanced protection against subsequent infection with the bacterial pathogen *Citrobacter rodentium* (240). Importantly, regulatory T cells (Tregs) probably

play a critical role in limiting and regulating IL-17A-producing cells and preventing unnecessary or uncontrolled mucosal inflammation.

IL-17A in infection

Studies using blocking antibodies or mice deficient in IL-17A or IL-17R have demonstrated that IL-17A is a crucial component in immunity to a variety of infections. Early production of IL-17A after infection leads to the production of proinflammatory chemokines and cytokines that can recruit and activate neutrophils and other innate immune cells. IL-17A production has been observed during multiple infections that require rapid neutrophil recruitment (within 4-8 hours) for bacterial control or clearance, including *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Candida albicans* (241). The speed of this initial proinflammatory response suggests that early IL-17A production might be mediated by innate cells, innate-like T cells, and memory $CD4^+$ T cells rather than newly generated Th17 cells, as the generation of antigen-specific Th17 cells can take 3-5 days. In fact, multiple reports suggest that γδ T cells may be the predominate source of IL-17A during the early stages of certain infections. Antigen-specific Th17 cells may play a pivotal role in later stages of infection, however. In many cases, initial infection leads to induction of both IL-17A and IFN-γ, which appear to have unique and complementary functions in bacterial immunity. In general, it appears as if IL-17A promotes local inflammation while IFN-γ has more systemic effects.

Perhaps the best evidence for an essential role for IL-17A in infection is found during infection with the gram-negative extracellular bacterium *Klebsiella pneumoniae*, a

model of acute bacterial pneumonia. IL-17A$^{-/-}$ and IL-17R$^{-/-}$ mice are both more susceptible to infection with *K. pneumoniae* (238, 242), and the resulting absence of IL-17A signaling leads to a reduction in chemokine production and deficiencies in early neutrophil recruitment during infection (242). Stimulation of dendritic cells with *K. pneumoniae* leads to TLR4-dependent production of IL-23, which is important for IL-17A production in this model (183). IFN-$\gamma^{-/-}$ mice also have increased mortality to *K. pneumoniae* (243). Further supporting a role for both IL-17A and IFN-γ in *K. pneumoniae* immunity, mice deficient in the IL-23 p19 subunit, which have substantially diminished IL-17A production, and mice deficient in the IL-12 p35 subunit, which have limited IFN-γ production, have significantly enhanced mortality to infection (244). Interestingly, IL-23p19 mice have markedly reduced local inflammation in response to infection, while mice deficient in the IL-12 p35 subunit have specific deficits in systemic immune responses. Early studies characterizing Th17 cells identified a role for these cells in immunity to the extracellular bacterium *Citrobacter rodentium* (221). Infection with *C. rodentium* induces the differentiation of a large percentage of Th17 cells, as assessed by their ability to secrete IL-17A after restimulation. Interestingly, Th17 cells developed normally in mice deficient in the IL-23p19 subunit, but the Th17 cells that were generated in these mice did not function normally, and infected IL-23p19$^{-/-}$ mice displayed significantly increased morbidity and mortality. More recent work has suggested that the IL-17-associated cytokine IL-22 may actually play a more pivotal role in immunity to *C. rodentium* than IL-17A (245).

γδ T cells appear to be a more significant source of IL-17A than canonical Th17 cells during pulmonary infection with *Mycobacterium tuberculosis* (246). Interestingly,

IL-17A induced by vaccination with *M. tuberculosis* antigens leads to the production of chemokines that are able to recruit protective Th1 cells (247, 248), further suggesting that cooperation between IL-17A and IFN-γ secreting cells may be a crucial component of antibacterial immunity. IL-17A is also induced during *Bordetella pertussis* infection, and immunization with the whole cell pertussis vaccine leads to induction of both Th17 and Th1 cells, which cooperate to mediate vaccine-induced protection (249). Intraperitoneal challenge with *Escherichia coli* or *Salmonella enterica* also leads to IL-17A production (250, 251), and in both models resident γδ T cells were found to be the major source of IL-17A and contribute to an early influx of neutrophils after infection. IL-17A is produced during cutaneous infection with *Staphlococcus aureus*, and also appears to be produced predominately from γδ T cells (252). $\gamma\delta^{-/-}$ and IL-17R$^{-/-}$ mice, but not $\alpha\beta^{-/-}$ mice had larger skin lesions and impaired neutrophil recruitment during *S. aureus* infection, which was demonstrated to be dependent on the secretion of IL-17A by resident epidermal V$\gamma5^{+}$ γδ T cells

IL-17A is also induced during infections with fungal species. Clearance of *Crypotococcus neoformans* is delayed in IL-23 p19$^{-/-}$ mice, and a greater percentage of these mice succumb to disease (253). IL-17R$^{-/-}$ mice also have increased mortality after *Candida albicans* infection, which is characterized by reduced mobilization and recruitment of neutrophils and higher fungal burdens in the kidney (254). Similarly in a model of oral candidiasis, mice deficient in IL-23 p19, IL-17R or RORγt are much more susceptible to disease (255). In contrast, IL-23p19$^{-/-}$ mice were less susceptible to intragastric infection with *C. albicans* and intranasal infection with *Aspergillus fumigatus*

(256), suggesting that route of infection may be a critical determinant in determining the efficacy of IL-17-mediated immune responses during fungal infections.

IL-17A in autoimmune disease

Many studies have focused on the role of IL-17A in the development and propagation of organ-specific autoimmunity and chronic inflammatory conditions. The inflammatory response initiated by IL-17A, including production of proinflammatory mediators and recruitment of innate cells, can be pathogenic if dysregulated. The earliest studies to hint at the presence of an additional lineage besides Th1 and Th2 were completed in the mouse model of EAE. In a pivotal paper, it was demonstrated that mice deficient in the p19 subunit of IL-23 are resistant to the development of EAE while mice deficient in the p35 subunit of IL-12 had exacerbated disease (257). This discovery of the role of IL-23 ultimately led to the identification of Th17 cell lineage. Confirming these earlier findings, it was later demonstrated that mice deficient in IL-17A and IL-17R or mice that had been treated with neutralizing antibody to IL-17A were also protected from the development of EAE (232, 257, 258). Although Th17 cells are thought to be the main source of IL-17A during EAE, there is evidence that γδ T cells can also produce this cytokine and may act to amplify IL-17A production from Th17 cells (213, 232). Studies in humans have demonstrated that $CD4^+$ T cells from MS patients secrete higher levels of IL-17A than controls following *in vitro* restimulation (259). IL-17A has also been implicated to play a role in collagen-induced arthritis (CIA), a mouse model for rheumatoid arthritis. In this model, γδ T cells were found to be a major source of IL-17A, and to secrete cytokine in response to IL-1β and IL-23 signaling (260, 261).

IL-17A has also been implicated in the development of inflammatory bowel disease (IBD) (262). IL-17A was induced in three mouse models of colitis, one induced by transfer of $CD4^+$ T cells to $Rag^{-/-}$ mice, the second by administration of anti-CD40 to $Rag^{-/-}$ mice, and the third by infection of $Rag^{-/-}$ mice with the bacterium *Helicobacter hepaticus* (199, 263). All three colitis models were abrogated in the absence of IL-23. Interestingly, the second and third systems are models of innate colitis, in which $Rag^{-/-}$ mice were used. Thus, in these models, the IL-17A produced was presumably derived from a source other than adaptive T cells or innate-like T cells. Indeed, recent reports have revealed that innate lymphoid cells produce IL-17A in both innate and adaptive colitis models, and play a pathogenic role in mediating inflammation (201). There is also evidence for a role of IL-17-associated immune responses in human IBD. In a genome-wide association study, humans with allelic variants in IL-23R were protected from the development of Crohn's disease and ulcerative colitis (264).

Allergic disorders, such as asthma and atopy, are generally believed to be caused by dysregulation of type 2 immune responses and overexpression of type 2-associated cytokines such as IL-4 and IL-13, but there is accumulating evidence that IL-17A may also contribute to the pathogenesis of these diseases (265). Levels of IL-17A are increased in the lungs, sputum, and bronchoalveolar lavage (BAL) of asthmatic patients (266). Overexpression of IL-17A or IL-17F can lead to local pulmonary inflammation characterized by a slew of inflammatory mediators such as TNF-α, IL-1β, and IL-6, which have many downstream effects such as mucus production and recruitment of neutrophils to the airways (265, 267). Mice deficient in IL-17A demonstrated reduced neutrophil recruitment and inflammation in an OVA/alum-induced allergy model (268).

In this same model, IL-17A was shown to promote activation of allergen-specific Th2 cells, serum IgE production and eosinophil accumulation. A subsequent study, however, demonstrated a negative regulatory role of IL-17A on established asthma, as administering anti-IL-17A antibodies at the later phase of disease led to increased IL-5 production and eosinophilia (269).

The relative importance of IL-17A versus IFN-γ in the development of EAE and other autoimmune diseases is controversial. Mice deficient in IFN-γ, IL-12p35 subunit and IFN-γR are more susceptible to the development of EAE (270-272) whereas mice that lack T-bet, a transcription factor necessary for IFN-γ production, are protected from disease (273). Other studies demonstrated that EAE could be induced in $Rag^{-/-}$ mice by the transfer of $CD4^+$ T cells that have been polarized under either Th1 or Th17 conditions (274). Complicating matters further, studies have suggested that $CD4^+$ T cells can simultaneously express IL-17A and IFN-γ in the central nervous system of mice with EAE (213, 220), and that Th17 cells may represent an unstable population that can convert to an IFN-γ producing phenotype under specific inflammatory conditions (275). It has been similarly demonstrated that either Th1 or Th17 cells can induce experimental autoimmune uveitis (276). In a model of colitis, it was demonstrated that Th17 cells were not required, but that IL-23 was required and acted by inhibiting Tregs, and thus indirectly promoting Th1 responses (277). $CD4^+$ T cells from the intestines of patients with Crohn's disease were found to secrete both IL-17A and IFN-γ (180). Taken together, these studies suggest that the relationship between IL-17A and IFN-γ and autoimmunity is complicated, and it is possible that either cytokine has the capability to play either pathogenic or regulatory roles in specific inflammatory contexts.

CHAPTER II: SYSTEMICALLY DISPERSED INNATE IL-13-EXPRESSING CELLS IN TYPE 2 IMMUNITY

Abstract

Type 2 immunity is a stereotyped host response to allergens and parasitic helminths that is sustained in large part by the cytokines IL-4 and IL-13. Recent advances have called attention to the contributions by innate cells in initiating adaptive immunity, including a novel lineage-negative population of cells that secretes IL-13 and IL-5 in response to the epithelial cytokines IL-25 and IL-33. Here, we use IL-4 and IL-13 reporter mice to track lineage-negative innate cells that arise during type 2 immunity or in response to IL-25 and IL-33 in vivo. Unexpectedly, lineage-negative IL-25 (and IL-33) responsive cells are widely distributed in tissues of the mouse and are particularly prevalent in mesenteric lymph nodes, spleen, and liver. These cells expand robustly in response to exogenous IL-25 or IL-33 and after infection with the helminth *Nippostrongylus brasiliensis*, and they are the major innate IL-13–expressing cells under these conditions. Activation of these cells using IL-25 is sufficient for worm clearance, even in the absence of adaptive immunity. Widely dispersed innate type 2 helper cells, which we designate Ih2 cells, play an integral role in type 2 immune responses.

Introduction

Type 2 immune responses are important for the control of infections at mucosal barriers and the development of allergic inflammation. These responses are characterized by eosinophilia, elevated IgE, goblet cell metaplasia with enhanced mucus production, and smooth muscle hyperreactivity, all of which rely critically on production of the canonical type 2-associated cytokines IL-4, IL-5, and IL-13 (22, 142, 147). Although adaptive Th2 cells and follicular T cells are important sources of these cytokines (142), various innate cells, including eosinophils, basophils, and mast cells, have also been implicated as producers of these cytokines in various model systems (22, 66, 104, 147). More recently, the cytokines IL-25 and IL-33, members of the IL-17 and IL-1 cytokine families, respectively, were found to induce type 2 cytokine production when administered to mice, implicating these cytokines in the initiation of type 2 immune responses (90, 92). IL-25 and IL-33 are expressed by epithelial cells, macrophages, and possibly other cell types (107), and they are expressed at elevated levels during infection with parasitic helminths (91, 124) or after challenge with allergens (91, 123). Administration of exogenous IL-25 or IL-33 to mice leads to markedly enhanced levels of IL-4, IL-5, and IL-13 and many of the tissue features of a type 2 immune response (90, 92). Conversely, deficiency in IL-25 leads to diminished IL-4, IL-5, and IL-13 production and variable delays in worm clearance in different helminth models (93, 278). Similarly, mice unable to respond to IL-33 because of deficiency in the T1–ST2 subunit of the IL-33 receptor display diminished Th2-associated cytokines and decreased granuloma formation after injection of *Schistosoma mansoni* eggs (114).

Some of the original descriptions of these cytokines as well as more recent reports have noted the capacity of exogenous IL-25, IL-33, or helminth infection to induce the proliferation of a novel non-T/non-B cell population (90, 91, 93-95, 279). Although the surface phenotype of these cells has not been firmly established, there seems to be a consensus that these cells are negative for standard lineage markers and have a size and morphology that suggests a lymphoid origin. Multiple reports also describe a role for these cells in producing and secreting the Th2-associated cytokines IL-4, IL-5, and IL-13, and function marking using cytokine reporter mice has contributed directly to investigations of these cells (93, 95, 101, 279).

Here, we characterize these non-T/non-B lineage-negative cells at rest, after administration of exogenous IL-25 and IL-33, and during the course of infection with the helminth *Nippostrongylus brasiliensis*. We show that these cells are present in many organs at rest, expand after the addition of cytokines or during infection, and possess a distinct surface phenotype and gene-expression pattern. Furthermore, we show that these lineage-negative cells are the major innate IL-13–producing cells in each of these models and thus, are poised to play a significant role in type 2 immune responses.

Materials and Methods

Mice IL-4 reporter (4get, KN2) (101, 102), mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ (280), and eosinophil-deficient ΔdblGATA (281) mice have been described. $Rag2^{-/-}$ and common-$\gamma^{-/-}Rag2^{-/-}$ mice on the C57BL/6 background were purchased (Taconic) and bred to the 4get background as noted. IL-13 reporter mice, designated YFP-enhanced transcript with Cre recombinase at the il13 gene (YetCre-13), were generated after amplification of a 2.5-kb fragment of the il13 locus containing partial exon 1 (downstream of the BglII site) through exon 4 of the protein-coding sequence from 129/SvJ genomic DNA to serve as the 5′ homologous arm of the targeting vector. A 1.8-kb PCR amplicon containing the complete il13 3′ UTR plus the endogenous polyadenylation signal and downstream intergenic sequence was used as the 3′ homologous arm. A loxPNeor-loxP-internal ribosomal entry site (IRES)-YFP-Cre cassette was introduced into pKO915 (Lexicon) polylinker followed by sequential insertions of both homologous arms. The targeting construct was completed by adding a diphtheria α chain (DTA)cassette from pKO-Select-DT (Lexicon) as a negative selection marker. Insertion of this bicistronic IRES-YFP-Cre reporter/recombinase cassette between the il13 translational stop codon and the 3′ UTR preserves the capacity of cytokine production and concomitant expression of the YFP-Cre fusion protein in the setting of the endogenous regulatory sequences. The NotI-linearized targeting vector was electroporated into PrmCre embryonic stem (ES) cells (282) and selected in 300 ug·mL−1 G418. Resistant ES cell clones were screened for homologous recombination by Southern blot, and two independent clones were injected into C57BL/6 blastocysts to generate chimeras. The floxed Neor cassette was deleted in

the male germline after breeding male chimaeras to wild-type B6 females. Heterozygous N1 animals were backcrossed to B6 mice, and N2 offspring were screened for the presence of the reporter/recombinase and the absence of the PrmCre transgene. YetCre13 mice were backcrossed onto BALB/c background for 10 generations. YetCre-13 mice were then crossed to C57BL/6 Rosa-YFP amplifier mice (Jackson Laboratories) or Rosa-DTA deleter mice previously generated in the laboratory to achieve targeted deletion (12). Mice were maintained in the specific pathogen-free animal facility at the University of California San Francisco according to institutional guidelines.

Cytokine injections Mice were injected intraperitoneally on 4 consecutive d with 500 ng IL-25 or IL-33 (R&D Systems) where designated.

***N. brasiliensis* infection** Infection with *N. brasiliensis* third-stage larvae (L3) was as described (12). Where noted, mice received an i.v. injection of 5×10^5 lineage-negative cells sorted from IL-25-stimulated mice 2 d before infection and/or were treated with daily i.p. injections of 500 ng IL-25 over the first 4 d of infection.

Cell preparation Blood was collected from the tail vein. Peritoneal lavage was performed after injecting 10 mL of PBS into the peritoneal space and withdrawing 5 mL for cell analysis. For tissue samples, mice were perfused transcardially with 20 mL PBS, and the mesenteric lymph nodes, spleen, liver, lung (left lobe), and bone marrow (left femur) were removed. Organs were mechanically dissociated, and cells were passed through a 70-μm filter to generate single-cell suspensions. Bone-marrow cells were

collected by flushing excised femurs with PBS. Liver samples were resuspended in 30% Percoll, underlaid with 100% Percoll, and spun at room temperature for 30 min, and the cells were collected from the interface.

Flow cytometry Single-cell suspensions were washed in FACS buffer (PBS, 3% FCS, 1 mg·L−1 NaN3), and the cell pellets were incubated for 10 min with anti-CD16/CD32 monoclonal antibodies (University of California San Francisco Antibody Core Facility). Cells were incubated for 30 min on ice with antibodies to surface markers, including allophycocyanin (APC)- or APCAlexa Fluor 780–anti-CD4 (RM4-5; BD Biosciences and ebioscience), phycoerythrin (PE)-Cy7– or APC–anti-CD49b (DX5; ebioscience), APC-Alexa Fluor 750-anti CD8 (5H10; Invitrogen), PE–anti-Siglec F (E50-2440; BD Biosciences), PE–anti-IgE (23G3;Southern Biotech), PE anti–IL-5 (TRFK5; BD Biosciences), APC anti–c-kit or APC-Alexa Fluor-750 anti–c-kit (2B8; BD Biosciences and Invitrogen), anti-mouse lineage mixture, and isotype control—including antibodies against CD3ε, clone 145–2C11, Ly-6G/Ly-6C, clone RB6-8C5; CD11b, clone M1/70; CD45R/B220, clone RA3-6B2; TER-119/Erythroid cells, clone Ter-119 (BioLegend), PE anti–Sca-1 (E13-161.7; BD Biosciences), APC-Alexa Fluor 780–anti-Ly5.2 (104; ebioscience), APC–anti-Thy1.2 (53-2.1; BD Biosciences), biotin–anti-CD122 (TM-β1; BD Biosciences) followed by PE-Streptavidin (Invitrogen), APC–anti-CD44 (1M7; BD Biosciences), and PE–anti-huCD2 (555; Invitrogen). Cells were washed inFACSbuffer and resuspended in 1 mg/mL−1 DAPI to exclude dead cells. Cell counts were performed using Count-Bright absolute counting beads (Invitrogen). Samples were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cell sorting Lineage-negative cells (DAPI−side-scatter (SSC)lo-4get+CD4−CD8−SigF−IgE− CD49b−), Th2 cells (DAPI−SSClo4get+CD4+), eosinophils (DAPI−SSChi4get+CD4−SigF+), and basophils (DAPI−SSClo4get+CD4−IgE+CD49b+) were sorted using a FACSAriaII (BD Biosciences). In some cases, cells were stained with biotin-conjugated antibodies to mouse CD4 (RM4-5; BioLegend), CD8 (53-67; BD Biosciences), CD11b (M1/70; BD Biosciences), CD19 (1D; BD Biosciences), and NK1.1 (PK136; BD Biosciences) and passed through a MACS column (Miltenyi Biotech) before staining and sorting.

Intracellular cytokine staining Sorted lineage-negative cells were resuspended in media and stimulated for 2.5 h in the presence of phorbol myristate acetate (500 ng/mL) and ionomycin (750 ng/mL). Monensin (3 μM) was added for the final 1.5 h. Cells were stained for surface markers, fixed, and permeabilized, and then, they were stained for intracellular markers. Dead cells were excluded using a violet live/dead fixable stain (Invitrogen).

Microarray analysis RNA was isolated from sorted lineage-negative cells, Th2 cells, and basophils (after IL-25 immunization or on day 5 of Nippostrongylus brasiliensis infection) using the RNeasy Plus Mini kit (Qiagen). Total RNA quality was assessed using a Pico Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was amplified using the Sigma whole transcriptome amplification kits following the manufacturer's protocol (Sigma-Aldrich). Cy3-cytidine triphosphate labeling was

performed using NimbleGen one-color labeling kits (Roche-NimbleGen). The size distribution and quantity of the amplified product was assessed using the Agilent 2100 Bioanalyzer and the Nanodrop ND-8000 (Nanodrop Technologies), the labeled DNA was assessed using the Nandrop 8000, and equal amounts of Cy3-labeled target were hybridized to Agilent Whole Mouse Genome Microarrays (4 °— 44 K format). Hybridizations were performed for 14 h, according to the manufacturer's protocol. Arrays were scanned using the Agilent microarray scanner, and raw signal intensities were extracted with Feature Extraction v10.1 software. Sample preparation, labeling, and array hybridizations were performed using standard protocols from the University of California San Francisco Shared Microarray Core Facilities and Agilent Technologies. Median feature pixel intensity was normalized using quantile normalization (283). A one-way ANOVA linear model was used to estimate the mean log2-fold change and calculate the moderated t statistic and false-discovery rate for each gene for the comparisons of interest. All procedures were carried out using functions in the R package limma in Bioconductor (284, 285).

Results

Lineage-negative cells are systemically distributed in resting mice

We used IL-4 reporter mice (4get) (101) to determine the phenotype and distribution of IL-4–expressing cells in resting mice. In these mice, the 3′UTR of the *il4* gene was modified to include an internal ribosomal entry site (IRES) followed by *egfp*, resulting in 5′ cap-independent translation of GFP when the locus is activated, thus effectively marking IL-4–competent cells in situ (286). Cell types that are constitutively GFP+ in these mice include eosinophils, basophils, mast cells, natural killer T (NKT) cells, and Th2 cells (66, 88, 104). On closer analysis, an additional GFP+ cell type was apparent in multiple tissues of resting mice (Figure 1A). These cells were small, side-scatter low cells resembling lymphocytes and were negative for surface markers that characterize T and NKT cells (including CD3, CD4, and CD8), eosinophils (Siglec-F and CD11b), basophils (CD49b, IgE, and CD131), and mast cells (IgE) (Figure 1A).

