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Type 2 Cytokines in Immunity and Metabolic Regulation

by

Davina Wu

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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by

Davina Wu

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Figures 1A, 1C, 2, 3A, and 4A of Chapter 2 are based on data included in the manuscript entitled “Tissue-restricted basophil interleukin-4 secretion in primary anti-helminth immunity” (currently under revision). The authors are Brandon M. Sullivan, Davina Wu, Hong-Erh Liang, P’ng Loke, James H. McKerrow, David Voehringer, and Richard M. Locksley.

With the exception of Figures 1, 9B, 9C, and 10, Chapter 3 was submitted as a manuscript entitled “Eosinophils sustain alternatively activated macrophages in visceral adipose tissue”. The authors are: Davina Wu, Hong-Erh Liang, Ari B. Molofsky, Roberto R. Ricardo-Gonzalez, Ajay Chawla, and Richard M. Locksley.

Abstract

TYPE 2 CYTOKINES IN IMMUNITY AND METABOLIC REGULATION

Davina Wu

In metazoan organisms ranging from simple to the complex, the innate immune system is the first line of defense against damage or infection. Vertebrates have the particular challenge of dealing with parasitism and allergen exposure, to which Type 2 responses are critical at mucosal barrier sites. While there is some overlap between innate and adaptive Type 2 effector cell functions, their unique contributions are unclear. Additionally, the broader role of innate immunity in tissue regulation as it relates to the conserved mechanisms of metabolic homeostasis is unexplored.

We targeted the IL-4/13 locus in mice to generate a Th2-specific model of IL-4/13 deficiency (CD4Cre x IL-4/13 fl/fl). Consistent with prior studies, we verified that CD4 T cell-derived IL-4/13 was not required for the clearance of acute *Nippostrongylus brasiliensis* helminth infection. However, in response to chronic conditions, such as OVA-induced airway inflammation and *Schistosoma mansoni* infection, conditional knockout mice had an intermediate phenotype. The conditional CD4Cre x IL-4/13 fl/fl model suggests that while innate IL-4/13 can mediate the clearance of acute helminth infection, they may interact with Th2 cells in chronic inflammation.

As IL-4/13 have been implicated as important for adipose tissue macrophage alternative activation in the lean metabolic state, we identified eosinophils as the major IL-4-producing cells in white adipose tissue. In eosinophil-deficient mice, adipose tissue macrophage alternative activation was impaired but could be restored by reconstituting

the eosinophil lineage. Additionally, eosinophil-deficient mice, when maintained on a high fat diet, developed increased adiposity and glucose intolerance. Thus, we find that eosinophils play a role in metabolic regulation by sustaining alternatively activated macrophages that promote the lean phenotype.

Our findings confirm that Type 2 innate effector cells are important for the clearance of acute helminth infection, but may be interacting with Th2 cells during chronic infection and airway inflammation. We have also uncovered a surprising observation that eosinophils, a major feature of Type 2 immune responses, sustain macrophage alternative activation in adipose tissue. This suggests that innate cells have evolved functions not only for immunity, but for also for metabolic homeostasis as part of a highly integrated survival strategy.

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CHAPTER I:

INNATE TYPE 2 RESPONSES IN INFLAMMATION AND METABOLIC HOMEOSTASIS

Parasitism and the evolution of innate immunity

The development of the innate immune system long predates that of the adaptive immune system, and can be found at almost every level of metazoan evolution (Cooper and Herrin, 2010). It is not until about 500 million years ago, coinciding with vertebrate evolution, do we begin to see the development of the adaptive immune system consisting of lymphocytes that develop receptors that recognize specific pathogens or toxins in a targeted fashion. In contrast to adaptive immunity, innate immunity relies on the allorecognition of general patterns characteristic of pathogens or signals indicative of damage to self (Janeway and Medzhitov, 2002). Early innate immune mechanisms likely arose as a strategy for survival as metazoans increased in complexity and encountered parasitic organisms in their surrounding environment. Thus, in many organisms, we can commonly find components of innate immunity at the mucosal interface between tissues of the host and parasitic organisms. Indeed, as far back as we can trace in historical record, vertebrates, including humans, have had a longstanding co-evolutionary relationship with parasitic helminth infection. The frozen corpse of the Tyroleon (Otzi) Iceman dating back to the Neolithic age (about 3300 B.C.) indicated that he was infected with whipworm (*Trichuris trichiura*) (Dickson et al., 2000). The Ebers papyrus, a written document of Egyptian medicine dating back to around 1500 BC, contains references to parasitic infections. Additionally, *Schistosoma hematobium* eggs have been found in Egyptian mummies dating back to around 1200 B.C. (Cox, 2002)

Currently, helminth infection continues to pose a major global health problem, with devastating effects on much of the world's poorest inhabitants. According to Hotez et al. (2008), it is estimated that "one-third of the almost three billion people that live on

less than two US dollars per day in developing regions of sub-Saharan Africa, Asia, and the Americas are infected with one or more helminth." Despite such a long history with parasitic helminthes, we have only just begun to clearly define the specific mechanisms by which the coordination between the innate and adaptive immune responses is highly orchestrated. Newer insights that we have gained in recent decades have begun to suggest that the immune system serves a critical role not just in inflammation, but in other evolutionary survival strategies of tissue repair and homeostasis as well.

Models of Type 2 immune responses: helminth infection and allergic airway inflammation

The stereotyped Type 2 response characterizes common features seen in helminth infection, allergy, and asthma. Hallmarks of a Type 2 immune response include polarization of CD4 T cells to the Th2 subtype, the production of cytokines (IL-4, IL-5, IL-9, IL-13), recruitment of innate effector cells (eosinophils and basophils) to tissue, and B cell immunoglobulin class switching to the IgE and IgG1 serum isotypes. IL-4 can bind both the type I IL-4 receptor (IL-4Rα and the common γ chain) or the type II IL-4 receptor (IL-4Rα paired with IL-13Rα1); IL-13 is thought to bind only the type II IL-4R complex (LaPorte et al., 2008). Signaling through IL-4Rα is mediated by downstream intracellular JAK1 and STAT6 and is required for cytokine effector function (Takeda et al., 1996). These effectors induce changes in target organs resulting in goblet cell hyperplasia and mucus secretion, smooth muscle contraction, and often fibrosis that either eliminate or sequester the parasite, or cause symptoms in the host.