Further examination of these cells revealed a surface phenotype characterized as lineage-negative for classic T, B, NK, myeloid, and dendritic cell markers (Figure 1B) and c-kit low, Sca-1-negative, CD122 (IL-2Rβ) low, Ly5.2+, Thy1+, and CD44 high (Figure 1C). As such, these resting cells resembled c-kit+ lineage-negative cells that were elicited in response to IL-25 or IL-33 in previous studies (90, 93-95). In resting mice, we identified populations of these lineage-negative cells in all organs and tissues that we examined, except for the blood (Figure 1D). The highest numbers of lineage-negative cells were present in the mesenteric lymph nodes, spleen, liver, and bone marrow, with fewer cells in the lung and peritoneum. Consistent with previous reports, lineage-negative

cells were readily recovered from tissues of $Rag2^{-/-}$ mice lacking adaptive immune cells, $Kit^{W\text{-}sh/W\text{-}sh}$ mice lacking mast cells, and ΔdblGATA mice lacking eosinophils (Figure 2). However, and as originally noted (90), these cells were completely absent in mice lacking the lymphocyte common-γ chain receptor, suggesting that these cells require signaling through a common-γ chain cytokine for their development and/or survival (Figure 1E). Although more extensive analysis is required, we observed no cytokine expression or proliferation of these cells in vivo after administration of 500 ng of the individual γc-binding cytokines IL-2, IL-4, IL-9, IL-13, IL-15, or IL-21 to 4get mice. Thus, lineage-negative IL-4–competent cells are distributed widely throughout tissues of resting mice and share phenotypic and developmental features with earlier described populations of lineage-negative innate immune cells that expand in response to IL-25.

Lineage-negative cells expand in response to exogenous IL-25 or IL-33 and during helminth infection

The previously described lineage-negative cells were identified initially in lung and spleen (90, 91) and later in mesentery (94) by their capacity to expand in vivo in response to exogenous IL-25 or IL-33 and release IL-13 and IL-5 implicated in epithelial goblet cell metaplasia and eosinophilia, respectively. It was unclear whether the cells we describe in multiple organs share this common effector capacity. We administered 500 ng IL-25 or IL-33 on 4 consecutive d to 4get mice and monitored the kinetics of cell expansion and eosinophil accumulation in the respective tissues. After four doses of cytokine, we documented dramatic increases in the numbers of lineage-negative cells and eosinophils in all organs examined (Figure 3A). With kinetic studies, we showed that

lineage-negative cells peaked in the mesenteric lymph node, spleen, and liver between days 3 and 4, whereas eosinophils continued to increase in the various organs throughout the 8-d time course (Figure 4), suggesting that eosinophil recruitment is likely downstream of activation of these lineage-negative cells and presumably, reflects their IL-5– and IL-13–producing capacity. Importantly, these studies link these widely dispersed lineage-negative cells by their common responsiveness to IL-25 and IL-33 and by their effector capacity as revealed by eosinophil recruitment into multiple tissues throughout the body.

N. brasiliensis is an intestinal helminth that models the migratory pathway of human hookworm (287); s.c. injected larvae enter the vasculature, migrate to the lungs, molt, penetrate the alveoli, and ascend the trachea, where the worms are swallowed to complete maturation in the small bowel. Adult worms of this rat parasite are cleared in immunocompetent mice after 8–10 d by a Th2-orchestrated type 2 immune response (33). After infection with *N. brasiliensis*, the lineage-negative GFP+ cell population also increased in all organs examined, peaking in the mesenteric lymph node 5 d after infection and in the spleen and lung 2 d later (Figure 3B). Thus, lineage-negative cells respond to both exogenous cytokines and helminth challenge by marked increase in numbers, consistent with a shared lineage for all of these cells, even in disparate organs. Furthermore, the eosinophil infiltration induced in each of these organs in response to IL-25 or helminth infection supports their systemic activation to a common functional state after these challenges.

Lineage-negative cells express a distinct transcriptome compared with other type 2 immune cytokine-secreting cells

Prior investigations have identified Th2 cells and basophils as the major type 2 cytokine-expressing cells in tissues from mice challenged with helminths (65). We sorted Th2 cells, basophils, and lineage-negative cells from tissues of *N. brasiliensis*-infected 4get mice and used microarray to compare their transcriptional profiles (Figure 5A). By this analysis, lineage-negative cells could be independently grouped as a separate lineage and were readily distinct from Th2 cells or basophils. Each population had sets of uniquely expressed transcripts not expressed by the other lineages (Figure 5A & B). Conversely, using stringent criteria, some effector populations also shared a core set of transcripts, consistent with their concordant activities in type 2 immune responses.

Lineage-negative cells are the major innate IL-13–expressing cells after administration of IL-25 and during *N. brasiliensis* infection

Despite the constitutive GFP fluorescence of the lineage-negative cells from 4get mice, it remained unclear whether these cells produce the cytokine IL-4 in vivo. To address this question, we used 4get × KN2 mice, which express one 4get allele and one allele in which a modified human CD2 gene replaces the *il4* gene at the endogenous IL-4 start site (102). Cells from these mice can be used to track IL-4–secreting cells in vivo without the need for restimulation. Whether assessed at rest, after IL-25 challenge, or during the course of *N. brasiliensis* infection, however, we were unable to document significant human CD2 expression on lineage-negative cells from 4get × KN2 mice, despite our ability to show robust human CD2 expression by Th2 cells collected from the

same tissues (Figure 6). Thus, we infer that lineage-negative cells do not produce IL-4 in vivo under physiologic conditions.

IL-13 plays an important and nonredundant role in type 2 immune responses (24, 33). After infection with *N. brasiliensis*, mice deficient in IL-4 have diminished Th2 cytokine responses but are able to clear worms (288). In contrast, mice deficient in IL-13 have a more profound deficit in worm clearance and show defects in goblet cell hyperplasia (33, 289). To gain insight into the expression and distribution of IL-13–producing cells in vivo, we generated IL-13 reporter mice, designated YFP-enhanced transcript with Cre recombinase at the *il13* gene (YetCre-13), by introducing an IRES followed by YFP-Cre recombinase fusion protein at the start of the 3′UTR of the *il13* gene. To enhance detection of lineage-negative cells that activated the IL-13 locus, we crossed YetCre-13 mice to Rosa-floxed-Stop-YFP mice (11). In this way, any cells having activated the YFP-Cre fusion protein at any time will flox and activate the Rosa-YFP in a constitutive fashion, thus leaving a lineage mark that defines the prior activation of the *il13* gene.

Using the YetCre13 Rosa-YFP amplifier reporter mice to follow IL-13–expressing cells in response to IL-25, we observed only small numbers of YFP+ CD4+ T cells and substantial populations of innate YFP+ cells in multiple organs with the same surface and size phenotype as the lineage-negative cells defined using the 4get allele (Figure 7A and B). Induction of IL-13+ lineage-negative cells also occurred after infection with *N. brasiliensis*, with similar kinetics and numbers of cells as previously shown using 4get mice (Figure 7C). Using these IL-13 reporter mice, we found that, after IL-25 administration or during *N. brasiliensis* infection, the only significant population of

innate cells that expressed IL-13 besides the lineage-negative cells was a minor population of mast cells in the peritoneum (Figure 7D). Eosinophils and basophils did not express the IL-13 marker under these conditions (Figure 8). Taken together, these data suggest that a substantial portion of the lineage-negative cells express IL-13 when activated in vivo during type 2 immune responses and that these cells are the major innate IL-13–producing cells in multiple tissues in the mouse. Additionally, when taken directly from mice after expansion and assayed for intracellular IL-5 production, a substantial proportion of lineage-negative cells also produced IL-5, consistent with their effects on tissue eosinophils after activation (Figure 9).

Lineage-negative cells contribute to worm clearance and tissue eosinophil accumulation

To confirm that IL-13 production constitutes a stereotyped response by the lineage-negative cells in all organs, we crossed YetCre-13 mice to Rosa-flox-Stop-diphtheria toxin α (Rosa-DTA) mice (12). In cells from these animals, activation of *il13* transcription generates YFP-Cre fusion protein that will excise the flox-stop cassette and lead to the production of diphtheria toxin α, thus killing IL-13–producing cells. These mice were additionally crossed onto a heterozygous 4get background, enabling the tracking of the total numbers of lineage-negative cells using surface markers and GFP fluorescence. After administration of IL-25, we noted a substantial decrease in the numbers of recovered lineage-negative cells in the YetCre13-Rosa-DTA mice compared with littermate controls in all organs assayed, including the mesenteric lymph nodes, spleen, liver, and peritoneum (Figure 10A and B). Furthermore, the decrease in lineage-

negative cells was accompanied by a marked decrease in the numbers of infiltrating eosinophils. Although the deleter allele will similarly delete IL-13–producing Th2 cells, the small numbers of these cells induced after IL-25 suggest that lineage-negative cells are the major mediators of eosinophil recruitment after administration of this cytokine.

To establish that lineage-negative cells alone could reconstitute functional aspects of antihelminth immunity, we corroborated prior findings (93, 95) that exogenous IL-25 could mediate worm clearance and eosinophil tissue infiltration in Rag $2^{-/-}$ mice infected with *N. brasiliensis* (Figure 10C). In contrast, administration of IL-25 to common-$\gamma^{-/-}$ × Rag$2^{-/-}$ mice, which lack both adaptive immunity and lineage-negative cells did not lead to worm clearance or significant increases in tissue eosinophils. To confirm that the failure to mediate these effects was caused by the absence of lineage-negative cells, we adoptively transferred 5×10^5 lineage-negative cells into the common-$\gamma^{-/-}$ × Rag $2^{-/-}$ mice 2 d before infection with *N. brasiliensis*. When assessed 5 d after infection, lineage-negative cells alone had no effect on worm numbers or eosinophil tissue infiltration, consistent with the need for an additional signal regulating their activation during infection. Indeed, adoptively transferred lineage-negative cells substantially rescued IL-25–dependent worm clearance and eosinophil tissue infiltration in common-$\gamma^{-/-}$× Rag 2 $^{-/-}$ mice, showing that these cells are necessary and sufficient to integrate IL-25 signals that mediate these downstream pathways.

Discussion

IL-4 and IL-13 play central roles in the orchestration of antihelminth immunity and allergic responses. Originating from a gene duplication, these cytokines bind to shared and disparate receptors and mediate many overlapping downstream effector pathways (27, 143). Despite these observations, these cytokines also have more dedicated functions during a type 2 response, with IL-4 facilitating humoral IgG1 and IgE production (142) and IL-13 contributing to epithelial hyperplasia and eosinophil recruitment in peripheral tissues (290). As such, efforts to understand where and how IL-4 and IL-13 are produced in tissues will be important in understanding the dichotomous roles for these cytokines in barrier and allergic immunity.

Here, we use knockin reporter mice to identify IL-4– and IL-13–expressing cells that accumulate in tissues in response to exogenous cytokines, IL-25 and IL-33, and intestinal worm infection. We confirm prior findings that a unique lineage-negative cell is a target of IL-25 and IL-33, that activation of these cells is accompanied by their expansion and their expression of IL-13 and IL-5, and that activation of these cells is sufficient to mediate the major peripheral effects of IL-25, including eosinophilia and worm clearance (90, 91, 93-95). We extend these findings to show that cells of this same phenotype populate many organs in the resting mouse and are particularly prevalent in the mesenteric lymph node, spleen, and liver. Importantly, all of these populations share a similar surface phenotype and functional response, suggesting that they represent a common lineage of cells that is distributed throughout the body where they can integrate signals mediated by IL-25, IL-33, and possibly other cytokines. In deference to the

original observations of these cells by investigators at the prior DNAX Research Institute as well as the historical designation of Th1 and Th2 cells at that institute, we designate these cells innate helper type 2 cells (Ih2 cells), although alternative names have been suggested for similar cells by other investigators (94, 95, 279).

Despite the potent production of IL-13 and IL-5, we could not show production of IL-4 by Ih2 cells in vivo. We used sensitive reporter mice to show that these cells do not produce IL-4 in vivo, even under conditions where Th2 cells in tissues can be readily shown to be IL-4–secreting. After administration of IL-25, IL-33, or during *N. brasiliensis* infection, Ih2 cells were the predominant IL-13-expressing cells in all tissues examined. As such, the widespread effects of IL-13 deficiency on tissue manifestations of type 2 immunity may be explained by the functional deficit conferred on these peripherally arrayed Ih2 cells.

The widespread distribution of Ih2 cells raises questions regarding their trafficking and survival in peripheral tissues. Despite a sensitive marker for these cells, we did not detect Ih2 cells in blood, consistent with rapid transit through blood to tissue or local expansion from tissue-restricted precursors. Further work will be required to address these possibilities. Additionally, the sources of IL-25 or IL-33, or possibly other cytokines, that lead to activation of these cells during physiologic responses will be important areas of investigation. Lastly, the potential exists for a fundamental homeostatic role for these cells in regulating the status of peripheral organs and tissues by cytokine-mediated interactions with resident and recruited cells. As such, aberrant activation of tissue-resident Ih2 cells may contribute to the chronic nature of allergic

diseases such as atopy and asthma, and further assessment of their role in such diseases will be aided through use of the reporter mice that we describe.

Finally, it will be important to determine the relationship of Ih2 cells to innate cells associated with type 2 immunity defined in prior reports. The functional identification of Ih2 cells as a major IL-13–producing population is consistent with the original description of these cells (90) as well as more recent studies using IL-13 reporter mice (95). We have not noted organization of Ih2 cells into the adipose-associated structures reported to be present in the mesentery (94), although further study will be required to more precisely determine the localization of Ih2 cells in peripheral tissues. A report by Saenz et al. (279) described cells with a similar surface phenotype in mesenteric lymph nodes that may have a precursor relationship with myeloid lineages involved in type 2 immunity. In contrast, the IL-13–producing cells we and others describe have properties suggesting a lymphoid lineage, including, as shown here, the shared expression of Aiolos with Th2 cells (291). Further work is certainly needed to understand the interrelationships between these intriguing innate cell populations.

Acknowledgements

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Footnotes

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Author contributions: A.E. Price, D.J. Erle., and R.M. Locksley designed research; A.E. Price, B.M. Sullivan, and R.L. Reinhardt performed research; H.-E. Liang contributed new reagents/analytic tools; A.E. Price, C.J. Eisley, and D.J. Erle analyzed data; and A.E. Price and R.M. Locksley wrote the paper.

Figures

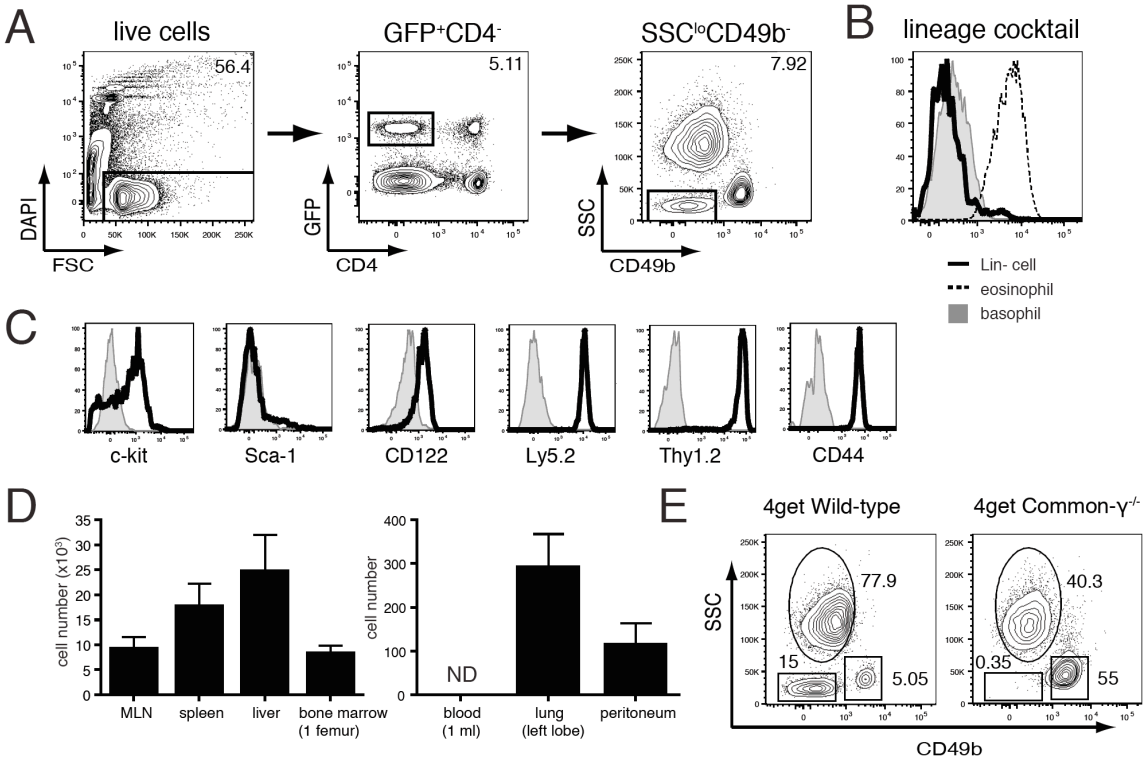

Figure 1. Lineage-negative cells are systemically distributed at rest and possess a unique surface phenotype.

(*A*) Gating scheme for lineage-negative cells in 4get mice. Example shown is resting liver. Box denotes lineage-negative gate. Numbers denote percentage of boxed cells of total. (*B*) Staining with a lineage-marker antibody mixture (CD3ε, Ly-6G/Ly-6C, CD11b, CD45R, and TER-119). Eosinophils are used as a positive control, whereas basophils are used as a negative control. (*C*) Surface-marker antibody staining (black lines) compared with isotype control (gray) on lineage-negative cells from resting liver. (*D*) Numbers of lineage-negative cells in organs and tissues at rest. Experiment was independently

repeated three times, and data were combined for graph (n = 3–14 mice per group). (*E*) Gating of lineage-negative cells in 4get mice compared with 4get × common-$\gamma^{-/-}$ mouse liver. Lineage-negative cells collect in side-scatter (SSC)-lo, CD49b-negative gate. Numbers are percentages of total non-CD4 GFP-positive cells.

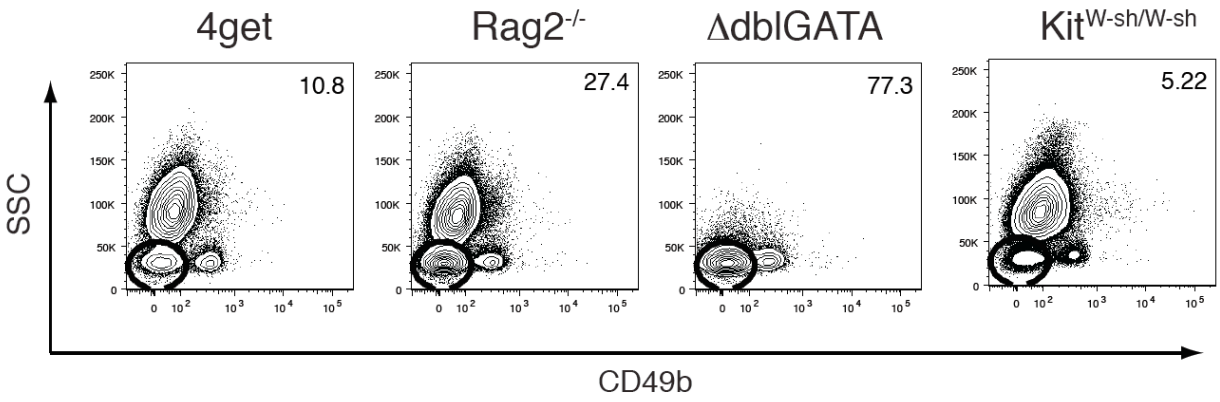

Figure 2. Lineage-negative cells in immunodeficient mice.

GFP+CD4– cells from livers of designated immunodeficient strains crossed onto 4get background. Percent shown is percent of lineage-negative cells.

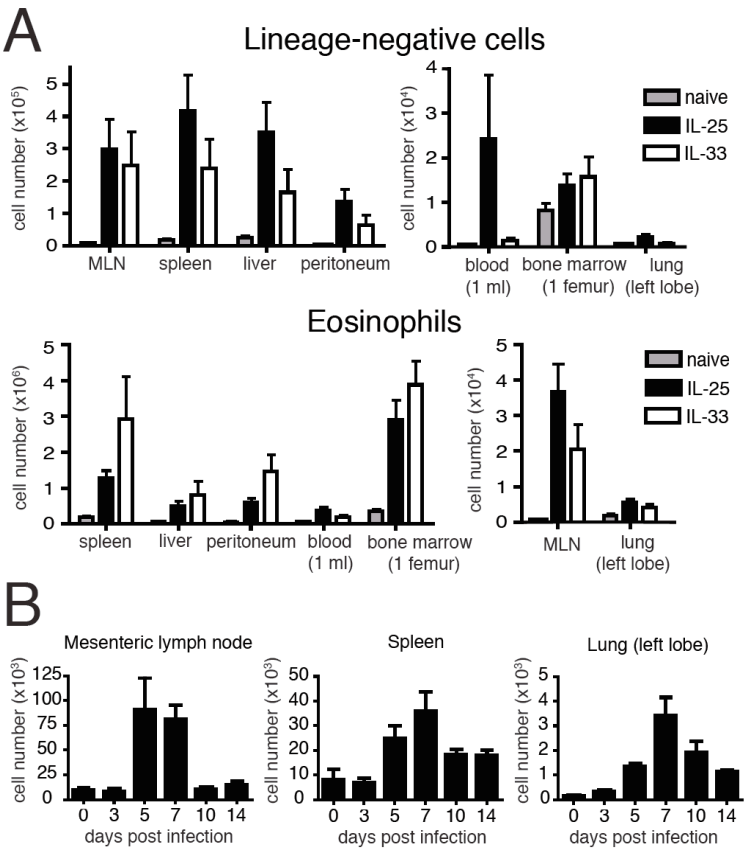

Figure 3. Lineage-negative cells increase in number after IL-25 administration and during infection.

(*A*) Numbers of lineage-negative cells and eosinophils in designated organs after four daily doses of 500 ng IL-25 or IL-33. (*B*) Numbers of lineage-negative cells after infection with *N. brasiliensis*. Experiments were repeated three times, and data were compiled for graphs (n = 3–14 mice per group). Bars are means with SEM.

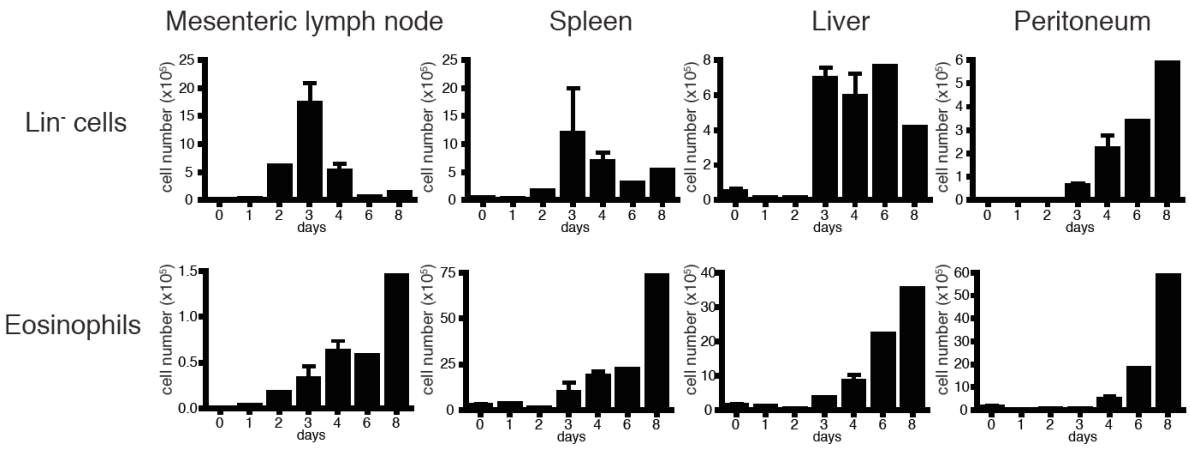

Figure 4. Lineage-negative cells and eosinophils increase in number after IL-25 administration.