Nippostrongylus brasiliensis infection

One experimental model commonly used to study acute Type 2 immune responses *in vivo* is *Nippostrongylus brasiliensis* intestinal helminth infection. A natural parasite of rats, *N. brasiliensis* has been adapted in the laboratory for infections in mice and the migration of *N. brasiliensis* in the host closely mimics the pattern seen in human infections with Old World and New World hookworms (Gause et al., 2003). After subcutaneous inoculation, L3 *N. brasiliensis* larvae enter venules and travel via the circulatory system to the lung. In the lung, the larvae enter the alveoli and migrate up the trachea. The larvae are coughed up, swallowed, and enter the digestive tract to mature into adult worms in the small intestine where eggs are produced and excreted in the host's feces (Ogilvie and Hockley, 1968). The Type 2 response necessary for efficient worm expulsion depends on IL-4 with contributions from IL-13, IL-5 and IL-9 (Fallon et al., 2002). Effector cells producing these cytokines include Th2 cells, eosinophils, and basophils (Voehringer et al., 2004), and are recruited to sites of inflammation in the lung and small intestine. One suggested mechanism by how these cells and cytokines function in worm expulsion is by inducing alternatively activated macrophages that in turn stimulate smooth muscle hypercontractility in the intestine (Zhao et al., 2008). While we know that these cytokines are produced and coordinated by a number of innate and adaptive sources, the relative contribution of each cellular component remains to be elucidated.

Schistosoma mansoni infection

Over 200 people worldwide are estimated to be infected with one of the *Schistosoma* trematode species (DeFranco et al., 2007). Infection occurs upon exposure to freshwater contaminated with cercariae that penetrate the skin, develop into schistosomula, and migrate via the vasculature to the liver where they mature into worms in the microvasculature. Adult male and female worms mate to form pairs that then migrate against portal venous blood flow to release eggs into the veins of the intestine or bladder depending on the species of *Schistosoma.* Eggs are excreted in the stool or urine where, in fresh water, they hatch to release miricidia. The miricidia infect snails to develop into cercariae to complete their life cycle. While the early stages of infection involving schistosomula elicit a Th1-associated type of response, the eggs produced at later stages initiate a granulomatous Th2-associated response characterized by elevated IgE levels and eosinophilia. Using *S. mansoni* infections in mice to model the human disease, IL-4 and IL-13 have been demonstrated to be important, as signaling via IL-4Rα in the hematopoietic compartment is required for protection from acute schistosomiasis. When IL-4 receptor signaling was specifically deleted in the macrophage lineage, mice had increased Th1 inflammation, impaired egg expulsion, and increased mortality (Herbert et al., 2004, 2008). While it does not seem to play a role in granuloma formation (Jankovic et al., 1999), IL-4 is important in mediating survival during infection possibly by maintaining tissue integrity to prevent septicemia (Fallon et al., 2000). The extensive collagen deposition as a result of the pro-fibrotic action of IL-13 has been suggested to be detrimental to survival, as IL-13-deficient mice had improved survival correlating with less fibrosis. IL-4 and IL-13 have been suggested to be mediating their effects by

inducing macrophage alternative activation. CD4 T cells are important for granuloma formation (Mathew et al., 1986); however, its contribution of IL-4 and IL-13 compared to the contribution from innate sources is incompletely defined.

Allergic airway inflammation

Allergic airway disease is chronic inflammation that results when environmental allergens initiate a Type 2 response at the mucosal barrier of the lung. The Type 2 response induces airway remodeling which is composed of inflammatory cell infiltration, mucus metaplasia, and smooth muscle hypertrophy. The narrowing of airways then leads to wheezing and breathlessness, the hallmark symptoms of asthma. Experimental models have demonstrated that IL-4 and IL-13 are critical to the development of the phenotypic features of the disease. IL-4 is important for inflammatory cell recruitment and the development of airway hyperreactivity (Brusselle et al., 1995; Corry et al., 1996). IL-13 can independently induce the asthma phenotype (Grunig et al., 1998), reflecting its ability to also bind IL-4Rα. By binding to the IL-4Rα-IL-13Rα1 complex on airway epithelia, IL-13 induces goblet cell hyperplasia and mucus production (Kuperman et al., 2002, 2005). IL-5 promotes lung eosinophilia to a degree where eosinophil numbers in the circulation and in BAL fluid has been correlated with asthma severity. The eosinophilia has also been suggested to contribute to airway remodeling (Flood-Page et al., 2003). IL-9 recruits mast cells to the lung where they promote many of the features of asthma (Yu et al., 2006). In studies of human asthma, mast cells have been observed in bronchial smooth muscle, thus potentially contributing to airway hyperresponsiveness (Brightling et al., 2002).

As the site of contact with environmental allergens, the airway epithelium is beginning to be appreciated for its contributions to the Type 2 inflammatory response. Thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 have been observed to be derived from, among other sources, lung epithelial cells upon allergen challenge; their specific roles with respect to the other aspects of Type 2-associated airway inflammation need further elucidation (Barrett and Austen, 2009). With many of the asthma studies in animal models thus far using OVA sensitization and challenge, we are just beginning to understand how environmental allergens such as chitin (Reese et al., 2007; van Dyken et al., manuscript in preparation) contribute to lung inflammation via interactions with the immune system at mucosal barriers.

Newly described components of Type 2 immunity

New cytokine mediators in Type 2 responses have recently been identified to interact with the components described above. IL-25, also known as IL-17E in the IL-17 family, was first described to be expressed by Th2 polarized CD4 T cells, and is sufficient to induce IL-4, IL-5, and IL-13 gene expression leading to eosinophilia, goblet cell hyperplasia, and increased serum levels of IgE, IgG1, and IgA (Fort et al., 2001). However, more recent evidence suggests that IL-25 can be derived from a variety of other activated cells. Intriguingly, IL-25 elicited from the spleen an IL-5- and IL-13-producing non-T/non-B cell population that was MHC class II^{hi}, $CD11c^{dull}$, and lineage-negative. Fallon et al. (2006) demonstrated that in IL-25-deficient mice infected with *N. brasiliensis*, worm expulsion was delayed compared to wildtype controls. Additionally, the administration of recombinant IL-25 could induce rapid worm expulsion independent

of T and B cells. With *N. brasiliensis* infection or recombinant mIL-25 administration, these cells were observed to accumulate in high numbers in mesenteric lymph nodes and could be further distinguished as ckit^{+} and FcεRI^{-}. In addition to its role in mediating the clearance of helminth infection, other studies further suggest that IL-25 can also play a role in limiting inflammation at mucosal sites during chronic helminth infection (Owyang et al., 2006). Recently, these cells have been suggested to be "natural helper cells" (described below). Thus, further study is needed to better define this cell population in the context of Type 2 responses.

IL-31 has been shown to be expressed by Th2 cells and was initially reported as being able to drive epithelial pathology in dermatitis and allergic airway hypersensitivity when overexpressed in mouse models (Dillon et al., 2004). In one study of human atopic dermatitis and allergic contact dermatitis biopsy specimens, mRNA levels of IL-31 were elevated compared to samples from healthy patients. The levels of IL-31 mRNA also correlated with IL-4 and IL-13 expression in skin, further supporting its role in Type 2 immunity (Neis et al., 2006). The IL-31 receptor is expressed on T cells, monocytes, and epithelial cells; therefore, IL-31 may function at other mucosal sites. Studies in *S. mansoni* egg-induced pulmonary inflammation and *Trichuris muris* intestinal infection have instead suggested that IL-31 functions at these sites to limit the magnitude of the Type 2 inflammatory response (Perrigoue et al., 2007, 2009). Whether IL-31 drives or limits inflammation may reflect its tissue-specific effects and further study is needed to better understand this relatively new cytokine.

IL-33 is another recently identified cytokine associated with Type 2 responses such as helminth infection (Humphreys et al., 2008). IL-33 protein is expressed by

fibroblasts, epithelial cells, and endothelial cells, particularly in high endothelial venules (Liew et al., 2010). It binds the ST2 receptor expressed on Th2 cells, mast cells, eosinophils, and basophils. IL-33 can polarize naïve T cells to produce IL-5 and IL-13 independent of IL-4, as well as enhance IL-5 and IL-13 from already polarized Th2 cells (Kurowska-Stolarska et al., 2008). Basophils are activated by IL-33 to produce IL-4, IL-6, and IL-33 (Suzukawa et al., 2008a). IL-33 induces eosinophilia *in vivo*, and, enhances human eosinophil adhesion and survival (Suzukawa et al., 2008b). Studies also suggest that IL-33 promotes IL-13-mediated macrophage alternative activation in airway inflammation (Kurowska-Stolarska et al., 2009). IL-33 has been demonstrated to activate mast cells, as well as induce degranulation after sensitization with IgE (Kurowska-Stolarska et al., 2008); thus, IL-33 has been considered as a potential therapeutic target in treating anaphylaxis and atopic disease. Interestingly, IL-33-induction of the Type 2 phenotype is protective against atherosclerosis by skewing away from the inflammatory IFNγ-producing Type 1 phenotype found in plaque formation (Miller et al., 2008).

Innate components of Type 2 immunity

Eosinophils

Eosinophils are granular leukocytes known for their secretory function and association with Type 2 immune responses. It has recently been suggested that eosinophils compose one of the earliest components of innate Type 2 immunity to evolve as a vertebrate host response to parasitism. While phagocytic cells resembling monocytes/macrophages and neutrophils can be found in invertebrates, granular cells resembling mast cells and eosinophils first appear in jawed fishes. Eosinophilic rodlet

cells of jawed fishes morphologically contain a characteristic crystalline core in their cytoplasm and can degranulate their contents upon stimulation. Rodlet cells locate to epithelial surfaces such as the gills and the intestine, where numbers increase and correlate in response to helminth infection and/or tissue damage (Reite and Evenson, 2006). Therefore, these rodlet cells are likely earlier versions of eosinophils that exist in mammals, and that have acquired additional functions over time in response to continued selective pressure from parasitism.

The eosinophils we now find in mammals retain such secretory granules, but now also contain a myriad of mediators and cytokines implicated in disease and host defense. Eosinophil granules contain major basic protein (MBP) and eosinophil cationic protein (ECP) that have been suggested to have anti-helminth function (Hogan et al., 2008). IL-5 is critical for eosinophil maturation, expansion, and mobilization from the bone marrow, as IL-5 transgenic mice are highly eosinophilic (Dent et al., 1990). Eosinophils express chemokine receptors CCR3 and CCR1 and migrate in response to chemokines such as eotaxin-1 and RANTES. They can also be recruited to tissue in response to leukotrienes such as LTB_4, LTD_4, and LTE_4 (Hogan et al., 2008). L-selectin mediates eosinophil rolling on vascular endothelial surfaces, while adhesion and transmigration is mediated by eosinophil integrins such as $\alpha 4\beta 1$, $\alpha L\beta 2$, and $\alpha 4\beta 7$ that interact with endothelial VCAM, ICAM, and Mad-CAM-1 adhesion receptors, respectively (Forbes et al., 2002; Tachimoto et al., 2002).

Helminth infection induces blood and tissue eosinophilia, where recruitment to tissue is STAT6-dependent (Voehringer et al., 2004). In IL-4 cytokine reporter mice (4get), eosinophils are transcriptionally active at the IL-4 locus, yet produce low levels of

protein compared to basophils and CD4 T cells (Khodoun et al., 2004). Despite dramatic eosinophilia in helminth infection, they are dispensible for IgE production and worm expulsion during primary *N. brasiliensis* infection (Voehringer et al., 2006). In *S. mansoni* infection, prominent eosinophilia is observed to peak between 8 and 10 weeks post-infection (Davies et al., 2005). However, in eosinophil-deficient mice infected with *S. mansoni*, liver pathology, worm burden, and egg deposition were unaffected (Swartz et al., 2006). While the role of eosinophils in primary helminth infection remains unclear, it is has been suggested that eosinophils may instead have roles in secondary re-infection and tissue remodeling (Hogan et al., 2008). In addition to parasitic helminth infection, eosinophils are one of the prominent cell types recruited to the lung in asthma and in animal models of the disease. Studies in eosinophil-deficient mouse models suggest that eosinophils exacerbate airway inflammation by contributing to the increase in mucus secretion and airway remodeling by driving collagen deposition (Lee et al., 2004; Humbles et al., 2004). However, due to differences in the experimental protocols and the genetic background of animal models used in published studies, our understanding of eosinophils in asthma remains incompletely defined. Despite being a prominent feature of Type 2 inflammation, we still do not have a clear understanding of the exact role that eosinophils play, and therefore, this cell type remains somewhat enigmatic.