Numbers of lineage-negative cells and eosinophils in designated tissues during and after administration of IL-25 on days 0–3. Bars are means with SEM.

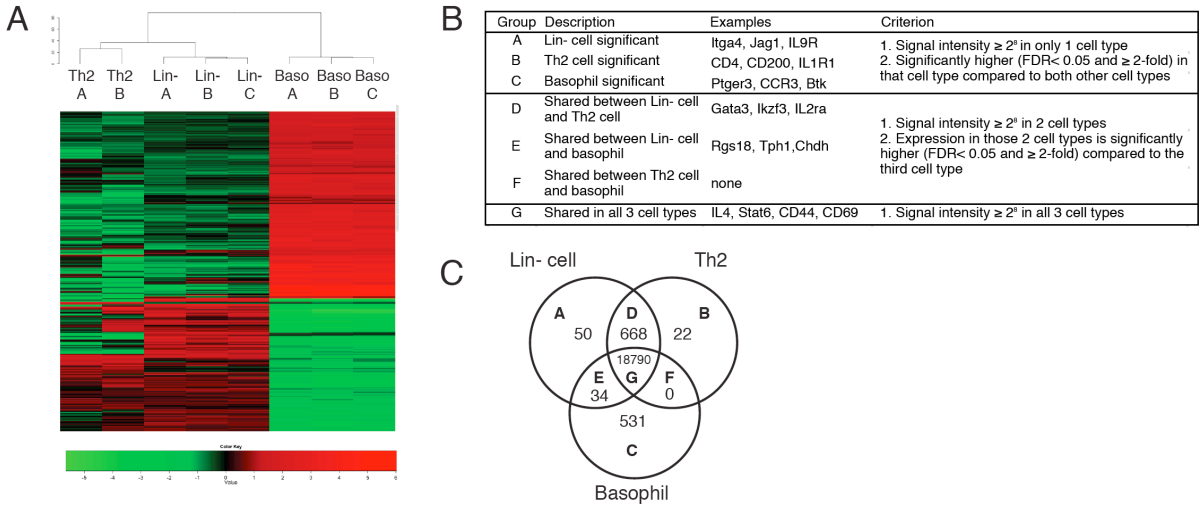

Figure 5. Lineage-negative cells are a distinct Th2-associated cell type.

(*A*) Relative gene expression in Th2 cells (two samples), lineage-negative cells (three samples), and basophils (three samples) isolated from *N. brasiliensis*-infected mice. Heat map shows log2 (sample intensity/mean intensity for all eight samples) for all microarray probes with differential expression (false discovery rate (FDR) < 0.05) in any pairwise comparison between cell types. (*B*) Grouping of microarray probes based on their expression in lineage-negative cells, Th2 cells, and basophils. Signal intensity cutoff of 2^8 is ~2^3-fold higher than the median signal obtained with randomized negative-control probes. *, Itga4, integrin alpha 4; Jag1, Jagged 1; IL9R, IL-9 receptor; IL1R1, IL-1 receptor type 1; Ptger3, prostaglandin E receptor 3; CCR3, chemokine receptor 3; Btk, Bruton agammaglobulinemia tyrosine kinase; Gata3, GATA binding protein 3; Ikzf3, IKAROS family zince finger 3 (Aiolos); IL2ra, IL-2 receptor alpha chain; Rgs18, regulator of G protein signaling 18; Tph1, tryptophan hydrolase 1; Chdh, choline

dehydrogenase; Stat-6, signal transducer and activator of transcription 6 (*C*). Diagram showing the numbers of probes in each of the seven groups listed in *B*.

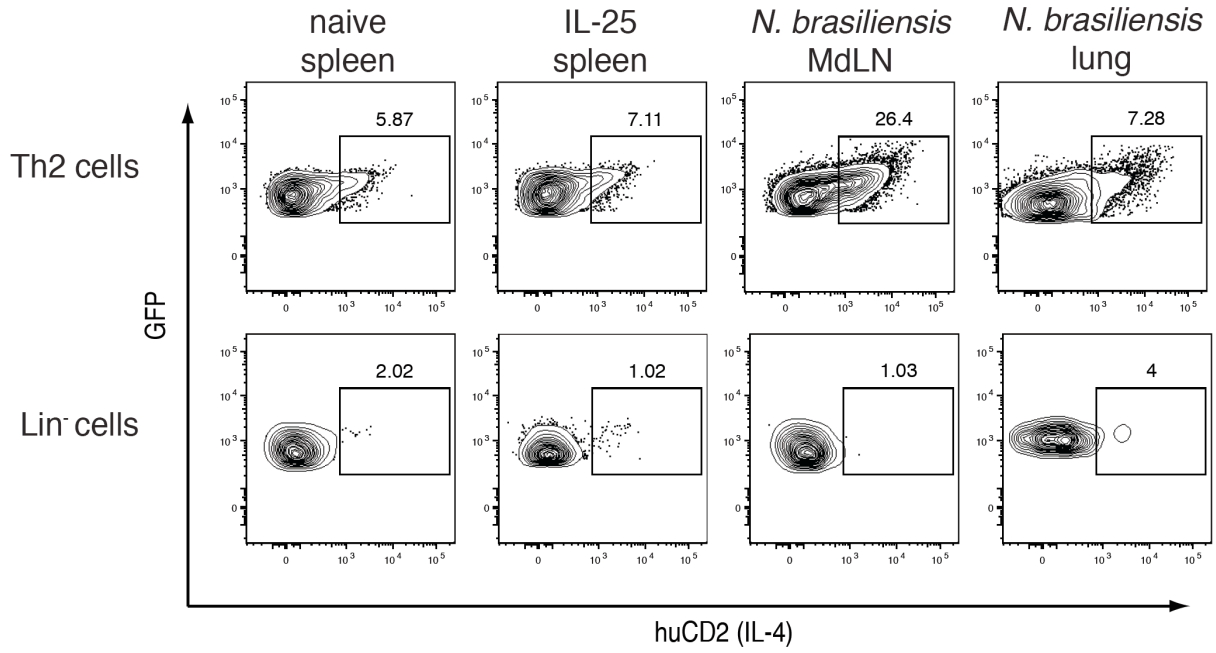

Figure 6. Analysis of IL-4–producing cells in tissues using 4get - KN2 mice.

Percentage of huCD2+ (IL-4–producing) cells in 4get KN2 dual reporter mice at rest, after administration of IL-25, and in mediastinal lymph nodes (MdLN) or lung at day 9 of infection with N. brasiliensis. Gated GFP+ CD4 Th2 cells (Upper); gated lineage-negative cells (GFP+SSCloCD49b–SiglecF–IgE–; Lower). Box denotes human CD2+, IL-4–secreting cells within the designated cell populations. Gates were set using isotype controls.

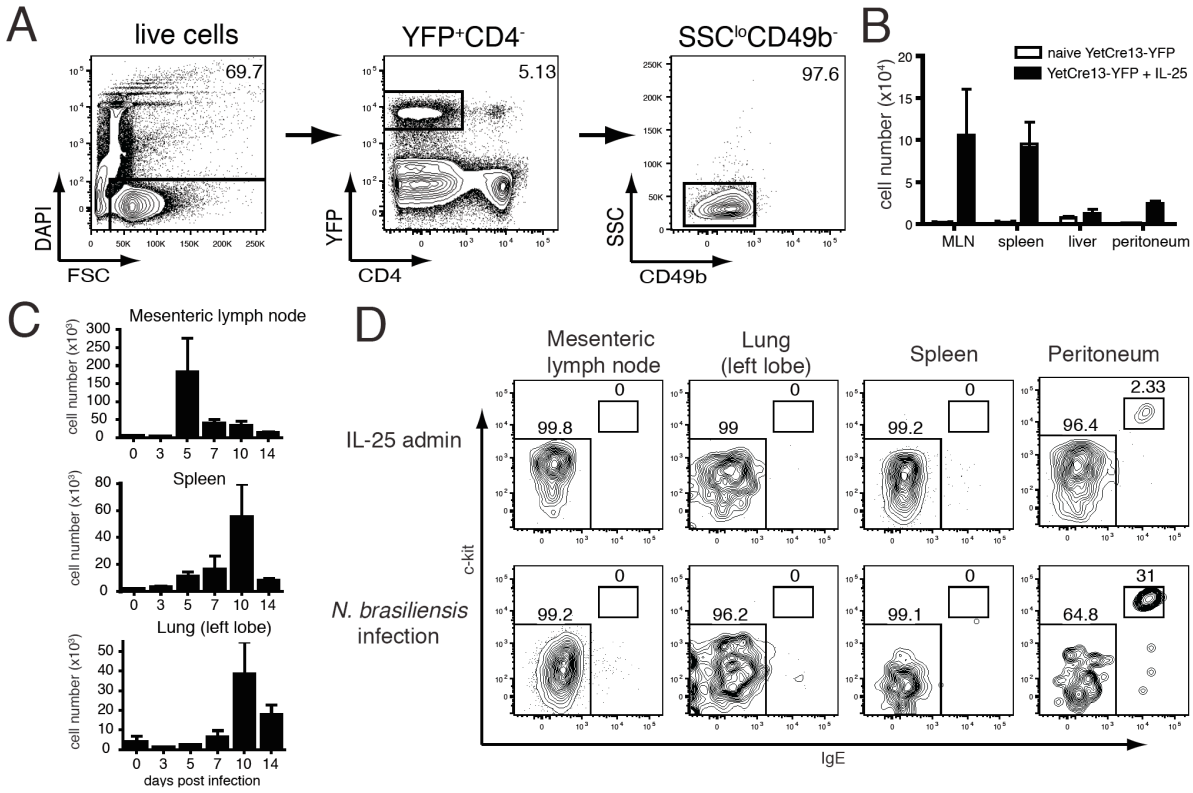

Figure 7. Lineage-negative cells are major innate IL-13–expressing cells.

(*A*) Gating of YFP+ lineage-negative cells from YetCre13 Rosa-YFP amplifier mice. Example shown is of liver after administration of IL-25. Gates and percentages are as in Figure 1 (*B*) Numbers of YFP+ lineage-negative cells after IL-25 administration. Experiment was repeated two times, and results were compiled (n = 2–3 mice per group). Bars are means with SEM. (*C*) Time course showing YFP+ lineage-negative cells from designated organs during infection with *N. brasiliensis*. Experiment was repeated two times, and results were compiled (n = 3–5 mice per group). Bars are means with SEM. (*D*) Gating of non-CD4+ YFP+ cells showing percentages of mast cells (c-kit^{hi}IgE^{+}) and

lineage-negative (c-kit^{lo}IgE^{-}) cells in multiple organs after IL-25 administration (*Upper*) or after infection with *N. brasiliensis* (*Lower*).

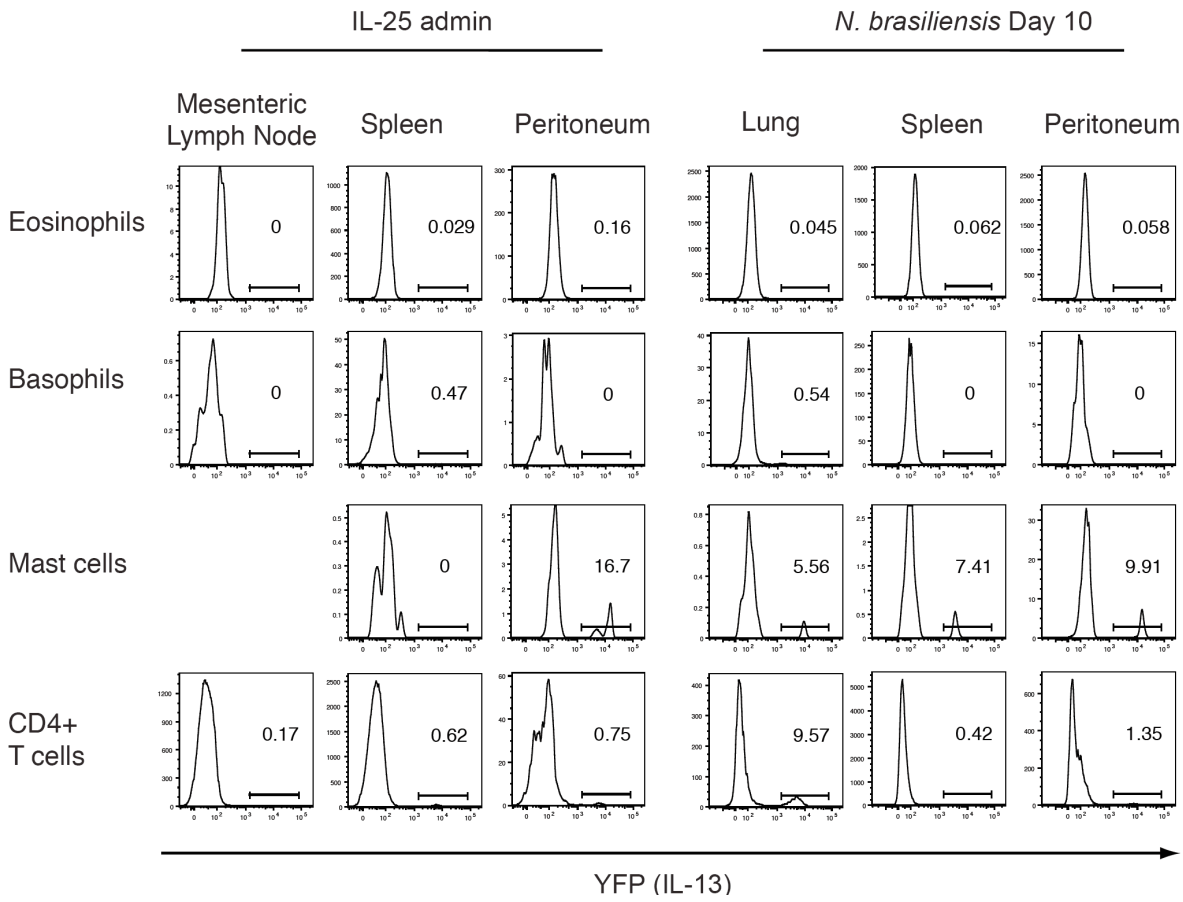

Figure 8. IL-13 is only expressed by certain type-2-associated cell types.

YFP expression as a marker of IL-13 expression in diverse populations of type 2-associated immune cells induced by administration of IL-25 (Left) or N. brasiliensis infection (Right). Expression of YFP in eosinophils ($SSC^{hi}SigF+$), basophils ($SSC^{lo}CD49b+IgE+$), mast cells ($SSC^{hi}c\text{-}kit^{hi}IgE^{hi}$), and CD4+ T cells after administration of IL-25 or at day 10 of *N. brasiliensis* infection.

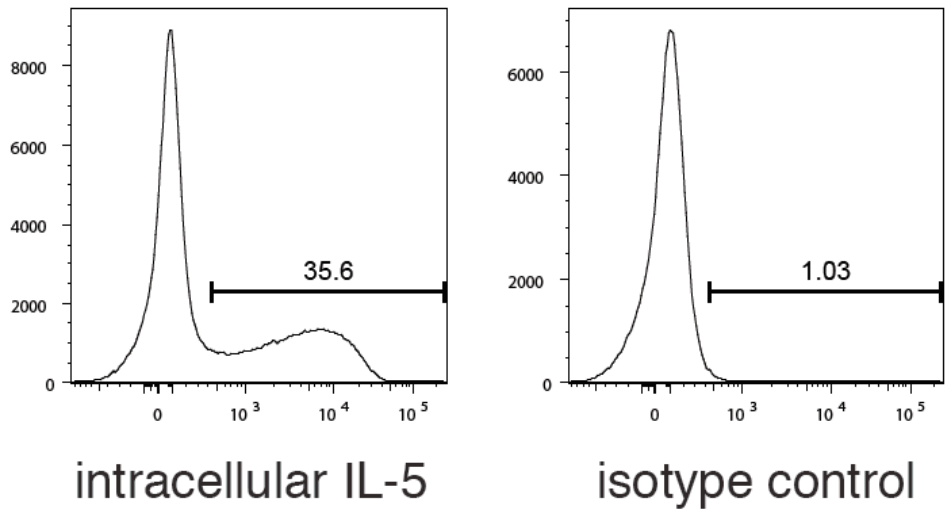

Figure 9. Lineage-negative cells express IL-5.

Percentage of IL-25–induced sorted lineage-negative cells producing IL-5 compared with isotype control.

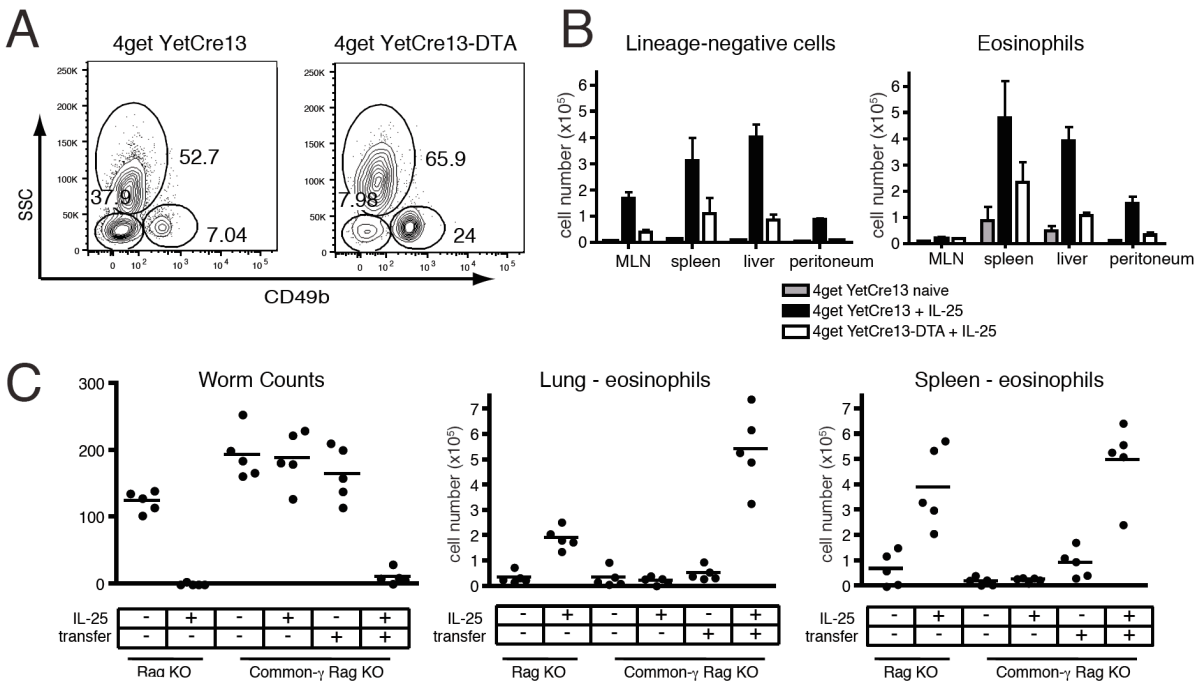

Figure 10. Lineage-negative cells contribute to eosinophilia and mediate IL-25–dependent worm clearance.

(*A*) Gating of GFP+CD4- cells in IL-25–treated 4get YetCre13-Rosa-DTA deleter mice or 4get YetCre13 littermates. Percentages shown are eosinophils ($SSC^{hi}CD49b^{lo}$), basophils ($SSC^{lo}CD49b^{+}$), and lineage-negative cells ($SSC^{lo}CD49b^{-}$). (*B*) Numbers of lineage-negative cells and eosinophils in multiple organs in 4get YetCre13-DTA deleter mice or 4get YetCre13 controls after administration of IL-25. Experiment was repeated two times, and results were compiled (n = 3–5 mice per group). Bars denote means with SEM. (*C*) $Rag2^{-/-}$, common-$\gamma^{-/-}$ × $Rag2^{-/-}$, or common-$\gamma^{-/-}$ × $Rag2^{-/-}$ mice adoptively transferred with 500,000 lineage-negative cells 2 d previously were infected with *N*.

brasiliensis. Five mice from each group received IL-25 on days 0–4. Worm counts from the small intestine and numbers of eosinophils ($SSC^{hi}CD11b^{+}Siglec\text{-}F^{+}$) were quantified on day 5. Experiment was repeated two times, and a representative experiment is shown.

CHAPTER III: GENETIC MARKING OF IL-17A-PRODUCING CELLS *IN VIVO*

Abstract

Interleukin (IL)-17A plays an important role in host defense against a variety of pathogens and may also contribute to the pathogenesis of autoimmune diseases. However, the cells that produce this cytokine *in vivo* remain incompletely characterized. Here, we use novel reporter mice to demonstrate that IL-17A is not expressed by immune cells under resting conditions. After challenge with known IL-17A-inducing conditions, including *Klebsiella pneumoniae* or experimental autoimmune encephalitis, a proportion of γδ T cells, invariant (i)NKT cells, and other $CD4^-$ $CD3\varepsilon^+$ cells became marked with the reporter *in vivo*. A smaller percentage of marked $CD4^+$ T cells were also recovered. Production of IL-17A by non-T cells was not evident. Administration of IL-1β, IL-23 or a combination of these cytokines elicited rapid expression of the reporter by resident γδ T cells, iNKT cells and other $CD4^-$ $CD3\varepsilon^+$ cells *in vivo*, demonstrating that these innate-like T cells are poised for rapid IL-17A production and likely comprise major sources of this cytokine during acute immunologic challenges.

Introduction

The cytokine interleukin (IL)-17A has a key role in immunity, inducing the release of a variety of inflammatory cytokines as well as chemokines that mediate the rapid recruitment of neutrophils. Studies with knockout mice and neutralizing antibodies have revealed a role for IL-17A in immunity to various bacterial and fungal infections and also in the induction and propagation of several autoimmune diseases (292). In humans, genetic deficiencies in IL-17 receptor A or IL-17F are associated with susceptibility to mucocutaneous infection with *Candida albicans* and, to a lesser extent, *Staphylococcus aureus* (163). Autoantibodies to IL-17A and IL-17F are seen in patients with mutations in the autoimmune regulator (*AIRE)* and may contribute to mucocutaneous candidiasis (293). Patients with hyper-IgE syndrome associated with mutations in Stat3 have deficits in the induction of IL-17-producing $CD4^+$ T (Th17) cells that correlate with recurrent bacterial and fungal infections (164). Polymorphisms in the gene encoding the receptor to IL-23, a cytokine implicated in the generation and maintenance of Th17 cells and in the promotion of IL-17 secretion from innate cells (197, 232, 235, 294), are associated with protection against the development of inflammatory bowel disease (264). A more complete understanding of the biology of IL-17A, including the temporal and cell-specific patterns of expression, could aid in the generation of effective therapeutics targeted towards these infectious and autoimmune conditions.

Although early work focused on IL-17A production by Th17 cells, more recent studies suggest important contributions by innate-like lymphocytes, including γδ T cells

and NKT cells (191). However, most reports use *ex vivo* restimulation to identify IL-17A-producing cells, and thus potentially alter the pattern of cytokine secretion that occurs *in vivo*. Several groups have generated IL-17F reporter mice in order to bypass the need for restimulation, but while IL-17A and IL-17F are often co-expressed, they are differentially induced in certain models and can have distinct functional roles (168, 215, 216). More recently, IL-17A-cre reporter mice were developed to permanently mark cells that have activated the IL-17A locus, thus facilitating fate-tracking, and an IL-17A-eGFP knockout mouse was used to track Th17 cells during tolerance induced by a CD3-specific antibody and in a mouse model of sepsis (213, 214). Studies using IL-17F BAC transgenic mice and IL-17A-cre mice, as well as studies using adoptive transfer (295, 296), have raised the possibility that Th17 cells have an unstable phenotype, such that they lose the capacity to produce IL-17 and begin to produce interferon (IFN)-γ.

Here, we generated IL-17A reporter mice and used these mice to examine the expression of IL-17A at rest, after bacterial challenge, and during the development of autoimmune encephalitis. To establish the relationships between IL-17A-producing cells and IFN-γ-producing cells, we additionally crossed these IL-17A reporter mice to mice with an IFN-γ reporter allele. Together, these data offer insights into the expression of IL-17A in the mouse during health and disease as assessed without the need for *ex vivo* restimulation.

Materials and Methods

Mice C57BL/6 mice and RORγt-GFP reporter mice (297) were obtained from Jackson Laboratories. Great IFN-γ reporter mice have been described (142). Smart-17A mice were generated by first assembling a composite selection/reporter cassette using standard cloning procedures in the following order: 1) a floxed-neomycin-resistance gene (floxed Neo^r); 2) encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES); 3) low-affinity human nerve growth factor receptor (p75 LNGFR, also known as CD271) cDNA (OriGene); 4) bovine growth hormone (bGH) poly-adenylation signal (pA). This 3.1 kb selection/reporter cassette was cloned into a basal targeting construct pKO915-DT (Lexicon) containing diphtheria toxin (DT) α chain for negative selection. Both 5' and 3' homologous arms used to flank the cassette were obtained by high-fidelity PCR amplification of the *il17a* locus from 129/SvJ genomic DNA. The 5' arm consists of a 1.6 kb fragment covering intron 2 and coding sequence of exon 3. The 3' arm consists of a 2.2 kb fragment spanning the endogenous 3' UTR and downstream sequences. After sequence verification, the NotI-linearized construct was electroporated into PrmCre ES cells, which express Cre recombinase driven by the protamine promoter (282). G418-resistant ES clones were screened for homologous recombination by Southern blot. Two independent clones were injected into C57BL/6 blastocysts to generate chimeras. The neomycin-resistance cassette was deleted in the male germline by Cre-mediated recombination after breeding male chimeras to C57BL/6 females. Mice carrying the Smart17A allele were backcrossed 10 generations to the C57BL/6 background. In experiments utilizing Smart-17A/RORγt-GFP mice, the mice were homozygous for the

Smart-17A allele and heterozygous for RORγt-GFP. Smart-17A/Great mice were homozygous for both the IL-17A and IFN-γ reporter alleles and these dual reporter mice were used for all experiments described in Figures 3-6.

Th17 polarization $CD4^+$ T cells were isolated from the lymph nodes of Smart-17A and wild-type C57BL/6 mice using MACS beads (Miltenyi Biotech) and cultured with irradiated splenocytes from TCR-$C\alpha^{-/-}$ mice. Cells were stimulated under the designated conditions for 4 days: Th0 (50 U/ml IL-2), Th1 (50 U/ml IL-2, 5 ng/ml IL-12, 10 μg/ml anti-IL-4), Th2 (50 U/ml IL-2, 50 ng/ml IL-4, 10 μg/ml anti-IFN-γ), Th17 (3 ng/ml TGF-β, 20 ng/ml IL-6, 10 μg/ml anti-IFN-γ and anti-IL-4). Cytokines were purchased from R&D Systems. For intracellular cytokine staining, cells were restimulated with phorbol myristate acetate (5 ng/ml) and ionomycin (750 ng/ml) for 6 hr, with monensin (3 μM) added for the final 2 hr.

***Klebsiella pneumoniae* infection** *K. pneumoniae* (American Type Culture Collection #43816) were cultured in Nutrient Broth (Difco) with shaking overnight at 37°C. Cultures were diluted in Nutrient Broth and cultured for an additional 2-3 hr until bacteria reached log phase. Bacteria were pelleted by centrifugation, washed twice in PBS and diluted to a final dose of 500-1,000 bacteria in 50 μl PBS. This inoculum was administered intranasally after anaesthetizing mice with isofluorane. Doses were confirmed by plating the inoculum on Nutrient Broth agar plates and counting colonies the following day.

Induction of experimental autoimmune encephalomyelitis Mice were immunized with 200 μg of MOG35–55 emulsified in CFA containing 4 mg/ml *Mycobacterium tuberculosis* (Difco) subcutaneously and given 200 ng of pertussis toxin (List Biological Laboratories) intravenously on the day of and 2 days after immunization. Animals were graded daily according to their clinical severity as follows: grade 0, no abnormality; grade 1, limp tail; grade 2, limp tail and hind limb weakness (waddling gait); grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis; grade 5, moribund.

Cell isolation and preparation Mice were perfused with PBS and organs were removed. Mechanical dissociation was used to prepare single-cell suspensions from lymph nodes, spleens and Peyer's patches. Lungs were dissociated using a GentleMacs Dissociator (Miltenyi Biotech). Small intestines and colons were cut into small pieces and incubated in 5 mM EDTA in HBSS on stir plates 4 times for 15 min to remove the epithelial layer containing intraepithelial lymphocytes. Pieces were incubated at 37°C in 200 U/ml collagenase VIII (Sigma) in complete RPMI for a total of 4 30-min incubations. Cells from all incubations were pooled and lamina propria lymphocytes were purified over a 40%/100% Percoll gradient. Central nervous system lymphocytes were isolated as described (21) with modifications. Briefly, spinal cords and cerebellums were cut into pieces and digested in 300 U/ml Mandl units Collagenase D (Roche) and 50 U/ml DNase I (Roche) at 37°C for 30 min. Lymphocytes were enriched by separation on a 30%/70% Percoll gradient.

Flow cytometry Single-cell suspensions were washed in FACS buffer (PBS, 3% FCS, 1 mg/L NaN_3), and the cell pellets were incubated for 10 min on ice with anti-CD16/CD32 monoclonal antibodies (UCSF Antibody Core Facility). Cells were incubated for 30 min on ice with antibodies to surface markers. As necessary, cells were washed and stained with secondary antibodies for an additional 20 min on ice. Live cells were gated using DAPI exclusion. For intracellular cytokine staining, cells were stained for surface markers, fixed in 2% formaldehyde in PBS for 20 min at room temperature, washed and permeabilized in FACS buffer plus 0.5% saponin. Cells were stained at room temperature for 30 min in buffer containing 0.5% saponin buffer and 25% FCS. Dead cells were excluded using a violet live/dead fixable stain (Invitrogen). Antibodies were as designated and included: CD4 (BD, eBioscience), CD8 (BD, Biolegend), γδ (eBioscience), CD3 (eBioscience), CD11b (BD, Biolegend), CD19 (Biolegend), Gr1 (BD), CD11c (BD), Thy1.2 (eBioscience), Sca-1 (BD), IL-17A (BD, eBioscience), hNGFR (LabVision), Streptavidin-PE (Invitrogen), Streptavidin-APC (Biolegend). PBS-157-loaded CD1d tetramer was obtained from the NIH Tetramer Core Facility. Cell counts were performed using Count-Bright absolute counting beads (Invitrogen). Samples were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Quantitative PCR Whole lungs were homogenized and RNA was isolated using RNAzol (Molecular Research Center, Inc.). cDNA was prepared using the SuperScript III First Strand Synthesis System (Invitrogen). Primer sequences (PrimerBank) where designated were as follows: IL-1β: GAAATGCCACCTTTTGACAGTG,

CTGGATGCTCTCATCAGGACA; IL-23p19: CAGCAGCTCTCTCGGAATCTC, GCATGTGCGTTCCAGGCTA. Transcripts were quantified by incorporation of SYBR Green (Invitrogen) on a StepOne Plus Real-Time PCR System (Applied Biosystems) and quantified relative to the expression of *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase).

Results

Generation and validation of Smart-17A reporter mice

To assess IL-17A expression *in vitro* and *in vivo*, we generated IL-17A reporter mice, termed Smart (Surface marker for transcription)-17A mice (Figure 1A). In these mice, the 3' untranslated region (UTR) of the *il17a* gene was modified to include an internal ribosomal entry site (IRES) followed by a non-signaling form of the human nerve growth factor (hNGFR) gene, resulting in IRES-mediated translation of hNGFR when the IL-17A locus is activated. We verified the efficacy of the Smart-17A allele by demonstrating that hNGFR expression was specifically induced in $CD4^+$ T cells *in vitro* only under Th17 polarizing conditions and that intracellular IL-17A was found almost entirely within the $hNGFR^+$ population (Figure 1B, Figure 2A). Thus, the hNGFR reporter accurately marks 98% of Th17 cells identified using standard methods of *in vitro* restimulation and intracellular cytokine staining. Cells with the brightest staining for intracellular IL-17A were also those with the highest mean fluorescence intensity (MFI) for the hNGFR reporter. Approximately 30% of cells were $hNGFR^+$ but negative for intracellular IL-17A (Figure 1B). These cells tended to have the lowest MFI for hNGFR, consistent with their identification as cells that had previously secreted IL-17A and continued to be marked by the surface reporter. The half-life of the reporter on the cell surface was approximately 48 hr as assessed by decay under *in vitro* conditions (Figure 2B). Taken together, these results demonstrate that the Smart-17A reporter mouse sensitively and accurately marks cells that are induced to express IL-17A.

IL-17A expression in naïve mice

To characterize IL-17A expression *in vivo*, we crossed Smart-17A mice to RORγt-GFP reporter mice (297). Expression of the transcription factor RORγt has been shown to accurately identify both Th17 cells (220) and other lymphoid IL-17-producing cells (191). We anticipated that the inclusion of this second reporter would enhance the detection of rare populations of IL-17A-expressing cells by their concordant expression of RORγt. We first looked at CD3ε$^+$ cell populations in multiple organs of naïve mice for evidence of RORγt and IL-17A expression (Figure 3). CD3ε$^+$ cells were gated as indicated in Figure 4. Invariant (i)NKT cells were identified using a tetramer loaded with PBS-57 (an analogue of α-galactosylceramide) provided by the NIH tetramer facility, and "other CD3ε$^+$ cells" were defined as cells that were CD3ε$^+$ but negative for CD4, CD8, the γδ T cell receptor (TCR) and the CD1d-tetramer.

As previously reported, we observed constitutive expression of the RORγt GFP reporter in CD4$^+$ T cells in the lamina propria of the small intestine and colon (220). We also identified populations of GFP$^+$ γδ T cells in the spleen, lung, skin and small intestine, GFP$^+$ iNKT cells in the lung, and GFP$^+$ other CD3ε$^+$ cells in the lung and skin. Despite recovery of these RORγt$^+$ cells, however, we did not observe hNGFR$^+$ cells among the CD3ε$^+$ cell populations in any of the examined organs. In all populations, the small percentage of cells within the hNGFR$^+$ gate was similar to background levels present in naïve wild-type mice (data not shown). We were also unable to find evidence of hNGFR expression by dendritic cells, macrophages, neutrophils and a recently described, lineage-negative, Thy1$^+$ population of innate lymphoid cells (Figure 5) (201). We repeated these

analyses in Smart-17A mice without the RORγt-GFP allele and also in mice colonized by segmented filamentous bacteria and obtained identical results (data not shown). In all cases, none of the examined cell types, including cells from intestinal tissues, constitutively expressed hNGFR, suggesting that IL-17A is not expressed or is expressed at levels too low to be detected using this reporter in resting mice.

IL-17A expression during *Klebsiella pneumoniae* infection

Klebsiella pneumoniae is a gram-negative extracellular bacterium that is a cause of nosocomial and community-acquired pneumonia. IL-17A is rapidly produced in the lungs of mice during *K. pneumoniae* infection (298), and downstream signaling through the IL-17 receptor (IL-17R) leads to the induction of a variety of proinflammatory cytokines and chemokines that promote neutrophil accumulation (242). Mice deficient in IL-17A, IL-17R or the p19 subunit of IL-23 have increased bacterial dissemination and increased mortality (238, 242, 244), supporting the use of this model to investigate the cell types that produce IL-17A during acute bacterial challenge.

We intranasally inoculated Smart-17A mice with a dose of *K. pneumoniae* that led to mortality in a majority of infected mice by day 5. During the first 3 days after infection, we noted a substantial increase in the total number of $CD3\varepsilon^+$ cells in the lungs (Figure 6A). The vast majority of these cells were $CD4^+$ and $CD8^+$ T cells, with much smaller numbers of γδ T cells, iNKT cells and other $CD3\varepsilon^+$ cells. When reporter expression was assayed two days post-infection, the highest percentages of $hNGFR^+$ cells were found among γδ T cells, with lower percentages of $hNGFR^+$ iNKT cells and other $CD3\varepsilon^+$ cells (Figure 6B). A small but reproducible percentage of $CD4^+$ T cells were also

hNGFR$^+$, whereas percentages of reporter-positive CD8$^+$ T cells did not differ from background levels in wild-type mice. We did not observe hNGFR expression by any CD3ε$^-$ cell populations (data not shown). Although innate-like T cells comprised only a small portion of the total number of CD3ε$^+$ cells in the infected lung, they represented a substantial fraction of the total hNGFR$^+$ cells (Figure 6C). γδ T cells comprised over half of the hNGFR$^+$ cells (54%), followed by CD4$^+$ T cells (31%), other CD3ε$^+$ cells (9%) and iNKT cells (5%). Thus, innate-like T cells are major sources of IL-17A during *K. pneumoniae* infection.

Innate-like T cells are poised to produce IL-17A in response to proinflammatory cytokines

The discovery that a measurable percentage of innate-like T cells expressed IL-17A during acute *K. pneumoniae* infection led us to investigate the potential signals that mediate the production of this cytokine. It has been noted that IL-1β and IL-23 can induce TCR-independent IL-17A production from γδ T cells *in vitro* (232) and TCR/CD1d-dependent IL-17A production from iNKT cells *in vitro* and *ex vivo* (197). To determine if these findings applied to innate-like T lymphocytes *in vivo*, we administered IL-1β and IL-23 intranasally to Smart-17A mice and examined reporter expression from cells in the lungs 8 hr later (Figure 7A). The addition of either IL-1β or IL-23 individually elicited hNGFR expression in γδ T cells, iNKT cells and other CD3ε$^+$ cells, and the combination of both cytokines resulted in a synergistic increase in hNGFR levels to levels similar to those seen after *K. pneumoniae* infection. In contrast, we did not observe induction of IL-17A production as assessed by hNGFR expression in CD4$^+$ T

cells. The brief time between cytokine administration and IL-17A expression suggests that tissue resident innate-like T cells are poised to produce IL-17A rapidly after receiving signals from these cytokines. IL-1β and IL-23 subunit p19 mRNA were induced in the lung within 1 day after *K. pneumoniae* infection (Figure 7B), providing further evidence that these cytokines may play a role in inducing acute IL-17A production from innate-like T lymphocytes.

IL-17A expression during experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a mouse model of multiple sclerosis that is associated with the production of IL-17A. IL-17A$^{-/-}$ mice have reduced incidence and severity of disease (258). IL-1R$^{-/-}$ mice and IL-23p19$^{-/-}$ mice are also less susceptible to disease (232, 257). Although Th17 cells are thought to be the main source of IL-17A during EAE, there is evidence that γδ T cells can also produce this cytokine and may act to amplify IL-17A production from Th17 cells (213, 232). Most of these studies relied on *ex vivo* restimulation to assay IL-17A production, however, and thus the cells producing IL-17A directly *in vivo* during the development and propagation of EAE are not fully characterized.

To track IL-17A expression during EAE, we immunized Smart-17A mice with myelin oligodendrocyte glycoprotein peptide (MOG) emulsified in complete Freund's adjuvant (CFA) and treated mice with pertussis toxin to induce disease. We then looked at multiple CD3ε^{+} cell lineages in the draining lymph nodes (LN) at day 6 and in the spinal cord and cerebellum (referred to as CNS) at day 12 when mice were displaying symptoms of paralysis. At both sites, the vast majority of isolated T cells were CD4^{+} or

$CD8^+$ T cells, with smaller numbers of γδ T cells, iNKT cells and other $CD3\varepsilon^+$ cells (Figure 8A). Small but reproducible percentages (1-2%) of $CD4^+$ T cells expressed the hNGFR reporter in both the LN and the CNS (Figure 8B). As these percentages were substantially lower than what has been reported in the literature using *ex vivo* restimulation, we verified that *ex vivo* restimulation of cells isolated from the LN of Smart-17A mice led to an induction of hNGFR expression that very closely mirrored the percentages of IL-$17A^+$ cells found using standard intracellular cytokine staining methods (Figure 9). This confirms that cells from Smart-17A mice accurately report IL-17A production, and further suggests that *ex vivo* restimulation might induce IL-17A production from cells that do not actively produce the cytokine *in vivo*.

Among the remaining T cell populations, we observed hNGFR-expressing γδ T cells, iNKT cells and other $CD3\varepsilon^+$ cells (Figure 8B). The percentages of these $hNGFR^+$ innate-like T cells were greater in the LN than the CNS. Expression of hNGFR by $CD8^+$ T cells in both the LN and CNS was negligible when compared to wild-type controls, and we did not observe hNGFR expression by any $CD3\varepsilon^-$ cells (data not shown). Although the total numbers of γδ T cells, iNKT cells and other $CD3\varepsilon^+$ cells were substantially lower than the total numbers of $CD4^+$ cells, these innate-like T cells comprised a relatively large portion of the total number of IL-17A-expressing $hNGFR^+$ cells in both the LN and CNS (Figure 8C). In the LN, the greatest fraction of $hNGFR^+$ cells was other $CD3\varepsilon^+$ cells (36%), followed by γδ T cells (29%), iNKT cells (17%) and $CD4^+$ T cells (15%). In the CNS, the largest fractions of $hNGFR^+$ cells were γδ T cells and $CD4^+$ T cells (42% each), while other $CD3\varepsilon^+$ cells and iNKT cells together made up the remaining 16%. These data

suggest that innate-like T cells are a significant source of IL-17A during both the initiation and propagation phases of EAE.

Differential production of IL-17A and IFN-γ at effector sites during inflammation

IFN-γ is a cytokine that is expressed by multiple cell types and is generally associated with inflammatory immune responses. Mice deficient in IFN-γ or the IFN-γ receptor (IFN-γR) are more susceptible to a variety of bacterial infections including pulmonary infection with *K. pneumoniae* (243). IFN-γ has also been implicated in the pathogenesis of autoimmune diseases, including EAE, although the precise role of this cytokine is controversial. Thus, mice deficient in IFN-γ, IL-12 p35 subunit and IFN-γR are more susceptible to disease (270-272) whereas T-bet$^{-/-}$ mice are protected from disease (273). EAE can be induced in Rag$^{-/-}$ mice by the transfer of CD4^{+} T cells that have been polarized under either Th1 or Th17 conditions (274). Complicating matters further, recent studies have suggested that Th17 cells may represent an unstable population that can convert to an IFN-γ producing phenotype under specific inflammatory conditions (275).

To explore the relationship between IL-17A and IFN-γ production during *K. pneumoniae* infection and EAE, we crossed Smart-17A mice to Great reporter mice, which mark IFN-γ production by the coordinate expression of YFP downstream of an IRES introduced into the IFN-γ locus (142). The most striking observation in these dual reporter mice was that at the effector sites following both challenges, either in the lungs of mice infected with *K. pneumoniae* or in the CNS of mice during EAE, the percentages of YFP^{+} cells were substantially greater than the percentages of hNGFR^{+} cells among

$CD4^+$ T cells, iNKT cells and other $CD3\varepsilon^+$ cells (Figure 10A). In contrast, the populations of cells that expressed YFP and hNGFR was much more comparable among γδ T cells. As noted previously, we saw no significant expression above background of hNGFR among $CD8^+$ T cells, although we did note a substantial population of YFP^+ $CD8^+$ T cells, especially in the CNS during EAE. We observed some co-expression of YFP and hNGFR among $CD4^+$ T cells, especially in the CNS of mice during EAE, where approximately half of the $hNGFR^+$ cells also expressed YFP. However, among the innate-like T cells, IFN-γ-expressing and IL-17A-expressing cells segregated into distinct populations. The same trends in cytokine production were evident when we looked at total numbers of YFP^+ and $hNGFR^+$ cells isolated from these effector sites (Figure 10B). IFN-γ-producing cells outnumbered IL-17A-producing cells during the first three days of *K. pneumoniae* infection and during all stages of progressive EAE disease among all subsets of $CD3\varepsilon^+$ cells except for γδ T cells.

Discussion

We used IL-17A reporter mice to assess IL-17A expression in resting mice and during models of bacterial pneumonia and autoimmune disease. We did not observe constitutive IL-17A expression from cells in naïve mice. However, in both infectious and autoimmune models, we observed a small percentage of IL-17A-expressing $CD4^+$ T cells and greater percentages of IL-17A-expressing γδ T cells, iNKT cells and other $CD3\varepsilon^+$ cells. The other $CD3\varepsilon^+$ cells are defined by their expression of $CD3\varepsilon^+$ and lack of expression of CD4, CD8, γδ TCR and the CD1d-tetramer. It is possible that these cells are type II NKT cells that do not recognize α-galactosylceramide, or T cells that have downregulated the expression of CD4, CD8 or the γδ TCR. In all of these studies, IL-17A production as assessed by the reporter was limited to $CD3\varepsilon^+$ T cells and was not expressed by myeloid cell populations.

Although we identified populations of $ROR\gamma t^+$ cells in multiple organs of naïve mice, these cells did not constitutively express the hNGFR reporter, suggesting that IL-17A is not expressed *in situ* in resting mice. Segmented filamentous bacteria (SFB), *Candidatus arthromitis*, have been shown to induce RORγt-positive lamina propria T cells when introduced into the mouse intestinal flora (240). Cohousing Smart-17A mice with mice obtained from Taconic Farms led to colonization by SFB as determined by PCR, but did not induce hNGFR expression on cells in the lamina propria of the small intestine or colon (data not shown). In line with a previous report (299), we noted substantial populations of $ROR\gamma t^+$ γδ T cells in the skin and lungs of resting mice. We also noted smaller percentages of $ROR\gamma t^+$ iNKT cells in the lung and other $CD3\varepsilon^+$ cells in

the lung and skin. Resident dermal RORγt$^+$ γδ T cells have recently been shown to play a role in cutaneous immunosurveillance and to contribute to skin pathology during a mouse model of psoriasis (300-302). The localization of constitutive RORγt$^+$ innate-like T lymphocytes to the lung is intriguing as this mucosal tissue is relatively sterile and devoid of large numbers of commensal bacteria, a feature that is thought to be critical for the development of the RORγt$^+$ populations in the intestinal tract (206, 240, 303). Although lung-resident innate-like T cells did not constitutively express IL-17A as assessed by the hNGFR reporter, γδ cells, iNKT cells and other CD3ε$^+$ cells expressed hNGFR within 24 hr after *Klebsiella* infection or 8 hr after administration of IL-1β and/or IL-23. Taken together, these data suggest that RORγt$^+$ innate-like T cells accumulate at multiple epithelial barriers, where they are poised to respond rapidly to compromises in epithelial integrity with IL-17A production and the subsequent recruitment of neutrophils to the injured site.