Basophils

Basophils compose less than 1% of leukocytes found in circulation at rest, yet are increasingly recognized for their effector functions in Type 2 responses. For example, the crosslinking of FcεRI receptors via the binding of IgE is one of the major mechanisms by

which basophils release mediators such as histamine, granzyme B, and IL-4 that drive allergic inflammation. Using flow cytometric analysis of cytokine reporter mice, basophils can be identified as expressing IL-4 (Mohrs et al., 2001), and can be further distinguished by their cell surface expression of the high affinity FcεRI receptor, CD49b (DX5), as well as a characteristic forward-side scatter profile (Sullivan and Locksley, 2009). In helminth infection models, such as with *N. brasiliensis*, systemic basophilia is induced by mobilization from the bone marrow and into circulation. Recruitment of basophils to tissues has been shown to be CD4 T cell dependent, but independent of IL-4 and STAT6 signaling (Min et al., 2004; Voehringer et al., 2004). Additionally, CD4 T cell-derived IL-3 has been shown to be important for basophil development and expansion during helminth infection (Shen et al., 2008). Basophils produce high levels of IL-4 upon activation with IL-3 and IL-18 *in vitro* or by exogenous administration to helminth-infected mice (Yoshimoto et al., 1999). However, the functional contribution of basophil-derived IL-4 in helminth infection awaits further definition (Sullivan et al., manuscript in preparation).

Mast cells

Mast cells circulate as progenitors and become mature cells upon transendothelial migration into tissues. Much like the basophil, mast cells are activated by the crosslinking of surface FcεR by antigen-bound IgE. One of the major subtypes of mast cells, the intraepithelial mucosal mast cell, is also stimulated by Type 2 cytokines and are triggered to release a number of mediators that influence the phenotypic responses seen in allergy and helminth infection. Mast cells release cytokines (IL-4, IL-13, IL-18), histamine,

proteases, and lipid mediators that induce vascular leak, constrict smooth muscle, and inflammatory cell recruitment (Barrett et al., 2009). In rats, it was observed that the secretion of rat mast cell protease II coincided with the expulsion of *N. brasiliensis* (Woodbury et al., 1984). Additionally, elevated serum IgE correlated with elimination of primary schistosome worm infection from rats (Cutts and Wilson, 1997). Asthma studies in mast cell-deficient mice demonstrate decreased inflammatory cell infiltration into the lung, goblet cell metaplasia, and airway hyperreactivity (Yu et al., 2006). One recent study reported that mast cells in white adipose tissue produce IL-6 and IFN-γ as part of the inflammatory process that drives obesity (Liu et al., 2009). However, models of mast cell deficiency have been found to have hematologic abnormalities (Galli et al., 2005; Nigrovic et al., 2008), so the role of mast cells in these chronic diseases needs further investigation.

Alternatively activated macrophages

Macrophage polarization is generally divided into classical and alternative activation. Classically activated macrophages (CAM/M1) are a component of Th1-associated, Type 1 immune responses to microbial stimuli in which IFN-γ is a key signal in inducing macrophage production of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β (Gordon, 2003). IFN-γ induces nitric oxide synthase 2 (NOS2/iNOS) to generate nitric oxide and the respiratory burst, important for bacterial killing. Alternatively activated macrophages (AAM/M2), on the other hand, accumulate during Type 2 responses. Macrophages express receptors for IL-4 and IL-13; upon exposure to these cytokines, they undergo an activation pathway leading to the induction of arginase-1, which

counteracts the iNOS inflammatory pathway, leading to the production of polyamines and prolines, which are important for cell proliferation and collagen synthesis, respectively. Other characteristic features of alternative activation in macrophages include upregulation of mannose receptor (CD206) expression, and the production of RELMα (FIZZ1, a resistin-like secreted protein) and Ym1/2 (chitinase-like secretory lectins) (Gordon, 2003).

During helminth infection, such as with *N. brasiliensis* or *S. mansoni*, signature genes of alternative activation are induced such as RELMα. In the setting of RELMα deficiency, mice had exacerbated Type 2 responses, suggesting that RELMα has a suppressive effect (Pesce et al., 2009). This may be one mechanism by which AAM promote tissue repair and maintain tissue homeostasis. Other studies suggest that AAM are not necessary for clearance of primary *N. brasiliensis* infection; rather, AAM are immunoprotective against Th1 skewed pathology in *S. mansoni* infection (Herbert et al., 2004). In the chronic model of gastrointestinal helminth infection with *Heligmosomoides polygyrus*, AAM induced by memory CD4 T cells were important for mediating primary and secondary clearance of the parasite (Anthony et al., 2006). AAM have also been demonstrated to have protective anti-inflammatory effects against atherosclerotic plaques that contain Th1 skewed, proinflammatory M1 macrophages (Bouhlel et al., 2007). Taken together, the results from the various studies thus far indicate that AAMs are critical for host protection and tissue homeostasis, particularly in the setting of chronic infection.

Natural helper cells

"Natural helper cells" are a recently described cell type suggested to play a role in Type 2 responses (Moro et al., 2010). These are the cells induced by IL-25 as described above and are lineage-negative, c-kit+, Sca1+, IL7R+, and IL-33R+. Upon infection with *N. brasiliensis* or administration of IL-33, these cells produce high levels of IL-13 to induce goblet cell hyperplasia. Through their production of IL-5 and IL-6, natural helper cells are suggested to maintain B1 cells and IgA production (Moro et al., 2010). Additionally, these cells have been described to be associated with mesenteric adipose tissue, but their function there is currently undefined.