Earlier reports used *ex vivo* restimulation to identify Th17 cells induced during a variety of bacterial and fungal infections, particularly those initiated by mucosal challenges (221, 304-307). Although our studies using *K. pneumoniae* in Smart-17A mice revealed activation of reporter expression in both innate-like T cells and CD4$^+$ Th17 cells, earlier studies demonstrated increased mortality in mice lacking γδ T cells but no difference in mortality in mice lacking αβ T cells (242, 307), suggesting that γδ T cells may be a more important source of IL-17A during this infection. Similar observations were made in experimental *Mycobacterium tuberculosis* infection (246) and in intraperitoneal infection with *Escherichia coli* (250), suggesting a more generalized role for IL-17A production by γδ T cells in immunity to bacterial infections.

Somewhat unexpected were the small percentages of IL-17A-expressing $CD4^+$ T cells recovered from lymph nodes and spinal cords and cerebellums during the peak of EAE disease.

Despite the relatively small percentage of IL-17A reporter-positive $CD4^+$ T cells, the large numbers of $CD4^+$ T cells in the inflammatory milieu was such that $CD4^+$ T cells still comprised a sizeable portion of total IL-17A-producing cells, making up 15% of the total in the LN and 42% in the CNS. The vast majority of recovered $CD4^+$ T cells, however, were Th1 cells as assessed by expression of the Great IFN-γ reporter allele and, based on our *in vitro* studies of the decay of the reporter on Th17 cells, are unlikely to have secreted IL-17A over the prior 2-3 days. Our findings seem to contrast with those obtained using an IL-17A-cre reporter mouse (14). In that study, the authors crossed IL-17A-cre mouse to ROSA-flox-stop-YFP mice and observed that over half of the $CD4^+$ and γδ T cells were YFP^+ in the spinal cords of mice during the peak of EAE. However, cells positive for the IL-17A-cre/Rosa-flox-stop-YFP reporter were those that expressed the Cre recombinase from an activated IL-17A locus at any time during their development, whereas the Smart-17A reporter specifically marks cells that have recently expressed or are currently expressing IL-17A. Thus, differences in construction of these various reporter mice likely account for the differences in the experimental results.

Recent reports have raised the possibility that Th17 cells represent an unstable transient phenotype rather than a fully differentiated Th subset akin to that of Th1 and Th2 cells. During the development of EAE, IL-$17A^+$, IFN-γ^+ and IL-17A/IFN-γ double-positive $CD4^+$ T cells have been observed in the lymph nodes and spinal cords of immunized mice (220). Adoptive transfer of encephalitogenic Th17 cells purified from

an IL-17F-cre BAC transgenic mouse into RAG-2$^{-/-}$ or wild-type recipients revealed that a portion of these cells began to secrete IFN-γ during the progression of EAE (308). Similarly, studies using the IL-17A-cre/Rosa-flox-stop-YFP mice revealed that only half of the reporter-positive fate-marked CD4$^+$ T cells were positive for intracellular IL-17A in the spinal cords during the peak of EAE (213), providing further evidence for a switch from an IL-17A-producing to an IFN-γ-producing phenotype. However, all of these studies used *ex vivo* restimulation to assess IL-17A and IFN-γ production from isolated spinal cord cells. Our study, which relied solely on direct *ex vivo* detection of cytokine production using reporter mice, provides additional evidence for the marked predominance of IFN-γ production by CD4$^+$ T cells during each of the clinical stages of EAE. We saw only low numbers of IL-17A$^+$ cells as compared to IFN-γ$^+$ cells, and approximately half of the Th17 cells concordantly expressed IFN-γ. Taken together, these findings suggest that activation of the IL-17A locus may be important in the early differentiation of pathogenic CD4$^+$ T cells in EAE, but that actual production of IL-17A might not be a major mechanism driving the neurological manifestations of the disease. Indeed, recent reports have demonstrated critical contributions by GM-CSF rather than IL-17 in the pathogenesis of EAE (309, 310).

Another consistent observation was the pronounced segregation of IL-17A-expressing and IFN-γ-expressing cells within the innate-like T cell populations. This segregation occurred in both the infectious and autoimmune models, raising questions about the mechanisms of regulation that account for this exclusionary pattern of cytokine production. IFN-γ and IL-17 have been shown to limit differentiation of Th17 and Th1 cells, respectively, and this mutual inhibition could be operating in these innate

populations as well (176, 177, 311). It has also been suggested that antigen-naive γδ T cells predominately produce IL-17A when activated, whereas antigen-experienced γδ T cells produce IFN-γ (188), which could potentially explain the differences in cytokine-secreting populations seen in our models. Further characterization of the cells expressing these cytokines is needed to address this question more fully.

Cytokine reporter mice allow for the functional marking of cells during the course of an inflammatory challenge and have provided essential insights into the coordination of the immune response *in vivo*. The Smart-17A mice and Great mice used in this study permitted the detection of IL-17A-producing and IFN-γ-producing cells *in situ* without restimulation. Using these mice, we demonstrated that innate-like T cells, particularly γδ T cells, comprised major cell populations poised for acute IL-17A production. During both *K. pneumoniae* infection and EAE, models previously demonstrated to induce potent IL-17 expression, we show that the numbers of IL-17A-producing cells were far fewer than the numbers of IFN-γ-producing cells in the same tissues, suggesting different levels of regulation of these two inflammatory cytokines *in vivo*. Although IL-17A production has been elicited from a number of different cell types using restimulation, our reporter system suggests that cytokine production is limited to T cells in the models we studied. These cytokine reporter mice will be valuable tools for future studies investigating the full contributions of IL-17A-expressing cells to vertebrate immunity.

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Footnotes

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Figures

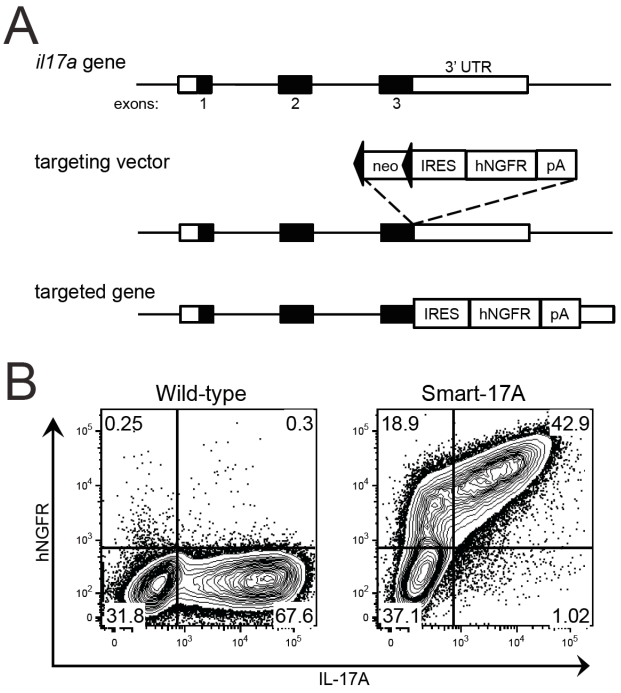

Figure 1. Generation of Smart-17A mice.

(A) Targeting strategy for the *il17a* locus. For detailed description, see Materials and Methods. (B) $CD4^+$ T cells were isolated from wild-type or Smart-17A mice and polarized under Th17 conditions for 4 days. hNGFR was detected using a surface antibody and IL-17A was assayed using intracellular cytokine staining after restimulation. A representative flow cytometry plot is shown from >5 comparable experiments.

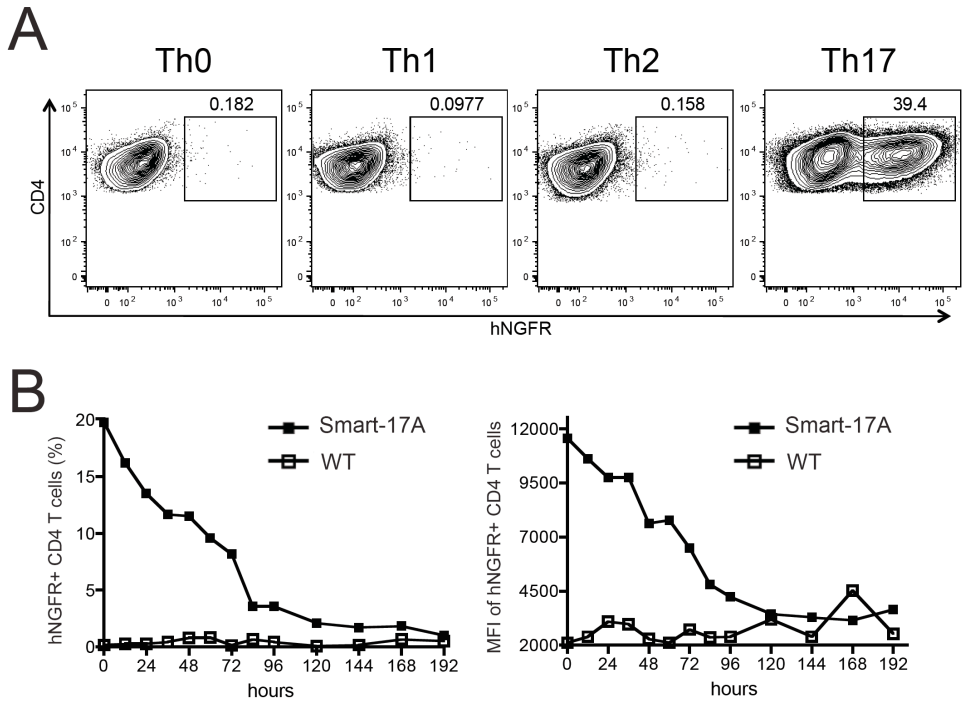

Figure 2. Polarization of Smart-17A CD4 T cells *in vitro*.

(A) $CD4^+$ T cells were isolated from Smart-17A mice using MACS beads and polarized under Th0, Th1, Th2 or Th17 conditions for 4 days, at which point surface hNGFR expression was assayed by flow cytometry. This experiment was repeated 3 times and representative flow cytometry plots are shown. (B) $CD4^+$ T cells from wild-type or Smart-17A mice were polarized under Th17 conditions for 4 days. Cells were then washed and re-plated in wells containing no cytokines. The percentage of $hNGFR^+$ cells and the MFI of $hNGFR^+$ cells were measured at indicated times to determine the rate of decay of the hNGFR reporter.

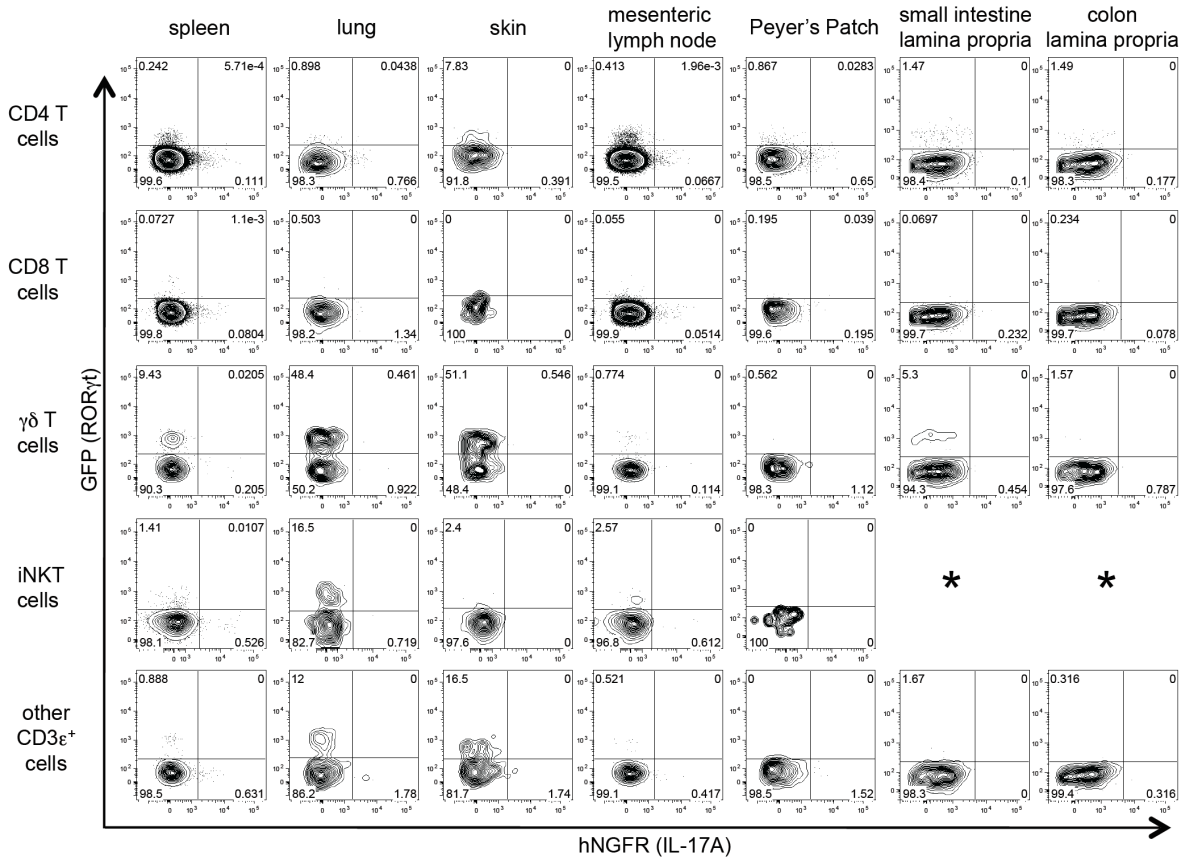

Figure 3. IL-17A expression in resting mice.

Cells were isolated from indicated organs of Smart-17A/RORγt reporter mice and levels of hNGFR were assayed. Populations were gated as described in Supplementary Figure 2. All gates were set using a wild-type mouse as a negative control. * denotes cell populations that were too few in number to reliably assess marker expression. Representative flow cytometry plots are shown from 1 of 3 comparable experiments, each including 2-3 mice.

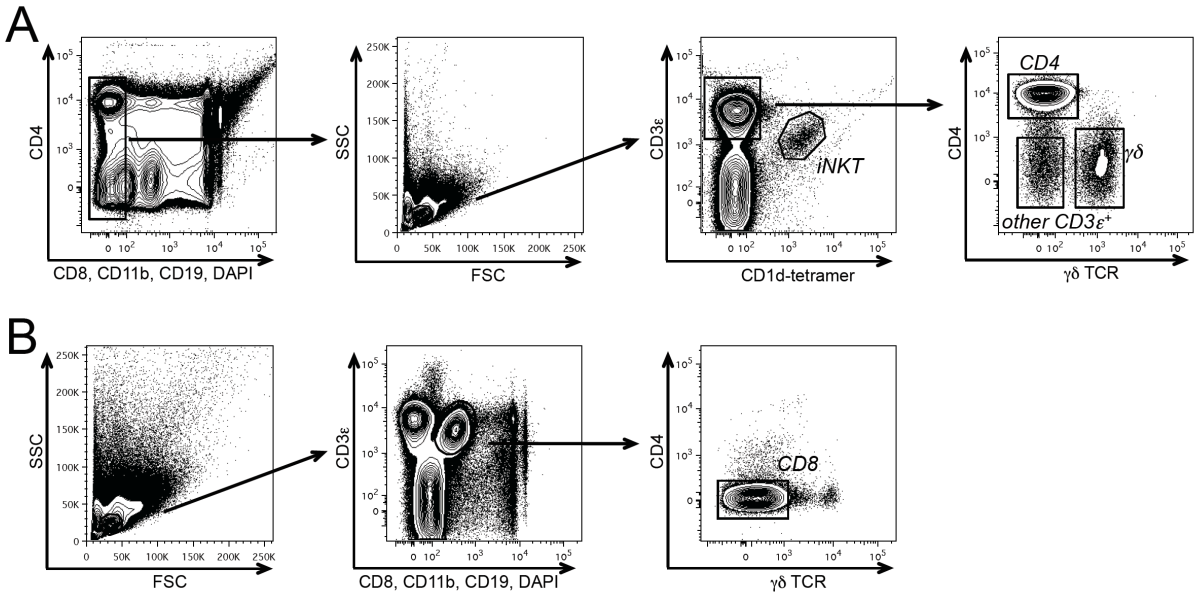

Figure 4. Gating of CD3ε$^+$ cell populations.

Flow cytometry gating schemes for CD3ε$^+$ cells used throughout this study. (A) Gating scheme for CD4$^+$ T cells, γδ T cells, iNKT cells and other CD3ε$^+$ cells. (B) Gating scheme for CD8$^+$ T cells. Plots shown are from the mesenteric lymph node of a naïve Smart-17A mouse.

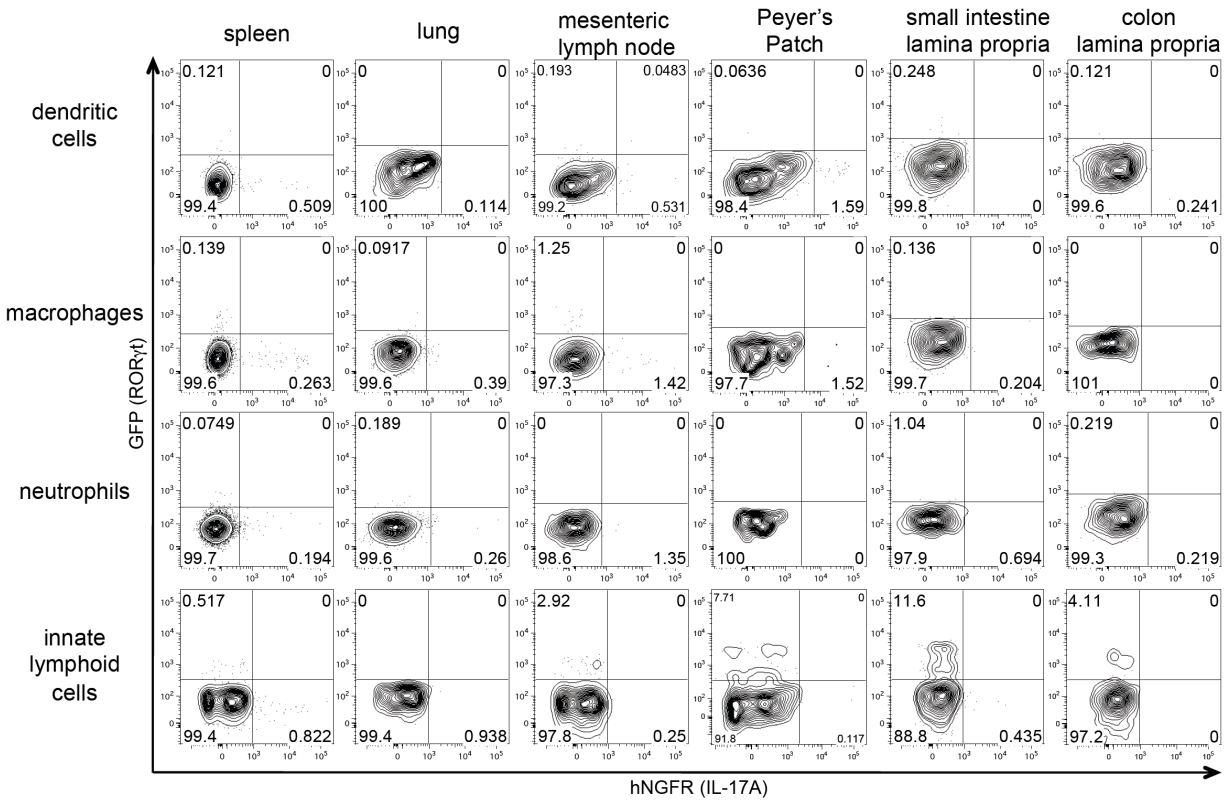

Figure 5. IL-17A expression in CD3ε$^-$ cell populations.

Cells were isolated from the indicated organs of Smart-17A/RORγt dual reporter mice and assayed for GFP and surface hNGFR expression. Dendritic cells were defined as CD11c$^+$, macrophages as CD11b$^+$, neutrophils as CD11b$^+$ andGr1$^+$, and innate lymphoid cells as lineage-negative (negative for CD3ε, CD8, CD19, CD11b, Gr1) and Thy1$^+$. The gated innate lymphoid cells included cells that were positive and negative for both CD4 and Sca-1. hNGFR expression was not seen using any gating scheme. All gates were drawn using a wild-type mouse as a control. The experiment was repeated twice and representative plots are shown.

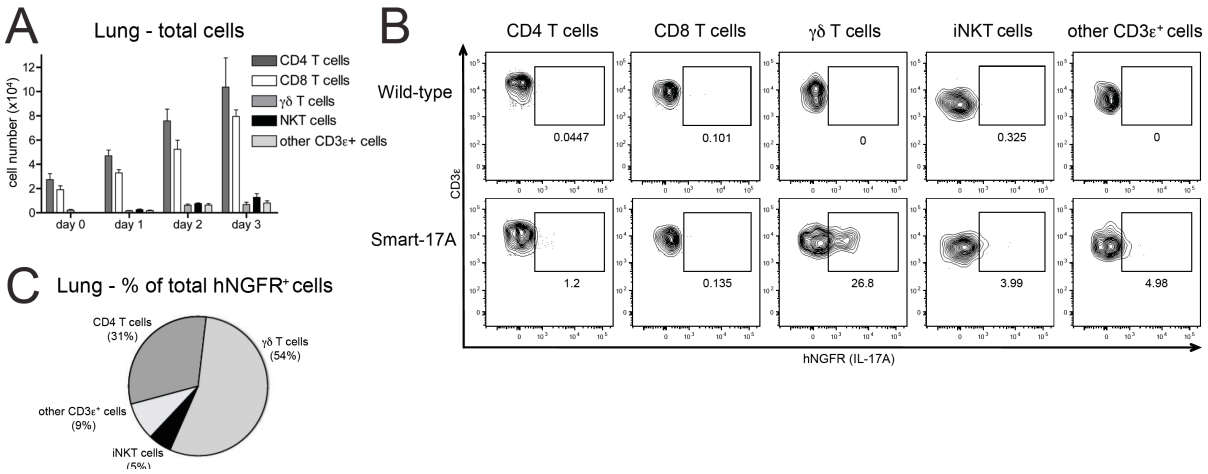

Figure 6. IL-17A expression during infection with *Klebsiella pneumoniae*.

Wild-type or Smart-17A mice were infected with 500-1000 *K. pneumoniae*. (A) At the indicated time points, cells were harvested from lungs and numbers of cells were enumerated. (B) Cells were isolated from the lungs of mice 2 days after infection and assayed for hNGFR expression. (C) The total number of $hNGFR^+$ and YFP^+ cells on day 2 post-infection were calculated and the percentage attributable to each cell population is shown in a pie graph. The percentage of background staining seen in a wild-type mouse under identical conditions was subtracted before performing all calculations to control for nonspecific staining. This experiment was repeated 3 times with $n > 3$ mice at each time point. For bar and pie graphs, data from independent experiments were compiled. For flow cytometry, representative plots are shown.

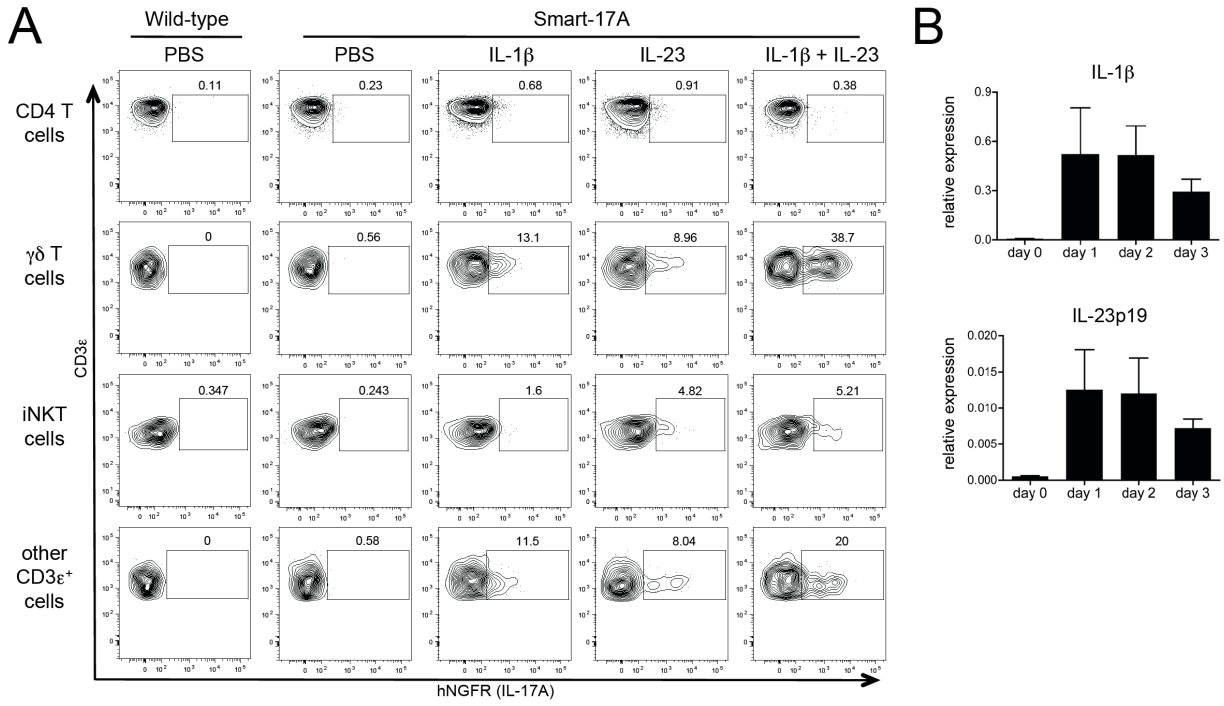

Figure 7. Expression of IL-17A from innate-like T lymphocytes cells can be induced by IL-1β and/or IL-23.

(A) Smart-17A mice were inoculated intranasally with PBS or with 500 ng IL-1β, IL-23 or both cytokines. Lungs were harvested 8 hr later and cells were analyzed for hNGFR expression. Gates were set using a wild-type control. The experiment was repeated 2 times and representative data are shown. (B) Wild-type mice were infected with *K. pneumoniae* and levels of IL-1β and the IL-23 subunit p19 mRNA in whole lung homogenate were measured using quantitative PCR. Expression of GAPDH was used as a reference to define relative expression. The experiment was done twice and a representative experiment is shown, n = 3 for all groups.

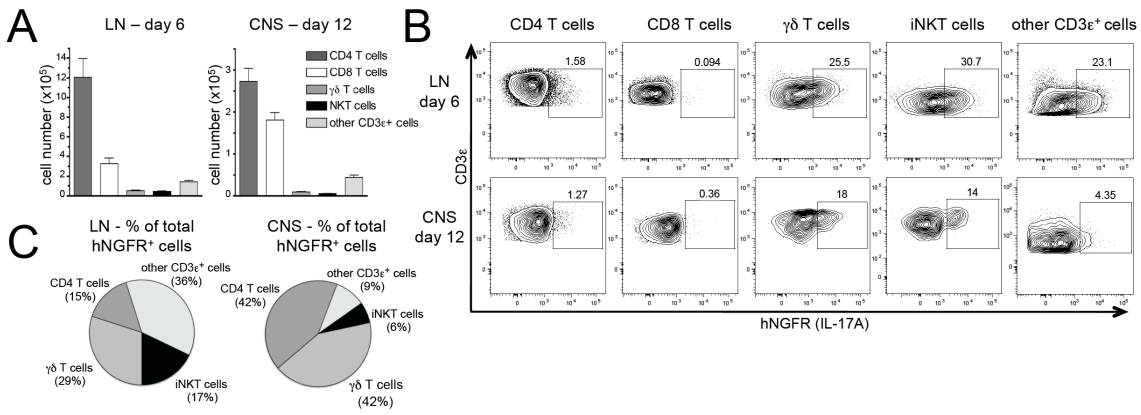

Figure 8. IL-17A expression during experimental autoimmune encephalomyelitis. Wild-type or Smart-17A mice were immunized with MOG-CFA to induce EAE. (A) At the indicated disease scores, cells were harvested from the draining axial, brachial and inguinal lymph nodes (LN) at day 6 or spinal cords and cerebellums (CNS) at day 12 and numbers of cells were enumerated. (B) Cells were assayed for hNGFR expression. (C) The total numbers of hNGFR$^+$ cells were calculated and the percentage attributable to each cell population is shown in a pie graph. The percentage of background staining seen in a wild-type mouse under identical conditions was subtracted before performing all calculations to control for nonspecific staining. For the CNS data, mice were excluded that did not display symptoms of paralysis. These experiments were repeated 3 times with $n > 2$ mice at each time point. For bar and pie graphs, data from independent experiments were compiled. For flow cytometry, representative plots are shown.

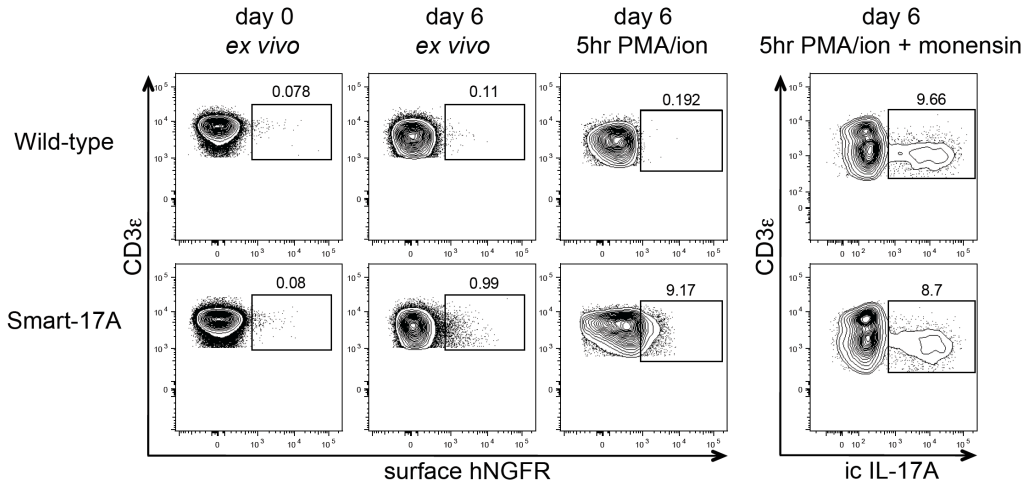

Figure 9. Induction of hNGFR expression after *ex vivo* restimulation of CD4⁺ T cells from the lymph nodes of mice during EAE.

Cells were isolated from the draining lymph nodes wild-type or Smart-17A mice either before induction of EAE or on day 6 after immunization. As indicated, cells were immediately stained for surface markers or restimulated for 5 hr with PMA/ionomycin and then stained for either surface markers or intracellular expression of IL-17A.

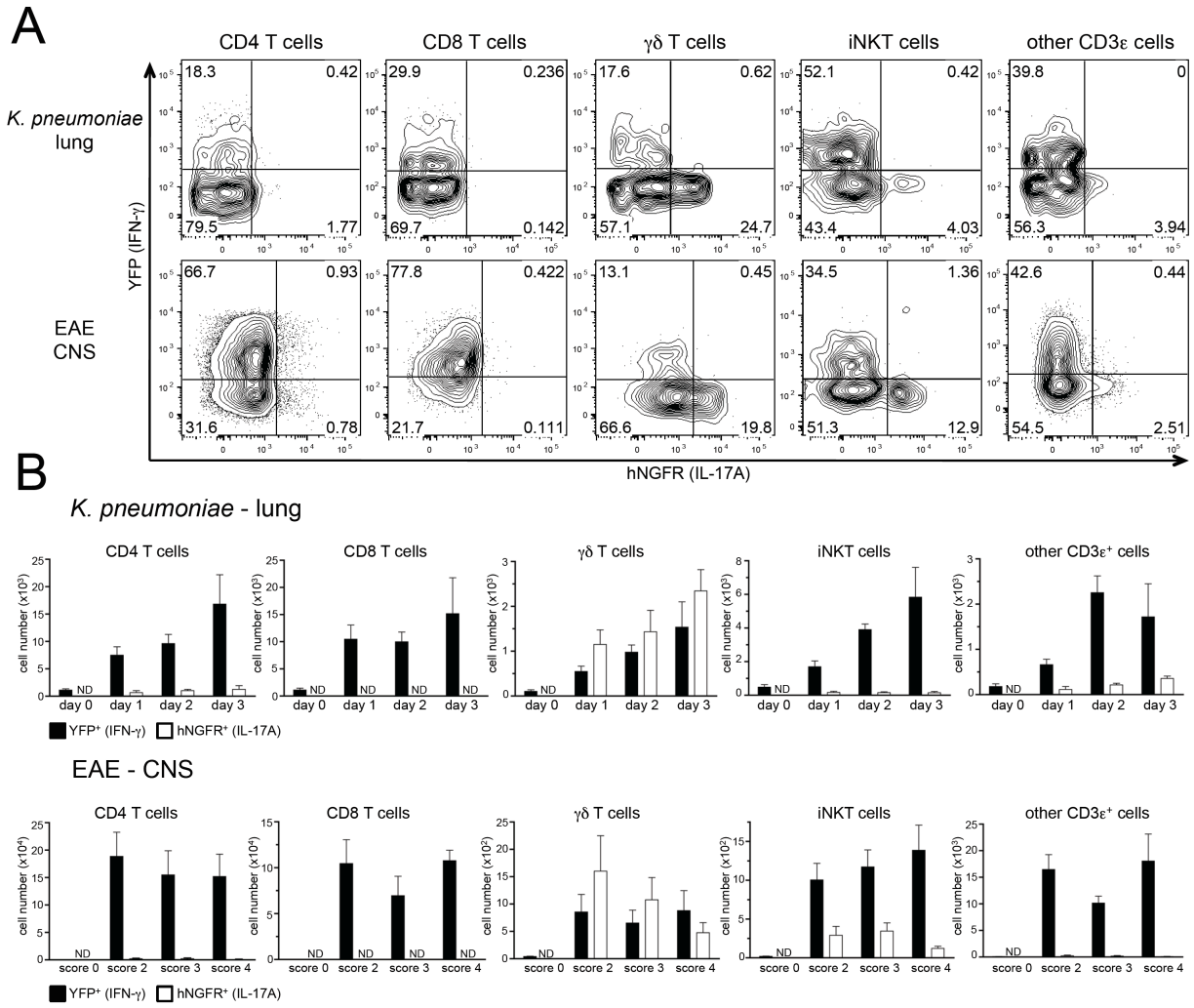

Figure 10: Differential production of IL-17A and IFN-γ in effector sites during inflammation.

(A) Expression of hNGFR and YFP was assessed in the lungs of mice at day 2 after infection with *K. pneumoniae* or in the spinal cord and cerebellum (CNS) of mice at day 12 after induction of EAE. (B) Numbers of $hNGFR^+$ and YFP^+ cells were enumerated

during *K. pneumoniae* infection or EAE disease course. EAE disease scores were determined as described in Materials and Methods. ND = not detected. These experiment was repeated 3 times with n > 2 mice at each disease score. For flow cytometry, representative plots are shown. For bar graphs, data from individual experiments were compiled.

CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

Part A: Cytokine production during Type 2 immune responses

Summary

Our work focused on type 2 immunity, described in detail in Chapter II, details the identification of a new innate cell type that participates in type 2 immune responses, which we termed innate helper type 2 or Ih2 cells. We used an IL-4 reporter mouse to identify these cells and to describe the distribution of this population in multiple organs at rest. We additionally demonstrated that these cells are negative for standard lineage markers and express several phenotypic surface markers, including CD45, IL-7R, c-kit, CD44 and Thy1. Using a microarray, we demonstrated that these cells express a unique subset of genes, including IL-9R and α4 integrin, and also express genes associated with type 2 immunity, including STAT-6 and GATA-3. We further demonstrate that Ih2 cells expand dramatically in response to the epithelial cytokines IL-25 and IL-33 and during infection with the helminth *Nippostrongylus brasiliensis*. Using a novel IL-13 reporter mouse, we showed that these cells are the major innate source of IL-13 after administration of IL-25 and IL-33 and during *N. brasiliensis* infection. Finally, we demonstrate that Ih2 cells are sufficient to mediate IL-25-induced eosinophilia and worm clearance during infection with *N. brasiliensis*.

Classification of Ih2 cells

As mentioned in Chapter I, multiple groups have identified and characterized populations of innate type 2-associated cells (90, 92-95). One of the most pressing needs

in the field is to determine whether these populations represent subsets of the same lineage, and if so, to establish a unifying nomenclature and series of phenotypic or functional markers to uniquely define this cell population. It appears clear that these separately identified populations are all negative for commonly used lineage markers and share the expression of certain surface markers, including low expression of c-kit and higher expression of CD45, IL-7R, and Thy1. These surface markers are not sufficient for definitive identification of innate type 2 associated cells, however, as these markers are also expressed by lymphoid tissue inducer (LTi) cells (312), recently identified innate lymphoid cells that produce IL-17 or IL-22 (200, 201, 206), subsets of bone marrow resident or migratory hematopoietic stem cells (313, 314), and potentially other populations of more specialized progenitor cells. Indeed, a report by Saenz et al. (279) identified an IL-25-inducible progenitor population that displayed this same surface phenotype and gave rise to basophils, mast cells and macrophages after *in vitro* culture. Innate type 2-associated cells also appear to express activation markers such as CD44, CD25, and inducible T cell costimulation (ICOS), although identification using these molecules is potentially problematic as their expression can be modified upon cellular activation and they can also be expressed by additional cell types during inflammation. A variety of additional proteins have been suggested to be expressed by innate type 2-associated cells, including MHC class II, T1/ST2, Sca-1 and a variety of chemokines and other homing molecules (90, 94, 100); however, the expression of these genes has not been verified in all identified type 2-associated subsets and may also exhibit some variability based on the tissue of origin or activation status of the cells (100). Multiple groups, including ours, have characterized innate type 2 associated cells functionally,

based on their expression or secretion of the type 2-associated cytokines IL-4, IL-5, IL-9 and/or IL-13 (90-95). Although any identification scheme has limitations, we propose identifying innate type 2-associated cells by the absence of lineage markers (including specific markers to eliminate basophils, mast cells, eosinophils and NKT cells), positive staining for CD45, IL-7R and Thy1, and the presence of mRNA or protein for one or more type 2-associated cytokines. We further propose adopting the nomenclature innate helper type 2 (Ih2) cells to define this lineage, as this naming convention corresponds to the classification of $CD4^+$ T cell subsets that originated at DNAX, which is where these type 2-associated cells were first described.

Mechanisms of Ih2 cell differentiation

Multiple pieces of evidence suggest that Ih2 cells are of lymphoid origin. Early experiments demonstrated that Ih2 cells have a morphology similar to lymphoid cells, as they are small in size and have a round shape, dark nucleus and limited cytoplasm (93, 94). In our Ih2 cell microarrays, we demonstrated that Ih2 cells expressed Aiolos, which is a transcription factor specifically associated with lymphoid cells. More recently, RAG-1Cre/ROSA26^{YFP} mice were used to demonstrate that Ih2 cells in the lung had a history of RAG-1 expression, which is induced during development in both common lymphoid progenitors and lymphoid primed multipotent progenitors (315). This group went on to demonstrate that the development of Ih2 cells required Flt3, which is a protein required for the generation of lymphoid progenitors and, most definitively, they demonstrated that Ih2 cells could develop from adoptive transfer of common lymphoid progenitors but not myeloid-erythroid precursors. Although this study represented an important first step in

the determination of the developmental origin of Ih2 cells, much more work is needed to elucidate the developmental pathways involved in generation of this lineage.

Interestingly, Ih2 cells are completely absent in mice that lack the helix-loop-helix transcription factor Inhibitor of differentiation 2 (Id2) (94). The Id family of transcription factors plays important roles in lymphopoiesis. $Id2^{-/-}$ mice are also deficient in other innate lymphoid populations, including NK cells, LTi cells, and innate lymphoid cells that secrete IL-17 or IL-22 (316), suggesting that these populations may share a common progenitor. The divergence from this common progenitor into separate lineages is likely promoted by unique combinations of transcription factors and cytokines. For example, NK cell differentiation requires the transcription factor E4BP4 and the cytokine IL-15, and the differentiation of LTi cells, IL-17-producing innate lymphoid cells and IL-22 producing $NKp46^{+}$ innate cells requires the transcription factor RORγt and the cytokine IL-7 (316, 317). Transcription factors required for the generation of Ih2 cells have not been identified, although our microarrays identified multiple transcription factors expressed by Ih2 cells, including GATA-3 and hepatic leukemia factor (HLF). Similarly, the cytokines required for Ih2 differentiation have not been fully elucidated, although IL-7 is likely required, as $IL\text{-}7R^{-/-}$ mice have no Ih2 cells (94). Ih2 cells are also completely absent in common-γ chain deficient mice (90, 94), and other common-γ chain cytokines such as IL-2 might also be involved in the differentiation of this lineage. Although IL-25 and IL-33 have a multiple effects on Ih2 cells, these cytokines do not appear to be absolutely required for the differentiation of this lineage, as Ih2 cells were still found at rest in mice lacking the ability to signal through the IL-25 or IL-33 receptors (95). Much further work is necessary to determine the factors required for Ih2 cell differentiation.

Recruitment, proliferation, and retention of Ih2 cells

Our work demonstrated that Ih2 cells are present in multiple organs at rest, with the highest concentrations of cells in the MLN and spleen. The mechanisms that contribute to the constitutive seeding of these organs with Ih2 cells have not been defined. Unlike other innate type 2-associated cells, such as eosinophils and basophils, we were unable to detect Ih2 cells in the blood of a resting mouse, suggesting that these cells do not freely circulate or circulate at very low levels and may preferentially home to peripheral tissues. Although Ih2 cells are derived from lymphoid progenitors, it remains to be determined whether these cells fully differentiate in the bone marrow or if they exit the bone marrow as progenitor cells that mature in the periphery, in a process similar to what is observed for mast cells. In either case, expression of a specific set of homing molecules would be required for the recruitment of Ih2 cells or Ih2 progenitors to the tissues in which they ultimately reside. Our array revealed that Ih2 cells express certain homing molecules such as α4 and β7, which are associated with homing to mucosal tissues. Neill et al. demonstrated that Ih2 cells from the mesenteric lymph node express CCR9 (95), although this expression was not observed in lung-resident Ih2 cells (100). Other groups have reported Ih2 cell expression of the chemokine receptors CXCR4 and CXCR6 and the adhesion molecules ICAM-1 and ICAM-2 (100). The full array of homing molecules expressed by Ih2 cells and the mechanisms that contribute to their recruitment is worthy of further study.

During helminth infection or after the administration of IL-25 or IL-33, the number of Ih2 cells in multiple organs increases dramatically. It is unclear whether this

increase is due to bone marrow proliferation and/or mobilization or is a result of local proliferation. The addition of IL-2 to cultures of Ih2 cells supplemented with stem cell factor and IL-7 induced rapid Ih2 cell proliferation (94). The addition of IL-25 or IL-33 to Ih2 cell cultures or administration of these cytokines *in vivo* also leads to rapid proliferation of Ih2 cells (93-95). Pilot experiments measuring Edu incorporation during a 30-minute pulse as a measure of active proliferation, revealed that over 50% of MLN and spleen-resident Ih2 cells were actively proliferating after three daily doses of IL-25 (data not shown), suggesting that local proliferation may be occurring. However, we also observed detectable numbers of Ih2 cells in the blood after administration of IL-25 and IL-33, which indicates that these cells might also be actively migrating from the bone marrow or other tissue reservoirs. It also remains to be determined whether local proliferation is a major factor contributing to the increase of Ih2 cell numbers in the MLN and lung during the course of helminth infection, as the Ih2 cell increase during infection is more gradual and also doesn't reach the same high levels seen after cytokine administration. Although IL-25 and IL-33 are often used interchangeably to induce Ih2 cell proliferation and cytokine production, including in our studies, it is likely that the production of these cytokines is induced by different stimuli *in vivo* and may also play different roles depending on the tissue examined. While we and others have shown that IL-25 appears to potently induce expansion of Ih2 cells in the MLN and spleen (90, 93, 95), IL-33 might be more important for inducing Ih2 cell expansion and cytokine production in the lung. Indeed, intranasal administration of IL-33 was found to induce 5 times more IL-13 reporter-marked Ih2 cells in the lung than similar administration of IL-25 (97). While it appears clear that IL-25 and IL-33 play an important role in inducing

Ih2 cell proliferation, it is likely that other factors induced during infection or inflammation can also influence this process. Elucidating the mechanisms leading to Ih2 cell recruitment and proliferation could potentially provide a means to therapeutically manipulate the number of these cells during helminth infection or the establishment of allergy and asthma.

Localization of Ih2 cells

Although our studies revealed a systemic distribution of Ih2 cells at rest and increases in Ih2 cell numbers in multiple organs during helminth infection or cytokine administration, we did not specifically localize these cells within tissues. The small number of cells observed in various organs at rest suggests that these cells may be playing a role as sentinels, poised to respond during infection or allergen challenge. It would be interesting to determine if these cells are scattered randomly throughout organs at rest, or if they reside in specific locations within tissues that contribute to their ability to sense and respond to infection or allergens. For example, within the lung, Ih2 cells may preferentially reside near branch-points in airways or near blood vessels. This localization would provide them with the ability to rapidly sense infection or allergen-induced signals from epithelial cells such as the release of IL-25 and IL-33. Additionally, localization near airways or blood vessels would allow the IL-13 and IL-5 released by Ih2 cells to locally promote mucus production by goblet cells, smooth muscle hyperreactivity, and the recruitment and survival of innate cells such as eosinophils. Similarly, in the intestine, Ih2 cells may be preferentially located in the lamina propria at sites adjacent to the epithelium, where the cytokines they produce can influence resident

goblet cells, eosinophils and smooth muscle. The ability to mark Ih2 cells with reporter mice, such as 4get mice, YetCre13 x Rosa-YFP mice, and the newly generated IL-5 reporter mice, allows for more detailed study into the localization of Ih2 cells and should provide further hints into the function of these cells under various conditions.