Immune dysregulation and inflammation link obesity and insulin resistance

We are just beginning to appreciate the closely intertwined relationship between the control of inflammation and metabolic homeostasis. Obesity is regarded as a chronic, subacute state of systemic inflammation. Increasingly recognized as more than merely a depot for energy stores in adipocytes, white adipose tissue deposits are major sites of obesity-related inflammation. Adipose tissue is a critical endocrine organ that produces and responds to physiologic signals that influence feeding and energy balance. Secreted bioactive mediators such as cytokines produced by adipose tissue are key components that drive the inflammatory process underlying the disease pathogenesis leading to type 2 diabetes-associated insulin resistance in peripheral tissues (Guilherme et al., 2008). These pro-inflammatory cytokines include TNF-α, IL-6, and IL-1β, and are secreted when adipocytes undergo cellular stress and hypoxia, such as in the state of chronic

overnutrition (Shoelson et al., 2006). These cytokines link obesity to insulin resistance by the induction of the JNK and NF-κB signaling pathways in target tissues. Increased JNK activity correlates with obesity, and a mouse model of JNK deficiency is protected from insulin resistance (Hirosumi et al., 2002). Additionally, targeted inhibition of the NF-κB pathway in myeloid cells protects mice from insulin resistance in response to high fat diet, obesity, and aging (Arkan et al., 2005). One consequence of the activation of the inflammatory pathways in adipose tissue is immune cell recruitment. In particular, macrophages are recruited to adipose tissue and can themselves produce pro-inflammatory cytokines, thus contributing to a feed forward inflammatory cascade affecting both adipose and peripheral tissues.

Macrophages in adipose tissue

A large proportion of the pro-inflammatory genes expressed in adipose tissue include those associated with macrophage recruitment and activation such as *monocyte chemoattractant protein-1* (MCP-1, also known as CCL2), *Csfr1*, and *Cd68* (Weisberg et al., 2003; Xu et al., 2003). Elevated expression of these genes correlates with an increase in adiposity. In addition to recruiting monocytes to adipose tissue, MCP-1 may play a role in insulin resistance; the addition of MCP-1 to cultured adipocytes decreases insulin-stimulated glucose uptake (Sartipy et al., 2003) and the overexpression of MCP-1 in adipose tissue causes insulin resistance (Kamei et al., 2006). *In vivo*, mice genetically deficient in CCR2, the receptor for MCP-1, exhibit reduced food intake and a partial protection from high fat diet-induced obesity (Weisberg et al., 2006).

Macrophages are phenotypically and functionally heterogeneous, depending on the signals in the local tissue microenvironment. In the setting of obesity, a subpopulation of macrophages that express the cell surface markers F4/80, CD11b, and CD11c have been observed to accumulate in adipose tissue. These obesity-associated macrophages have increased expression of the pro-inflammatory genes for IL-6 and iNOS, characteristic of classical M1 macrophage activation (Lumeng et al., 2007). Further, *ex vivo* stimulation with free fatty acids via binding to toll-like receptor (TLR)2 and TLR4 demonstrated increased IL-6 expression in F4/80+ CD11b+ CD11c+ macrophages, thus suggesting that these cells function as a link between adipocytes and the inflammatory process underlying metabolic dysregulation (Nguyen et al., 2007). Indeed, TLR4 signaling in hematopoietic cells plays a role in the induction of inflammation as mice lacking TLR4 specifically in the hematopoietic compartment are protected from hyperinsulinemia and have improved insulin sensitivity (Saberi et al., 2009)

In contrast to the pro-inflammatory phenotype of recruited adipose tissue M1 macrophages in obesity, those in the adipose tissue of lean mice express markers of alternative activation such as *Ym1, arginase 1* and *IL-10*. Decreased expression of these genes is associated with diet-induced obesity (Lumeng et al., 2007). This suggests that metabolic homeostasis is influenced by the balance between the populations of pro-inflammatory classically activated and alternatively activated macrophages in adipose tissue.

IL-4/IL-13 and macrophage alternative activation

IL-4 has been shown to be important for alternative activation by its induction of 12/15-lipoxygenase, which generates intracellular ligands for the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPARγ), a sensor of fatty acids (Huang et al., 1999). Additionally, IL-4 can upregulate PPARγ itself in macrophages. PPARγ has been demonstrated to be critical for AAM development; in a model of myeloid-specific PPARγ deletion, alternative activation is impaired (Odegaard et al., 2007). Mice with this absence of macrophage alternative activation exhibit metabolic defects such as diet-induced obesity and insulin resistance. This suggests that AAM in adipose tissue function to counteract the inflammatory mechanisms driving obesity and insulin resistance. Whether or not IL-4/IL-13 in adipose tissue plays a role is unclear, as the source of these exact signals that induce alternative activation in adipose tissue is poorly defined.

T cells in adipose tissue

T cells of the immune system recruited to adipose tissue have been recently implicated to be associated with metabolic homeostasis: CD8 T cells, CD4 T cells, and T_{regs}. Prior to the infiltration of inflammatory macrophages, the entry of CD8 T cells into adipose was observed to be an early event in the induction of diet-induced obesity (Nishimura et al., 2009). With the progression of diet-induced obesity, the CD4 T cell population recruited to adipose tissue is skewed toward an IFN-γ-producing Th1 phenotype that increase to numbers that outnumber the resident Th2 (GATA-3+) and Treg (Foxp3+) T cell populations found in lean adipose tissue prior to the onset of

obesity (Winer et al., 2009). This is consistent with an earlier study by Rocha et al. (2008) where adipose tissue T cells stimulated *ex vivo* from obese mice secreted greater amounts of IFN-γ compared to controls. Winer et al. (2009) also observed that Th2-polarized CD4 T cells, but not Th1 cells, when transferred into obese Rag1-null mice, could ameliorate weight gain and insulin resistance. Both studies by Nishimura et al. and Winer et al. report evidence of specific rearrangements in the T cell receptor repertoire, suggesting the presence of adipose tissue antigens capable of being recognized by the adaptive immune system. To complement the CD4 and CD8 T cell studies, Feurer et al. (2009) detected Foxp3+ T_{regs} present in lean visceral adipose tissue. These studies support a model of T_{regs} and Th2-biased cells present in lean adipose tissue under homeostatic conditions. But when obesity is established, CD8 T cells and M1 macrophages recruited to adipose tissue overwhelm the T_{regs} and Th2 cells, setting up a vicious cycle of inflammation. Additionally, whether these cells directly interact with macrophages and how they influence macrophage activation remains to be determined.