Cytokine production by Ih2 cells

One of the defining features of Ih2 cells is their ability to produce type 2 cytokines. Although the genes for IL-4, IL-5 and IL-13 are closely associated within 140 kb of each other mouse chromosome 11 or human chromosome 5, this locus appears to be coordinately regulated such that these cytokines can be expressed either together or separately. The mechanisms that account for this flexibility of expression within the type 2 cytokine locus remain incompletely defined, and elucidating the transcription factors and epigenetic modifications that contribute to this regulation is an important area for future research. Divergent production of type 2 cytokines is noted among both adaptive and innate immune cells. For example, follicular T helper cells, which reside in the B cell follicles and contribute to the production of high affinity antibodies by B cells, appear to produce predominately IL-4 during helminth infection while peripheral T cells appear to be capable of producing both IL-4 and IL-13 (318). Similarly, in a model of OVA-induced asthma, $CD4^+$ T cells in the mediastinal lymph node produced predominately IL-4, whereas lung-resident $CD4^+$ T cells expressed IL-13 or a combination of IL-4 and IL-13 but not IL-4 alone (97). The ability of peripheral T cells to produce IL-13 appears to be dependent upon higher levels of the transcription factor GATA-3 (318). Among innate cell populations, our work suggests that eosinophils are

not major sources of IL-4 or IL-13 despite the presence of IL-4 mRNA indicated by this population's constitutive positivity for the 4get reporter (141). Basophils are also constitutively 4get^{+}, and produce IL-4 but not IL-13 during helminth infection (105, 318). Mast cells appear to be capable of secreting IL-4 and IL-13 under certain conditions. Although some reports suggested that Ih2 cells produce low levels of IL-4 (94, 100), our studies using KN2 reporter mice and studies from other groups have suggested that while Ih2 cells may express IL-4 mRNA, they are likely not major sources of IL-4 during helminth infection or allergic lung inflammation. Instead, Ih2 cells appear to be potent sources of IL-13 and IL-5. Similar to what was observed among adaptive Th2 cells, high levels of GATA-3 are associated with IL-13-producing Ih2 cells (318). However, the full array of factors responsible for mediating divergent type 2 cytokine production among these various type 2-associated cells remain to be characterized.

Interestingly, our studies using various reporter mice, including 4get mice, YetCre13 mice, YetCre13 x Rosa-YFP mice, and IL-5-tomato reporter mice suggest that even among Ih2 cells there may be variation in cytokine production patterns. While we originally assumed that all Ih2 cells would be marked by the 4get reporter, as this reporter indicates activation of the IL-4 locus and is constitutively present in other innate type 2-associated cells such as basophils, eosinophils, and mast cells, it appears that a substantial proportion of Ih2 cells are actually negative for the 4get reporter. Instead, there seems to be a considerable number of cells in multiple organs that have the surface markers characteristic of Ih2 cells that are reporter positive for either the YetCre13 x Rosa-YFP lineage tracker or IL-5-tomato but are negative for the 4get reporter (data not shown). It is unclear whether these 4get^{-} cells marked with other reporters represent cells that have

expressed IL-4 at some earlier point in their development or if they have never activated the IL-4 locus. An IL-4-Cre reporter would be necessary to fully address this question. In any case, it would be very interesting to determine if the subsets of Ih2 cells differentially marked with IL-4, IL-5 and IL-13 reporters have different surface marker or transcription factor profiles and if they play unique roles in immunity to helminths or the establishment of allergic lung inflammation.

Downstream effector functions of Ih2 cells

Multiple groups have demonstrated that Ih2 cells expand and produce IL-13 and IL-5 during helminth infection and during allergic lung inflammation. Consequently, a majority of the work aimed at elucidating the function of Ih2 cells has focused on their production of type 2 cytokines. IL-13 production by Ih2 cells has been suggested to contribute to goblet cell metaplasia and mucus production and smooth muscle hyperreactivity during helminth infection and allergic lung inflammation (94, 95, 100), while Ih2 cell-derived IL-5 has been suggested to promote eosinophilia during these responses and also to support self-renewal and IgA production by mucosal B1 cells (94). Mice deficient in IL-13 have impaired immunity and worm clearance during *N. brasiliensis* infection and do not develop airway hyperreactivity in models of allergic lung inflammation (24, 140, 319, 320). In an elegant series of experiments by Andrew McKenzie's laboratory, it was demonstrated that transfer of wild-type IL-13-sufficient Ih2 cells was sufficient to restore normal worm clearance during *N. brasiliensis* infection in IL-4/13$^{-/-}$ mice (95) and airway hyperreactivity in IL-13$^{-/-}$ mice (97). This suggests that Ih2 cells can mediate the downstream effects of IL-13 during type 2 immune responses

even when they are the sole cellular source of this cytokine. IL-13 production by Ih2 cells may also have additional downstream functions, including the promotion of alternatively activated macrophages, although this remains to be validated. It is also possible that Ih2 cells may produce other factors that are important for type 2 immunity. Along those lines, Ih2 cell production of amphiregulin, an epidermal growth factor associated with wound healing, was shown contribute to the resolution of influenza infection by promoting lung function and tissue remodeling (321). It seems likely that Ih2 cells may play dual roles in both initiating type 2 immune responses and also in regulating and resolving inflammation, and that these cells probably utilize multiple downstream effector molecules and pathways to accomplish their varied functions in immunity.

Interactions between Ih2 cells and CD4$^+$ T cells

Accumulating evidence suggests that interactions between innate type 2-associated cells and adaptive Th2 cells are an important feature of type 2 immune responses. Although both innate cells and Th2 cells are capable of secreting type 2-associated cytokines, the establishment of antigen-specific Th2 cells generally takes 3-5 days. During helminth infection or the establishment of OVA-induced allergic lung inflammation, Ih2 cells accumulate and produce type 2 cytokines before their adaptive Th2 cell counterparts (95, 100), suggesting that these cells and perhaps other innate populations may be important early sources of type 2 cytokines. Cytokine production by innate cells may be directly or indirectly important for the development, differentiation or activation of Th2 cells. Indeed, the presence of IL-4 has historically been thought to be

required for the differentiation of Th2 cells, although recent work has raised questions about the absolute requirement of this cytokine (101, 322). Although perhaps not absolutely required, IL-4 from innate cells such as basophils may enhance Th2 differentiation and other innate cell factors may also play a role in this process. It has also been suggested that basophils may act as antigen-presenting cells for Th2 cells and thus more directly contribute to Th2 differentiation under certain conditions (323-325), although multiple researchers have disputed this assertion and argue that $CD11c^+$ dendritic cells are actually the relevant antigen-presenting cells for promoting type 2 immune responses (105, 326, 327). Early reports suggested that Ih2 cells may express MHC class II molecules (90), although this result has not been confirmed and, to our knowledge, a role for Ih2 cells in antigen presentation has not been explored. As mentioned previously, mice unable to signal through IL-25 and IL-33 receptors have reduced Ih2 cell expansion and proliferation, but they also have delayed $CD4^+$ T cell derived IL-13 secretion as assessed by *in vitro* antigen restimulation (95). Interestingly, transfer of wild-type or IL-$13^{-/-}$ Ih2 cells was sufficient to rescue IL-13 production by $CD4^+$ T cells in these mice, suggesting that Ih2 cells can mediate $CD4^+$ T cell activation and that this interaction does not require IL-13.

$CD4^+$ T cells also appear to play an important role in the accumulation and activation of innate immune cells. Although baseline numbers of eosinophils, basophils, and Ih2 cells appears to be normal in $Rag^{-/-}$ mice which lack T cells, there are significantly reduced numbers of these cells in effector organs such as the lung and MLN during infection with *N. brasiliensis* (95, 141), suggesting that $CD4^+$ T cells may be required for the recruitment, proliferation, and/or retention of these cells. Indeed, transfer

of wild-type or IL-4/13$^{-/-}$ CD4^{+} T cells was sufficient to rescue lung eosinophilia and basophilia during *N. brasiliensis* infection (141), suggesting further that CD4^{+} T cells are required for innate cell accumulation and that they mediate this accumulation independently of type 2 cytokine production. A recent study by our group demonstrated that CD4^{+} T cells were required for basophil production of IL-4 during infection, and further showed that this activation also did not require CD4^{+} T cell-intrinsic production of IL-4 or IL-13 (105). This study also used two-photon microscopy to demonstrate that basophils have prolonged contacts with CD4^{+} T cells in the lung during *N. brasiliensis* infection (105). Further study is needed to determine the factors produced by both CD4^{+} T cells and type 2-associated innate immune cells that mediate the interactions between these cell types.

It is interesting that each of the type 2-associated cytokines can be produced by either adaptive Th2 cells or one or more innate immune cell population. This division of labor likely evolved not only to enhance the efficacy of type 2 immune responses, but also to establish a system with inherent redundancy. This redundancy is supported by experiments showing that deletion of IL-4/IL-13 from either T cells or basophils had a minimal effect on worm clearance, while deletion of these cytokines from both cell types had a much more dramatic effect (105). This redundancy between adaptive and innate immunity might explain why deficiencies in basophils, eosinophils, mast cells or NKT cells do not dramatically impair anti-helminth immune responses (83, 105, 141, 328). It remains unclear whether Ih2 cells are similarly dispensable during type 2 immune responses. Multiple groups, including ours, have attempted to determine the function of Ih2 cells by adoptively transferring these cells into Ih2 cell-deficient mice to determine

their role during helminth infection or the development of allergic inflammation. However most of these investigators used either common-$\gamma^{-/-}$Rag$^{-/-}$ mice or Rag$^{-\backslash-}$ mice further depleted of Thy1^{+} cells as recipients for the Ih2 cell transfers, which does not allow for a dissection of the differential contributions or interactions between Ih2 cells and CD4^{+} T cells. A notable exception is a series of experiments done by Andrew McKenzie (95), which demonstrated a crucial role for Ih2 cells in worm clearance during *N. brasiliensis* by adoptively transferring wild-type Ih2 cells into IL-4/13$^{-/-}$ mice or mice deficient in signaling through IL-25 and/or IL-33. While these experiments highlight the fact that Ih2 cells can mediate worm clearance when they are the only cells able to produce IL-13 or respond to IL-25 and/or IL-33 during infection, they do not establish whether similarly capable CD4^{+} T cells or other innate cells could compensate for Ih2 cells under these circumstances. It would be very interesting to determine whether transfer of wild-type CD4^{+} T cells or other innate cells could similarly rescue worm clearance in IL-13$^{-/-}$ or IL-25/IL-33 receptor deficient mice. The redundancies built into the type 2 immune response pathways make it difficult to definitively determine the contributions of each of these cell types during the course of an immune response *in vivo*. The generation of mice deficient in Ih2 cells and/or specifically deficient in Ih2 cell production of type 2 cytokines, perhaps using a similar Cre-mediated strategy as was used to create basophil deficient and basophil-specific IL-4/13$^{-/-}$mice (105), would greatly enhance the ability to determine the function of these enigmatic cells in an intact mouse.

Ih2 cells in humans

Cells resembling Ih2 cells appear to exist in humans, but the role of these cells in parasitic immunity and in the development of allergy and asthma in humans remains to

be fully elucidated. A subset of $CD34^+$ progenitor cells in human umbilical cord blood was found to respond to IL-33 by producing IL-13 (329). Moro et al. (94) identified c-kit^+ IL-$7R^+$ cells in human mesenteric tissues. A similar population of human lineage-negative IL-$7R^+$ cells was identified by a different group (330) and further characterized by the expression of CD161 and CRTH2. These cells were found in the fetal and adult lung and gut and produced IL-13 after stimulation with IL-2 plus IL-25 or IL-33 *in vitro*. Lineage-negative IL-$7R\alpha^+CD25^+T1/ST2^+$ Ih2-like cells were also found in the human lung parenchyma and BAL (321). The presence of Ih2-like cells has been associated with allergic disease in humans, as these cells have been found in heightened levels in sputum of asthmatic patients (329) and in nasal polyps of patients with rhinosinusitis (330). Further support for a role for Ih2 cells in the development of human allergic inflammation is provided by studies showing that IL-33 is highly expressed in the lungs of patients with asthma (331), and that polymorphisms in the IL-33 gene were associated with asthma susceptibility (332, 333). Modulation of IL-25 or IL-33 or other aspects of Ih2 cell function might prove to be a beneficial strategy for the treatment of human asthma or allergy.

Part B: Cytokine production during IL-17-associated immune responses

Summary

Our studies focused on IL-17A-associated immunity, outlined in Chapter III, provide insight into cellular sources of IL-17A at rest, during a model of acute bacterial pneumonia, and during the development and propagation of central nervous system (CNS) autoimmunity. We generated IL-17A reporter mice, termed Smart-17A mice, which mark all cells that are currently or have recently produced IL-17A. Interestingly, using these reporter mice, we found no evidence for constitutive production of IL-17A by $CD3\varepsilon^+$ or $CD3\varepsilon^-$ immune cell populations in a resting mouse. However, during pulmonary infection with *Klebsiella pneumoniae*, we did note a substantial percentage of IL-17A-expressing γδ T cells, and smaller percentages of IL-17A-expressing invariant (i)NKT cells, other $CD3\varepsilon^+$ cells and $CD4^+$ T cells. Similarly, during a mouse model of experimental autoimmune encephalomyelitis (EAE) we noted substantial populations of IL-17A-expressing γδ T cells, iNKT cells and other $CD3\varepsilon^+$ cells and smaller percentages of $CD4^+$ cells in the draining lymph nodes and CNS. We demonstrated that substantial percentages of γδ T cells and iNKT cells are constitutively $ROR\gamma t^+$ in the resting lung, and further show that resident γδ T cells and iNKT cells are able to rapidly respond to IL-1β and IL-23 by producing IL-17A. Finally, we crossed our Smart-17A reporter mice to IFN-γ reporter mice and demonstrated that IFN-γ producing Th1 cells vastly outnumber Th2 cells in our two models. We also observed that IFN-γ-producing and IL-17A-producing innate-like T cells segregated into two distinct populations.

Redefining "Th17" cells

One of the most surprising series of findings from our studies was that $CD4^+$ T cells did not appear to express IL-17A at rest and that very small percentages (consistently less than 2%) of $CD4^+$ T cells expressed IL-17A during *K. pneumoniae* infection and after induction of EAE. These data appear to contradict results from the Littman lab (220, 240) and many other groups, which suggest that IL-17A is produced constitutively by $CD4^+$ T cells in naïve mice, especially in the lamina propria of the small intestine and colon. Additionally, an overwhelming majority of the literature has focused on IL-17A production from Th17 cells, and IL-17A-producing $CD4^+$ T cells have been identified and characterized in a wide range of infectious and autoimmune models. However, one major difference between our study and a vast majority of these earlier reports is that we used a reporter mouse and they used *ex vivo* restimulation and subsequent intracellular cytokine staining. Our ability to readily detect the hNGFR reporter on $CD4^+$ T cells *in vitro* and on innate like T cells *in vivo* suggest that our reporter is able to accurately report IL-17A expression. Furthermore, when we restimulated $CD4^+$ T cells isolated from the lymph nodes of mice with EAE, we were able to induce hNGFR expression to levels that were almost identical to levels of intracellular IL-17A found after a similar restimulation protocol. This suggests that *ex vivo* restimulation of cells may lead to production of IL-17A from cells that were not actively making the cytokine *in vivo*. However, we are unable to rule out the possibility that our reporter mouse are unable to detect very low levels of cytokine expression or that $CD4^+$ T cells make quantitatively less IL-17A than innate-like T cells *in vivo*, which may limit our ability to detect hNGFR on the surface of $CD4^+$ T cells.

Recent reports describe the generation and characterization of two additional IL-17A reporter mice, which both confirm and also appear to contradict our results. In a recent report by Esplugues et al. (214) utilizing a different reporter mouse engineered to report IL-17A expression with coexpression of GFP, the authors described constitutive expression of GFP in the intestine (2-6%), although, similar to our results, they observed minimal GFP expression at rest in other organs including the spleen, lymph node, and lung. The differences between our results and those of Esplugues et al. in resting mice could potentially be explained by differences in reporter genes and targeting constructs used. Esplugues et al. go on to demonstrate significant induction of the GFP reporter in the intestine during mouse models of CD3 antibody-induced tolerance, sepsis, and infection with H1N1 influenza. Importantly, administration of CD3 antibody leads to massive T cell activation and thus represents a nonphysiologic stimulus that is similar to some methods of *ex vivo* restimulation whereby cytokine production may be exaggerated. In the author's sepsis model, sepsis was induced by either intravenous infection with *Staphylococcus aureus* or intravenous injection with the superantigen *S. aureus* enterotoxin B (SEB). In either case, the authors observed significant GFP expression among Vβ8 $CD4^+$ T cells, which represent a small fraction of total intestinal T cells, while the increases among the total $CD4^+$ T cell pool were minimal. Similarly, the increase in GFP expression in the intestine after H1N1 influenza infection was also observed among Vβ8 T cells. Since we did not use the same models as Esplugues et al., it is difficult to draw direct comparisons. It is possible that a significant percentage of Vβ8 $CD4^+$ T cells do express IL-17A during intestinal infection or inflammation. However, even if this were true, the total percentage of IL-17A-producing Th17 cells in

the intestine would be relatively low, much less than has been reporter during some models of intestinal inflammation, such as *Citrobacter rodentium* (221). To confirm this, it would be interesting to infect the Smart-17A mice with an intestinal pathogen such as *S. aureus* or *Citrobacter rodentium*, and to assess reporter expression among total intestinal $CD4^+$ T cells and also among the $V\beta8$ $CD4^+$ T cell subset particularly.

The second IL-17A reporter mouse was designed by Hirota et al. (213) to report IL-17A expression with concordant expression of Cre recombinase. The authors then crossed these IL-17A-Cre mice to Rosa-YFP mice to allow for fate tracking. So, unlike our Smart-17A reporter mice, which mark cells that are actively or have recently expressed IL-17A, these reporter mice lead to the permanent marking of all cells that have ever expressed IL-17A. Using these mice, the authors observed YFP^+ $CD4^+$ T cells, $\gamma\delta$ T cells, NKT cells and innate lymphoid cells in a naïve mouse. This suggests that these cells expressed IL-17A at some point after their generation, but does not necessarily mean that they were actively producing the cytokine at the time of the assay. The authors also used models of EAE and subcutaneous *Candida albicans* infection. In both of these models, the authors find evidence that IL-17A production actually decreases during inflammation, such that a large number of the YFP^+ IL-17A-fate marked cells were no longer actively producing IL-17A as assessed by restimulation. The authors further show that many of the YFP^+ IL-17A-fate marked cells actually produce IFN-γ in the spinal cord of mice during the peak of EAE disease, providing support for the notion of plasticity between Th1 and Th17 lineages that had been suggested previously (216, 334).

Our results suggest that IL-17A is not expressed or is expressed at very low levels by $CD4^+$ T cells at rest. Furthermore, we show that IL-17A is expressed by a small

percentage of $CD4^+$ T cells during *K. pneumoniae* and EAE, levels much lower than reported in the same or similar models in the literature using *ex vivo* restimulation. The results from the fate-mapping study of Hirota et al. (213) suggest that a significant percentage of $CD4^+$ T cells have expressed IL-17 at some point during their development or proliferation, evidenced by the YFP mark, but that many of them are no longer actively making IL-17A at effector sites during EAE or *C. albicans* infection. Taken together, these findings suggest that perhaps Th17 cells activate the IL-17A locus at some point during their differentiation but that IL-17A production is not a major downstream effector mechanism of these cells. Our study and previous work using dual 4get/KN2 IL-4 reporter mice (102) show that it is possible for some cells, such as eosinophils and Ih2 cells, to activate the IL-4 locus and linked reporter gene, even though the cells themselves don't actively make IL-4 during inflammatory responses. Furthermore IL-4 and IFN-γ mRNA is detectable in resting NKT cells, although cytokine protein is not produced until these cells encounter their cognate antigen (88). Along these lines, it is possible that the Th17 cells that are detected by others using *ex vivo* restimulation actually represent cells that have activated the IL-17A locus and/or contain IL-17A transcripts but are not secreting IL-17A protein until artificially induced to do so by *ex vivo* restimulation.

In our view, Th17 cells do represent a unique lineage of cells that have undergone a distinct developmental event leading to activation of the IL-17A locus. These cells are readily detectable after *ex vivo* restimulation by their induced production of IL-17A, although identifying them in this fashion is misleading as these cells likely produce only low levels of IL-17A during inflammation. Instead, Th17 cells may represent an inherently plastic lineage, which is able to produce other cytokines

depending on the local inflammatory milieu (334). During infection, production of IFN-γ or IL-22 by Th17 cells might be more significant than production of IL-17A. Indeed, we saw substantially more $CD4^+$ T cells making IFN-γ than IL-17A during *K. pneumoniae* infection using our dual reporter system, and IFN-γ has been shown to be crucial for the resolution of multiple bacterial and fungal infections. IL-$22^{-/-}$ knockout mice are more susceptible than IL-$17A^{-/-}$ mice to *K. pneumoniae* and *C. rodentium* (238, 245), suggesting that IL-22 may play a more important role during mucosal bacterial infection than IL-17. IL-22 also has multiple important functions at epithelial barriers, including inducing production of antimicrobial peptides such as β-defensins, Reg3γ, S100A7, S100A8, and S100A9, and promoting epithelial cell proliferation, survival and repair (335). Specific cytokines and transcription factors may promote IL-22 production by Th17 cells. For example, IL-22 production is enhanced in conditions with limiting levels of TGF-β, while IL-17A levels are inhibited under these conditions(336). Additionally, IL-22 producing $CD4^+$ T cells, which some term Th22 cells, have been shown to express the aryl hydrocarbon receptor but significantly lower levels of RORγt than Th17 cells (337).

During the development of autoimmunity, production of IFN-γ as well as other inflammatory cytokines such as GM-CSF might be more important than production of IL-17A. In our studies, we observed vastly more IFN-γ-producing $CD4^+$ T cells than IL-17A-producing $CD4^+$ T cells during EAE disease. Similarly, the IL-17A-Cre fate-mapping experiments showed that many IL-17A-fate marked cells secrete IFN-γ in the spinal cords of mice during the peak of EAE (213). Limiting concentrations of TGF-β and the presence of IL-23 or IL-12 has been shown to promote production of IFN-γ by

Th17 cells, and this conversion involves the Th1-associated transcription factors STAT-4 and T-bet (213, 216). Two recent reports argued that GM-CSF might actually be the pivotal cytokine in promoting pathogenesis in the EAE model (309, 310). Both authors showed that blocking GM-CSF with a neutralizing antibody during disease onset prevents the development of the clinical features of EAE disease. Codarri et al. (310) further demonstrated that transfer of GM-CSF$^{-/-}$ T cells primed by MOG/CFA for 7 days and restimulated with MOG and IL-23 for 2 days failed to induce EAE in recipient wild-type mice, while transfer of similarly stimulated T cells from IL-17A$^{-/-}$ or IFN-$\gamma^{-/-}$ mice only led to mild reductions in disease onset and severity. These data suggest that GM-CSF may be more important than IL-17A or IFN-γ in promoting CNS inflammation, possibly by its role in enhancing the maturation and activation of monocytes and dendritic cells. Although the mechanisms leading to production of GM-CSF by Th17 cells remain incompletely defined, these authors suggest that it might involve the cytokines IL-23 and/or IL-1β and the transcription factor RORγt. (310).