Contributions of innate and adaptive IL-4 and IL-13 to immunity and beyond

The phenotypic Type 2 response is a complex interplay among many different cellular components and cytokine mediators. Cells that express IL-4 and IL-13 include CD4 T cells, eosinophils, basophils, mast cells, NKT cells, and γδ T cells (Brown et al., 1987; Moqbel et al., 1995; Yoshimoto et al.,1994). IL-4 and IL-13 have mostly been analyzed in models in which one or both are completely knocked out in all cells and tissues; therefore, the subtle contributions of specific cell populations, such as adaptive versus innate, have yet to be appreciated. Here we demonstrate a model of CD4 T cell-

specific IL-4 and IL-13 deficiency in the context of helminth infection and airway inflammation. Additionally, as IL-4 and IL-13 are most extensively studied in the context of allergy and helminth infections, their role in metabolism has been relatively unexplored to date. In support of a bias toward a Type 2 immune phenotype associated with AAM in lean adipose tissue, we propose that IL-4- and IL-13-expressing cells are present in adipose tissue. Here we present data identifying the cellular sources of IL-4 and IL-13 in adipose tissue that drive macrophage alternative activation, and thus the role of these cytokines in the regulation of metabolism.

CHAPTER II:

CELL-SPECIFIC DELETION OF IL-4/13 *in vivo*

Abstract

Conditional knockout mouse models provide powerful systems to identify the contributions that specific tissues or cells make in host defense and disease processes. We generated a model of conditional IL-4/13 deficiency, in which IL-4 and IL-13 are specifically deleted in CD4 T cells, to examine the role of adaptive and innate sources of IL-4 and IL-13 in helminth infection and allergic airway inflammation. We find that CD4 T cell-derived IL-4 and IL-13 are not required for host defense against an acute helminth infection with *Nippostrongylus brasiliensis*. However, IL-4 and IL-13 from CD4 T cells does appear to play a role in chronic conditions such as *Schistosoma mansoni* infection and in an OVA-induced model of airway inflammation. In response to *S. mansoni* infection, the conditional knockout mice had an intermediate survival phenotype, despite no differences in parasite maturation and fecundity. In the allergic airway model, conditional knockout mice had intermediate protection from airway hyperresponsiveness, decreased mucus production in the lung, and decreased eosinophil recruitment to the lung. Additionally, results from all three studies are consistent with IL-4 being necessary for IgE production. Taken together, these results demonstrate that IL-4 and IL-13 from Th2 cells are dispensable for acute helminth infection, with innate cells capable of redundant function. However, they may play a role in chronic inflammation, such as in survival during *S. mansoni* infection and airway hyperreactivity in response to inhaled antigen. This is possibly achieved through direct cell recruitment or indirectly through the maintanence of IgE production for innate cell activation.

Introduction

Animal models are powerful systems for modeling human disease, as they provide information regarding the organization and coordination of the cellular components that play important roles. Animal models with complete deficiencies in IL-4 and/or IL-13 have demonstrated that Type 2 cytokines are required for the efficient clearance and resolution of helminth infection (Fallon et al., 2002). However, the relative contributions of adaptive and innate IL-4- and IL-13-producing cellular subsets are not well defined. Studies by Voehringer et al. (2006) have demonstrated that while Type 2 cytokines from CD4 cells are important for cell recruitment and antibody class switching to IgE production, they were dispensable for the clearance of acute worm infection. IL-4 and IL-13 were still needed for worm expulsion, however, thus implicating a role for innate IL-4 and IL-13. The studies by Voehringer et al. were performed by transferring IL-4/13-sufficient or IL-4/13-deficient CD4 T cells into T cell-deficient Cα-/- recipients; however, one concern regarding this method is that transferred T cells may undergo homeostatic proliferation that could affect the cellular response during an infection. Thus, mouse models that would avoid this experimental manipulation could be advantageous. We applied a model of cell-specific cytokine deletion to study Type 2 responses in the context of helminth infection and allergen-induced airway inflammation. We demonstrate that a conditional knockout model of IL-4/13 is a useful tool to study cell-specific contributions to Type 2 immunity.

Results

Generation of the conditional double knockout model of IL-4 and IL-13

To generate a conditional double knockout model of IL-4/13, David Voehringer targeted loxP sequences to flank the IL-4 and IL-13 loci (Voehringer et al., 2009). BAC recombineering technology and dual antibiotic selection cassettes facilitated in inserting the loxP sites several kB apart in the genomic sequence of interest. In the presence of a Cre recombinase, IL-4 and IL-13 can be deleted in a cell- or tissue-specific manner as a result of recombination at the loxP sites (Fig. 1A).

In vitro confirmation of the conditional IL-4/13 knockout mouse model

Conditional IL-4/13 mice were crossed once with IL-4/13-/- (McKenzie et al., 1999) and then to CD4Cre (Lee et al., 2001) mice to generate CD4Cre x IL-4/13 fl/- mice, in which IL-4/IL-13 is specifically deleted in CD4 T cells. CD4 T cells isolated from lymph nodes were isolated from CD4Cre x IL-4/IL-13 fl/- mice and incubated under conditions for Th0, Th1, and Th2 polarization. We detected by intracellular cytokine staining that, under Th2 polarizing conditions, wildtype and IL-4/13 fl/- CD4 T cells were able to produce IL-4 protein. However, IL-4 production by CD4Cre x IL-4/13 fl/- T cells was impaired, consistent with CD4Cre efficiently excising the segment of IL-4 and IL-13 by recombining at the loxP sites (Fig. 1B). Additionally, we detected an absence of IL-13 in CD4Cre x IL-4/13 fl/- T cells cultured in the same conditions (data not shown). *Ex vivo* stimulation of CD4 T cells on plate-bound anti-CD3 and anti-CD28 induced cytokine production reflecting the intracellular cytokine staining findings, with IL-4 and IL-13 detectable in the supernatants of wildtype and IL-4 fl/- CD4 T cells and

Figure 1. Generation and analysis of cell-specific IL-4/13-deficient mice.