Tight regulation of IL-17A among adaptive immune cells makes sense physiologically, since this cytokine is such a potent activator of inflammation and dysregulation of IL-17A production is associated with the development of multiple autoimmune diseases. During most infections, the protective effect of IL-17A is thought to occur early and locally, by the recruitment of neutrophils and other innate immune cells to the site of inflammation. Antigen-specific Th17 cells would presumably take days to generate and thus arrive too late to participate in these early events, although they may have a role in more prolonged infections. Memory Th17 cells, however, could conceivably already be present in peripheral tissues and rapidly respond to the early

immune response to a secondary infection by production of IL-17A and other associated cytokines. Although one of the early descriptions of Th17 cells described IL-23-induced production of IL-17A from memory T cells in mice (175), much of the work aimed at characterizing memory Th17 cells has been done using T cells isolated from human peripheral blood. Human memory T cells that produced IL-17A after *in vitro* restimulation were shown to have a $CCR6^+$ $CCR4^+$ phenotype, and a subset of these putative memory Th17 cells were shown to respond to *in vitro* restimulation with *Candida albicans* but not *Mycobacterium tuberculosis* (338). Another group defined human Th17 cells as expressing CCR6, IL-23R, RORγt, and T-bet and isolated subsets from Crohn's disease patients that produced IL-17A or co-produced both IL-17A and IFN-γ upon restimulation (180). Further study is necessary to elucidate the mechanisms involved in the generation and maintenance Th17 memory. The IL-17A-Cre fate-mapping mice (213) would allow for more detailed tracking and characterization of Th17 memory cells, since they retain a permanent mark after activation of the Th17 locus. It would be very interesting to determine if fate-marked cells produce IL-17A upon encountering their cognate antigen during a secondary infection, or if, similar to newly-generated Th17 cells, they appear to function predominately by secretion of other effector cytokines.

Innate-like T cells as sentinels in mucosal tissues

Our studies highlight the fact that innate cells and innate-like T cells might actually be the predominant sources of IL-17A during inflammation. Although this idea has gained more traction among the immunology community in recent years (191), an

overwhelming majority of studies into IL-17A-mediated immune responses have focused on Th17 cells as the main source of IL-17A and associated cytokines. Our results mark the first time that researchers have used an IL-17A (or IL-17F) reporter mouse to fully characterize IL-17A production by innate cells or innate-like T cells during inflammation, and serve to corroborate recent findings using intracellular cytokine staining to demonstrate that innate-like T cells are major sources of IL-17A in multiple models (191). Although we were unable to detect IL-17A expression by innate lymphoid cells in our models, these cells have been described to reside primarily in the small intestine and colon. We did not detect IL-17A expression in these tissues at rest, but it would be interesting to use an intestinal infection model (such as *Citrobacter rodentium* or *Staphlococcus aureus*) to determine whether gut-resident innate lymphoid cells can produce IL-17A during intestinal inflammation. It appears that innate cells and innate-like T cells, by producing IL-17A in response to either conserved pathogen motifs or stress or damage-induced self ligands, might play a pivotal role in the induction of early proinflammatory responses.

Multiple groups have observed that innate-like T cells and innate lymphoid cells appear to reside preferentially in mucosal tissues, such as the intestine, skin and lung. A previous study (299) observed that approximately half of all RORγt$^+$ cells in the skin and lung of a resting mouse were $\gamma\delta$ T cells, although $\gamma\delta$ T cells make up a much smaller fraction of total lymphocytes at these sites. These authors further observed that a majority of constitutive RORγt$^+$ $\gamma\delta$ T cells (50-90%) produced IL-17A upon restimulation. Subsequent studies have identified similar populations of RORγt$^+$ dermal $\gamma\delta$ T cells that rapidly produce IL-17A after stimulation with IL-23 as assessed by intracellular cytokine

staining (300-302). We confirmed the presence of RORγt$^+$ γδ T cells in the lung and skin and also found populations of RORγt$^+$ γδ T cells in the spleen and small intestine, RORγt$^+$ iNKT cells in the lung, and RORγt$^+$ other CD3ε$^+$ cells in the lung and skin. After infection with *K. pneumoniae* or *in vivo* administration of IL-1β and/or IL-23, we observed that lung-resident innate-like T cells were able to rapidly produce IL-17A, as assessed by our reporter mouse. Although technical complications precluded us from directly demonstrating that the resident RORγt$^+$ population was the source of IL-17A upon infection or cytokine administration, it seems very likely that these resident RORγt$^+$ cells are the relevant cytokine-secreting populations in our models.

The circumstances leading to the accumulation of RORγt$^+$ cells poised for IL-17A production in mucosal tissues remain to be fully elucidated. These cells have been shown by others and verified by us (data not shown) to express the chemokine receptor CCR6 (191). This chemokine receptor binds to its ligand CCL20, which is preferentially expressed by multiple cell types in mucosal tissues (339). Thus, acquisition of CCR6 expression by these innate-like T cells presumably occurs before these cells enter peripheral sites. It would be interesting to determine if these constitutive RORγt$^+$ cells express any additional homing receptors, perhaps those that are more specific for different mucosal sites such as the skin and lung. It would also be informative to determine whether these cells are scattered throughout mucosal tissues at rest, or if they cluster in particular areas or interact with particular accessory cell types. These experiments could be done using immunohistochemical detection of the RORγt reporter, accompanied by exclusion of CD4$^+$ T cells. The induction of RORγt expression could occur during the development of these populations or, alternatively, could be promoted

by factors in the local microenvironment. Accumulating evidence suggests that the former mechanism might be more likely (see below). Perhaps simultaneously, these cells might acquire surface expression of IL-1β and IL-23 receptors, in order to be able to rapidly respond to these two proinflammatory cytokines. Further phenotypic characterization of IL-17A-poised and IL-17A-secreting innate like T cells using both RORγt and IL-17A reporter mice should provide further insights into the functions of these cells in mucosal immunosurveillance.

Segregation of IL-17A and IFN-γ production among innate-like T cells

One of the most striking observations from our studies was the strict segregation of IL-17A and IFN-γ-producing subsets among innate-like T cell populations. There are multiple explanations for this phenomenon, of which one or more might be occurring simultaneously: 1) The ability to exclusively produce either IL-17A or IFN-γ might be programmed during differentiation in the thymus; 2) IL-17A-producing or IFN-γ producing innate-like T cells might be recognizing unique subsets of activating ligands; 3) IL-17A or IFN-γ production might be encouraged by unique combinations of inflammatory cytokines or other mediators, which are specifically induced during infection or inflammation; and/or 4) IL-17A-producing and IFN-γ-producing innate-like T cells might reside in separate anatomical locations which predisposes them to production of their respective cytokines. We will explore each of these possibilities in detail, using both γδ T cells and iNKT cells as examples of innate-like T cells that display this marked cytokine segregation.

Thymic programming In our models, γδ T cells appear to a major source of IL-17A. During *K. pneumoniae* infection or induction of EAE, we consistently observed that 15-50% of γδ T cells expressed IL-17A, as assessed using our reporter. Interestingly, we observed similar percentages of γδ T cells that separately expressed IFN-γ under these same inflammatory conditions. Previous work has demonstrated that IL-17A and IFN-γ-producing γδ T cells express unique phenotypic markers, the expression of which could potentially be induced by programming events in the thymus. IL-17A-producing γδ T cells are $NK1.1^-$ and characterized by expression of IL-1R, IL-23R, CCR6, SCART2, and the transcription factor RORγt while IFN-γ-producing γδ T cells are $NK1.1^+$ and express IL-18R, IL-12R, CD122, and the transcription factor T-bet (191).

Recent evidence suggests that IFN-γ-producing γδ T cells may be programmed to adopt this IFN-γ-producing fate during differentiation and maturation in the thymus, while generation of IL-17A-producing γδ T cells might be a default pathway. Turchinovich et al. (340) demonstrated that Vγ5vδ1 γδ T cells interact with Skint-1 in the thymic epithelium, which induces these cells to adopt an IFN-γ-producing phenotype and also suppresses RORγt and IL-17A-producing capability. The authors hypothesize that Vγ6Vδ1 γδ T cells cannot respond to Skint-1 and therefore follow a default pathway towards RORγt expression and competence to produce IL-17A. In an earlier paper, this same group suggested that CD27 may operate in a similar fashion to promote IFN-γ production by γδ T cells (341). CD27 expressed by thymic γδ T cells engages its ligand CD70 in the thymus which leads to expression of T-bet and the ability to produce IFN-γ. Conversely IL-17A-producing γδ T cells do not express CD27 and therefore do not upregulate T-bet expression. An earlier report by Jensen et al. (188) further supports this

notion of default and active pathways of γδ T cell differentiation by showing that IFN-γ–producing T10/T22 γδ T cells encounter their antigen in the thymus while IL-17A-producing T10/T22 γδ T cells are antigen-inexperienced. Other IFN-γ and IL-17A producing subsets could be programmed in a similar fashion by Skint-1 or other thymic selecting determinants or the absence of such encounters.

A measurable percentage of iNKT cells also expressed IL-17A in both of our models. We found the greatest percentage of these cells from the lymph nodes (approximately 30%) and CNS (approximately 15%) of mice during EAE, and smaller percentages (approximately 4%) in the lungs of mice after *K. pneumoniae* infection. We also observed percentages of IFN-γ-producing iNKT cells that were consistently greater than the percentages of IL-17A-producing iNKT cells. Although we did not assess this with our studies, there is evidence that a subset of iNKT cells can produce IL-4 after stimulation *in vitro* and *in vivo* (87, 88). Although it appears that IL-4 and IFN-γ mRNA can be coexpressed by iNKT cells at rest and after stimulation (88), it is not clear whether IL-4 protein can be co-produced by cells that are also producing IFN-γ or IL-17A, or if production of this cytokine during inflammatory responses is also confined to a separate and distinct population. iNKT cells producing each cytokine appear to have unique phenotypic features, which could potentially be programmed into these subsets during thymic differentiation. Specifically, IL-17A-producing iNKT cells appear to be $NK1.1^-$ and $CD4^-$, and also express CCR6, CD103, IL-1R, IL-23R, and the transcription factor RORγt. IFN-γ producing iNKT cells are $NK1.1^+$, and additionally express CD122 and the transcription factor T-bet. IL-4-producing iNKT cells appear to be predominately $NK1.1^-$ and $CD4^-$ (342).

Similar to what has been hypothesized for γδ T cells, there is evidence that the cytokine producing ability of iNKT cells may be programmed in the thymus, with the generation of IL-17A and IL-4-producing iNKT cells occurring by a default pathway and IFN-γ-producing iNKT cells representing a more mature subset. Multiple groups have suggested that NK1.1$^-$ iNKT cells represent a more immature state that can ultimately mature into NK1.1$^+$ cells in the thymus or in the periphery (343). This is supported by experiments demonstrating that intrathymic injection of NK1.1$^-$ iNKT cells can lead to the generation of NK1.1$^+$ iNKT cells, but similar transfer of NK1.1$^+$ cells does not lead to recovery of NK1.1$^-$ cells (344). Michel et al (194) identified RORγt$^+$ iNKT cells in the thymus and further showed that these cells appear to give rise to all RORγt$^+$ IL-17A-competent cells in the periphery. In contrast, RORγt$^-$ iNKT cells do not differentiate into RORγt$^+$ cells or acquire ability to produce IL-17A, suggesting that this is also a separate and possibly more mature lineage. Much more work needs to be done to fully elucidate the mechanisms involved in the differentiation of IL-17A and IFN-γ producing subsets within both γδ T cell and iNKT cell lineages.

Recognition γδ T cells display limited TCR diversity, and thus it is possible that IL-17A-producing and IFN-γ-producing γδ T cells utilize unique combinations of Vγ and Vδ receptors. To date, multiple populations of γδ T cells have been suggested to be capable of making IL-17A, including Vγ1, Vγ4, Vγ6Vδ1, and skin Vγ5Vδ1 γδ T cell subsets (186). Multiple subsets of γδ T cells have also been shown to produce IFN-γ upon restimulation, including Vγ1, Vγ2, and Vγ4 populations. In our pilot experiments, some Vγ and Vδ γδ T cell subsets contained both IL-17A and IFN-γ-producing γδ T cells during *K. pneumoniae* infection and at the peak of EAE disease (data not shown), suggesting

that Vγ and Vδ usage might not be the sole determinant of differential cytokine production. These experiments need to be repeated and verified, however, which is made difficult by the availability of antibodies to certain Vγ and Vδ TCRs. These challenges could potentially be overcome by sorting IL-17A and IFN-γ reporter positive γδ T cells and assessing for Vγ and Vδ expression using quantitative PCR. Identifying the TCR usage of cytokine-secreting γδ T cells would be an important step in understanding their function.

However, even more relevant would be the identification of the specific antigens that are being recognized by IL-17A-producing and IFN-γ-producing γδ T cells. The full range of antigens that can be recognized by γδ T cells and the mechanisms of this recognition have not been fully elucidated, although multiple putative microbial and self ligands have been suggested. Microbial-derived molecules that can activate human γδ T cells include monoalkyl phosphates (including those expressed by *Mycobacterium tuberculosis*) (345) and a subset of alkylamines (including those expressed by *Salmonella typhimurium*, *Listeria monocytogenes,* and malaria parasites) (346). Murine γδ T cells have been shown to react to heat shock proteins present in *M. tuberculosis* and *Escherichia coli* (347). It is unclear how the recognition of these and other ligands contribute to γδ T cell activation *in vivo*. It would be interesting to determine which microbial-associated molecules are capable of inducing IL-17A production from γδ T cells, and to ascertain whether these molecules were expressed preferentially by pathogens associated with IL-17A-mediated immune responses such as extracellular bacteria and fungi. Along these lines, perhaps γδ T cell recognition of molecules associated with Th1-associated pathogens leads to the preferential production of IFN-γ.

Self ligands recognized by γδ T cells include the classical MHC I and II molecules as well as the non-classical MHC I-related molecules T10/T22 in mice, MHC I-like molecules MICA/B in humans, CD1c in humans, and CD1d in both mice and humans (348). Although it is an attractive hypothesis, it is unclear at present whether IL-17A production by γδ T cells is induced by stress or damage-induced self-ligands. If so, it is difficult to imagine how IL-17A production would be induced by some infections and not others. Perhaps the route of infection or particular characteristics of certain infections contributes to unique patterns of damage that contribute to IL-17A production.

Similar to the case with γδ T cells, the antigens that are recognized by NKT cells are not well characterized. Invariant NKT cells have limited TCR diversity, including an invariant α chain with a Vα14-Jα18 rearrangement paired with a limited set of β chains (including Vβ8, Vβ7 or Vβ2 in mice) (195). The first foreign antigen shown to activate NKT cells was α-galactosyl ceramide (αGalCer) from a marine sponge. Subsequently, iNKT cells have been shown to respond to other lipids including glycosphingolipids from *Sphingomonas* bacteria (85), and diacylglycerol antigens from *Borrelia burgdorferi* (343). Putative ligands from *K. pneumoniae* or most other IL-17A-inducing pathogens have not been identified. Although such ligands may eventually be discovered and characterized, it is also possible that iNKT cell activation during these infections is prompted by recognition of self ligands. Recognition of self ligands is also the most likely mechanism leading to iNKT cells during the development of EAE or other autoimmune diseases, as there is no foreign pathogen in these instances. iNKT cells have been show to recognize multiple self beta-linked lipids including β-galactosylceramide, β-glucosylceramide, isoglobotrihexosylceramide, disialoganglioside GD3, and β-D-

glucopyranosylceramide (349, 350). Intriguingly, it has been proposed that TLR signaling and other events during infection can lead to altered glycosphingolipid synthesis, which results in the loading of more stimulatory lipids onto CD1d and enhanced iNKT activation (349). The exact identity and nature of these infection-induced stimulatory lipids remains to be fully elucidated, but could potentially provide insights into the mechanisms of iNKT activation during infection, autoimmunity or cancer and also provide a means to therapeutically manipulate iNKT cell activation.

Although, by definition, all iNKT cells can bind the αGalCer-loaded tetramer and therefore have structurally similar TCRs, it is possible that slight variations in Vβ usage or in features of the CDR3β region can alter the affinity of different iNKT cell TCRs for different microbial or self-derived ligands. There is also some evidence that the quality of the TCR signal might influence the cytokines produced by activated iNKT cells. Stimulation of splenocytes with a modified αGalCer analog with a shortened sphingosine led to a higher ratio of IL-4 to IFN-γ secretion compared to αGalCer (351) through a mechanism that possibly involves poor induction of the c-Rel transcription factor (352). In contrast, a C-glycoside modified analog of αGalCer preferentially induced IFN-γ production (353). It is possible that the microbial and self-derived lipids described above may display different affinities for the iNKT TCR, which could lead to preferential induction of particular cytokines.

Inflammatory mediators A third possible factor that may contribute to differential cytokine production by γδ T cell and iNKT cells is the presence of certain inflammatory mediators that preferentially induce the production of either IL-17A and IFN-γ. As mentioned before, the proinflammatory cytokines IL-1β and IL-23 can induce

IL-17A production from γδ T cells in a TCR-independent fashion (232) and by iNKT cells through a process that is thought to be TCR/CD1d-dependent (197). Similarly, signaling through IL-12 and IL-18 receptors has been shown to promote IFN-γ production from γδ T cells (354) and iNKT cells (355, 356). These inflammatory mediators are differentially induced during infections that promote IL-17A-associated or Th1 responses, respectively, and likely play an important role in influencing downstream production of cytokines by associated innate-like T cells. It is possible that these cytokines or other specific inflammatory factors might induce production of either IL-17A or IFN-γ while simultaneously inhibiting expression of the receptors for the alternate array of inflammatory factors, thus prohibiting simultaneous production of the alternate cytokine. Alternatively, and probably more likely, the expression of unique subsets of IL-17A-promoting or IFN-γ promoting receptors might be programmed during differentiation of these lineages in the thymus and subsequent signaling through these receptors would promote and reinforce the production of either IL-17A or IFN-γ by these cells during inflammatory responses.

Localization It is possible that differential localization of γδ T cells or iNKT cells poised or programmed for production of IL-17A or IFN-γ could also affect cytokine production. The localization of RORγt$^+$ IL-17A-poised γδ T cells and iNKT cells to mucosal tissues might predispose them to respond to epithelial damage by promoting local inflammation. In contrast, T-bet$^+$ IFN-γ-poised γδ T cells and NKT cells might have a slightly different localization, such as a preference for lymphoid tissues that allow them to receive different inflammatory signals and to preferentially participate in systemic immune responses. Immunohistochemistry using IL-17A and IFN-γ reporter mice during

infection or autoimmunity would be very helpful in determining whether these cells are differentially located, and whether distinct patterns of localization contribute to the activation or functions of these cells. More generally, it would also be informative to use these reporter mice, and possibly also IL-4 reporter mice, to separate cytokine-poised or cytokine-producing populations and determine which unique receptors, transcription factors, and other genes they express in order to gain insight into their specialized functions in immunity.

IL-17A-producing "other CD3e$^+$ cells"

One of the biggest questions raised by our studies into IL-17A-mediated immune responses is the identity of the IL-17A-producing "other CD3ε$^+$" cells. These cells are CD3ε$^+$, but negative for CD4, CD8, γδ TCR and the CD1d-loaded tetramer. One possibility is that these cells are CD4$^+$ T cells, CD8$^+$ T cells or γδ T cells that have downregulated their surface markers upon activation. Indeed, it has been reported that γδ T cells rapidly downregulate their TCR after incubation with a TCR-specific antibody (357). To test for this, we could sort these cells and attempt to detect expression of the αβ or γδ TCR, CD4 or CD8 by intracellular staining or by quantitative PCR. Alternatively, we could use previously described Tcrd-H2BeGFP reporter mouse which effectively mark γδ T cells and γδ T cell progenitors with GFP, even in the absence of detectable surface γδ TCR (358). Another separate possibility is that these other CD3ε$^+$ cells are Type II NKT cells, which rely on CD1d for antigen presentation like iNKT cells, but have diverse TCR α and β chains (343, 359). These type II NKT cells cells do not bind the CD1d-loaded tetramer, which makes their identification difficult. Many researchers compare CD1d$^{-/-}$ mice that lack all NKT cells to Jα14$^{-/-}$ mice, which presumably lack only

iNKT cells, to ascertain functions of type II NKT cells. Identification of type II NKT cells using this method was too time consuming to be feasible for our studies, but could potentially help to determine if the other CD3ε$^+$ cells we identified were CD1d-dependent. Alternatively, we could sort the IL-17A-producing other CD3ε$^+$ cells and use quantitative PCR to determine whether they express TCR α and β chains, and if so, we could determine the receptor usage. An additional possibility is that these IL-17A-producing other CD3ε$^+$ cells represent a new population of CD3ε$^+$ cells that would need to be further characterized.

Therapeutic applications of IL-17A

Deficiencies in IL-17A or other components of the pathways involved in IL-17A-associated immune responses are associated with increased susceptibility to infections in both humans and mice. Conversely, some of these same deficiencies are associated with protection from the development of autoimmune diseases. Thus, therapeutic enhancement or blocking of this pathway is an attractive approach for the treatment of both infectious and autoimmune diseases. Manipulation of this pathway should be undertaken with care, however, as overabundance of IL-17A can result in excessive inflammation and tissue damage, while deficiency in IL-17A could impair the immune system's ability to control and eliminate subsequent infections. In fact, due to these considerations and the increasing evidence from our work and other studies suggesting that Th17 cells might actually switch to predominate production of other cytokines such as IFN-γ, IL-22 or GM-CSF, IL-17A itself might not be the most effective therapeutic

target. Modulation of IL-22 during infection and IFN-γ or GM-CSF during autoimmunity might be more efficacious and have less unwanted consequences.

Part C: Cytokines in immunity

New insights into cellular sources of inflammatory cytokines

While $CD4^+$ T cells indisputably play pivotal roles in Th1, Th2 and IL-17-associated immune responses, the results from our studies emphasize the fact that innate cells and innate-like T cells are also important sources of the signature cytokines associated with these responses. Further, our studies using reporter mice highlight the fact that alternate methods of accessing cellular sources of cytokines such as *ex vivo* restimulation and intracellular cytokine staining might overestimate percentages of cytokine-producing cells or even incorrectly identify entire lineages of cytokine-producing cells. Use of the 4get/KN2 mice to distinguish between cytokine mRNA and cytokine protein production has revealed that some cells, such as eosinophils, activate the IL-4 locus and produce mRNA but do not appear to actively make IL-4 protein at rest or during inflammatory responses. However, the constitutive cytokine mRNAs present in these cells can result in IL-4 protein production after restimulation, which has erroneously led some investigators to consider eosinophils as a major source of IL-4. We propose that similar discrepancies may have contributed to incorrect identification of cells secreting the type 2 associated cytokines IL-4, IL-5, IL-9, and IL-13 and the IL-17-associated cytokines IL-17A, IL-17F, and IL-22. The recent availability of cytokine reporter mice for most of these cytokines (with the exception of IL-22) should allow for more definitive identification of cellular sources of these cytokines.

Another unifying finding from our work was the discovery of cytokine-poised innate cells or innate-like T cells distributed throughout multiple organs at rest. These

cells appear to possess specific receptors that allow them to respond to unique combinations of cytokines induced during either type 2 or IL-17-associated immune responses by rapidly producing the cytokines associated with these respective responses. Specifically, epithelial cell production of IL-25 and IL-33 induced during helminth infection or allergen challenge can lead to the proliferation and activation of Ih2 cells and possibly other innate cells such as basophils, eosinophils, and mast cells. Similarly, infection or inflammation-induced production of IL-1β and IL-23 can lead to production of IL-17A by γδ T cells, iNKT cells, and other $CD3\varepsilon^+$ cells. These cytokine-producing innate cells or innate-like T cells are capable of producing cytokines before their adaptive $CD4^+$ T cell counterparts, which puts them in an ideal position to participate in the early stages of type 2 or IL-17-associated immune responses. Much further work is necessary to fully elucidate the contributions of innate cells, innate-like T cells, and adaptive $CD4^+$ T cells in type 1, type 2, and IL-17-associated immune responses, and cytokine reporter mice should prove to be valuable reagents for unraveling the complexities of these immune programs.

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