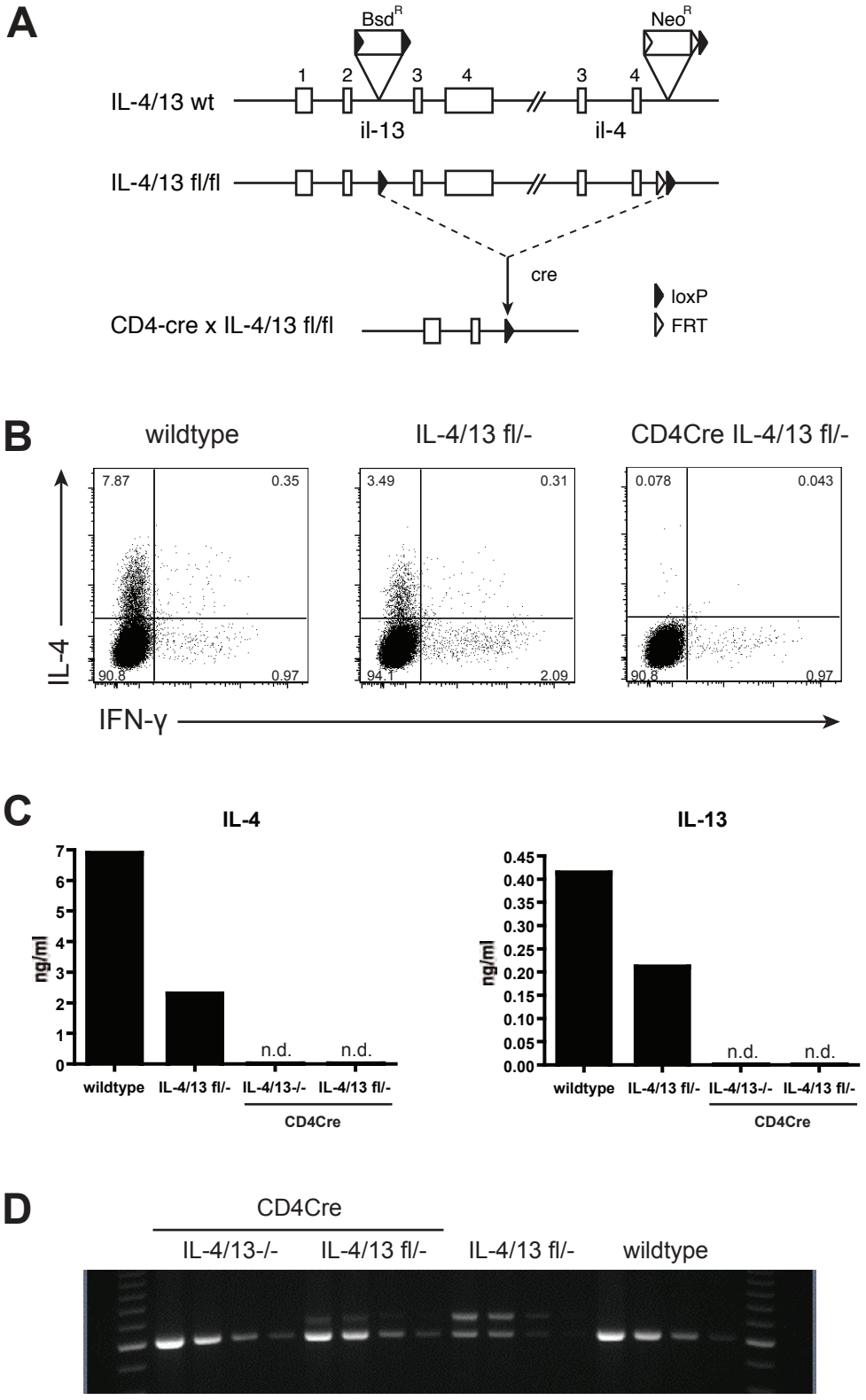
A. Schematic representation of the generation of conditional deletion of IL-4 and IL-13 in CD4 cells. BAC recombineering in *E. coli* was used to insert two antibiotic selection cassettes flanking IL-4 and IL-13. Using multiple replicate plates of transfected embryonic stem (ES) cell clones, IL-4/13 floxed mice were generated with loxP sites remaining after the removal of the selection cassettes. When the conditional knockout mice are crossed with CD4Cre mice, recombination takes place at the loxP sites in CD4 cells, resulting in cell-specific IL-4/13 deletion.

B. Intracellular cytokine staining of lymph node CD4 T cells isolated by magnetic beads and cultured in Th2 polarizing conditions (IL-2, IL-4, and anti-IFN-γ) on anti-CD3 and anti-CD28 for 10 days. Cells were re-stimulated with PMA and ionomycin and secretion blocked with monensin. Cells fixed and permeabilized followed by staining for IFN-γ, IL-4, and CD4. Values in quadrants represent CD4 cells as percent of live cells based on DAPI exclusion and forward- and side-scatter size characteristics.

C. ELISA quantification of IL-4 and IL-13 secreted by lymph node CD4 T cells isolated by magnetic beads and cultured in Th2 polarizing conditions and on anti-CD3 and anti-CD28 for 8 days. Secondary stimulation with anti-CD3 followed by supernatant harvest on day 9. n.d.= none detected.

D. Semi-quantitative PCR of lymph node CD4 T cell DNA to detect the FRT and loxP sites at the IL-4 locus. Detection of the FRT and loxP sites results in larger PCR product (upper band).

Figure 1


A
Bsd^R
Neo^R
1
2
3
4
3
4
IL-4/13 wt
il-13
il-4
IL-4/13 fl/fl
cre
loxP
FRT
CD4-cre x IL-4/13 fl/fl
B
wildtype
IL-4/13 fl/-
CD4Cre IL-4/13 fl/-
7.87
0.35
90.8
0.97
3.49
0.31
94.1
2.09
0.078
0.043
90.8
0.97
IL-4
IFN-γ
C
IL-4
IL-13
ng/ml
n.d.
n.d.
wildtype
IL-4/13 fl/-
IL-4/13-/-
IL-4/13 fl/-
CD4Cre
D
CD4Cre
IL-4/13-/-
IL-4/13 fl/-
IL-4/13 fl/-
wildtype

absent in the CD4Cre x IL-4/13 fl/- cultures (Fig.1C). Using primers to detect the FRT site (remaining after removal of the neomycin antibiotic selection cassette) and the loxP site at the IL-4 locus in targeted mice by semi-quantitative PCR, we confirmed the recombination excision event in CD4Cre x IL-4/13 fl/- T cells while the sites remained intact in IL-4/13 fl/- mice that did not express Cre recombinase (Fig. 1D).

N. brasiliensis: a model of acute helminth infection

To test the effect of CD4-specific IL-4/13 deletion *in vivo*, CD4Cre x IL-4/13 fl/fl mice were infected with 500 L3 stage *N. brasiliensis* larvae. On day 12 post-infection, intestinal worm burden and serum IgE levels were assessed. As expected, control wildtype mice efficiently cleared the intestinal worms, whereas CD4Cre x IL-4/13-/- were unable to (Fig. 2A). IL-4/13 fl/fl mice not on the CD4Cre background were also able to clear the worms, functioning similarly to the wildtype background, confirming that the insertion of loxP sites did not affect IL-4 and IL-13 production. The CD4Cre x Il-4/13 fl/fl mice were also able to clear intestinal worms, thus indicating that T cell-derived IL-4/13 are not necessary for intestinal worm clearance in this model. This is consistent with earlier studies in which adoptively transferred IL-4/13-deficient CD4 T cells were able to confer the ability to clear worms.

IL-4 is important for mediating antibody class switching to IgE in Type 2 responses (Finkelman et al., 1988). To investigate the contribution of CD4 T cell-derived IL-4/13 to IgE production, we collected blood from day 12, *N. brasiliensis*-infected CD4Cre x IL-4/13 fl/fl mice by cardiac puncture for serum analysis. While IgE production was at wildtype levels in infected IL-4/13 fl/fl control mice, IgE was absent in

Figure 2

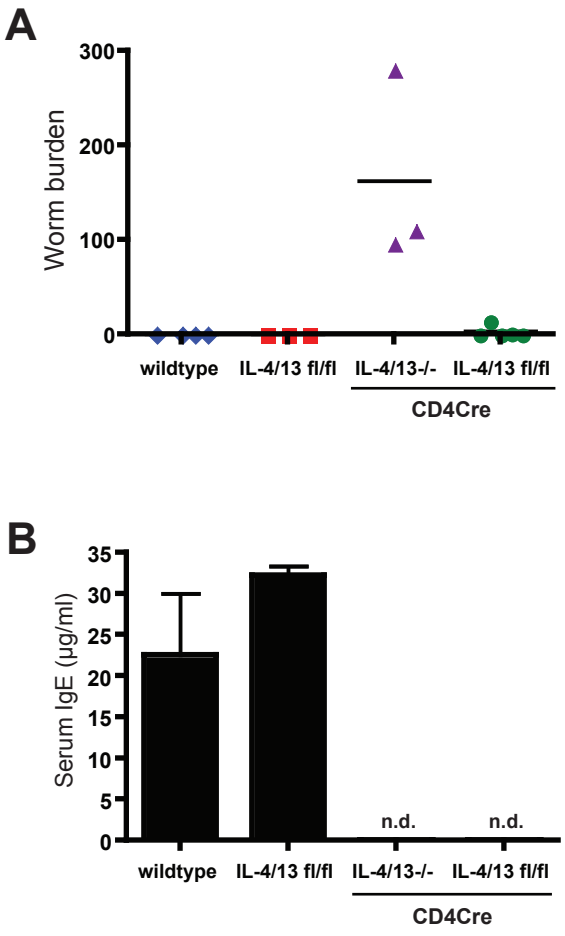


Figure 2. *N. brasiliensis* infection in CD4Cre x IL-4/13 fl/fl mice.

A. Day 12 post-infection intestinal worm burden in mice inoculated with *N. brasiliensis*. Dots represent individual mice. Bars represent the mean. Representative of 5 experiments.

B. Serum IgE levels in mice 12 days post-infection with *N. brasiliensis*. 3-5 mice per group. Representative of 5 experiments. Error bars represent SEM. n.d.= none detected.

the serum of infected CD4Cre x IL-4/13 fl/fl mice (Fig. 2B). Thus, IgE production during a Type 2 response is entirely dependent on IL-4 from CD4 T cells.

S. mansoni: a model of chronic helminth infection

To investigate the role of CD4 T cell-derived IL-4/13 in the setting of chronic infection, we infected CD4Cre x IL-4/13 fl/fl mice with *Schistosoma mansoni* cercariae. When monitored for survival, CD4Cre x IL-4/13-/- mice had a high rate of mortality, with a rapid decline in survival of the cohort as early as 48 days post-infection (Fig. 3A). This is consistent with published studies demonstrating the importance of IL-4/13 having a protective effect in *S. mansoni* infection (Herbert et al., 2008). IL-4/13 fl/fl mice survived similar to control wildtype Balb/c mice, surviving out to 56 days post-infection, approaching the termination of the experiment. Surprisingly, the CD4Cre x IL-4/13 fl/fl cohort had an intermediate survival phenotype compared to other groups.

To further investigate aspects of the CD4Cre x IL-4/13 fl/fl mice during chronic *S. mansoni* infection, we assessed serum IgE, cytokine production, egg production, the number of mature worms, and granuloma formation. We detected robust IgE production in *S. mansoni*-infected wildtype and IL-4/13 fl/fl mice at 6 weeks post-infection and even higher levels at 8 weeks (Fig. 3B). Although there appears to be differences in IgE levels when comparing wildtype and IL-4/13 fl/fl samples, this likely reflects small sample sizes especially at the late timepoint. It is possible that variability in IgE levels in individual mice may be influencing survival in the wildtype and IL-4/13 fl/fl groups. Similar to *N. brasiliensis*-infected mice, IgE production was absent in infected CD4Cre x IL-4/13 fl/fl mice. To assess the impact of IL-4/13-deficiency in the T cell compartment

Figure 3. *S. mansoni* infection in CD4Cre x IL-4/13 fl/fl mice.

A. Wildtype Balb/c, IL-4/13 fl/fl, CD4Cre x IL-4/13-/-, and CD4Cre x IL-4/13 fl/fl mice infected with 200-280 *S. mansoni* cercariae subcutaneously (5-8 mice per group). Survival monitored daily. CD4Cre x IL-4/13 fl/fl mice have intermediate survival compared to control groups.

B. ELISA of serum IgE of mice at 6 and 8 weeks post-infection of *S. mansoni*. 3-8 mice per group at 6 and 8 weeks, except for CD4Cre x IL-4/13-/- (1 surviving mouse at 8 weeks). Error bars represent SEM. n.d.= none detected.

C., D. ELISA analysis of IL-4 and IFN-γ in supernatants from splenocytes unstimulated or stimulated with PMA/ionomycin. Splenocytes taken from mice at 6 weeks of *S. mansoni* infection. 3-8 mice per group. n.s.= not significant.

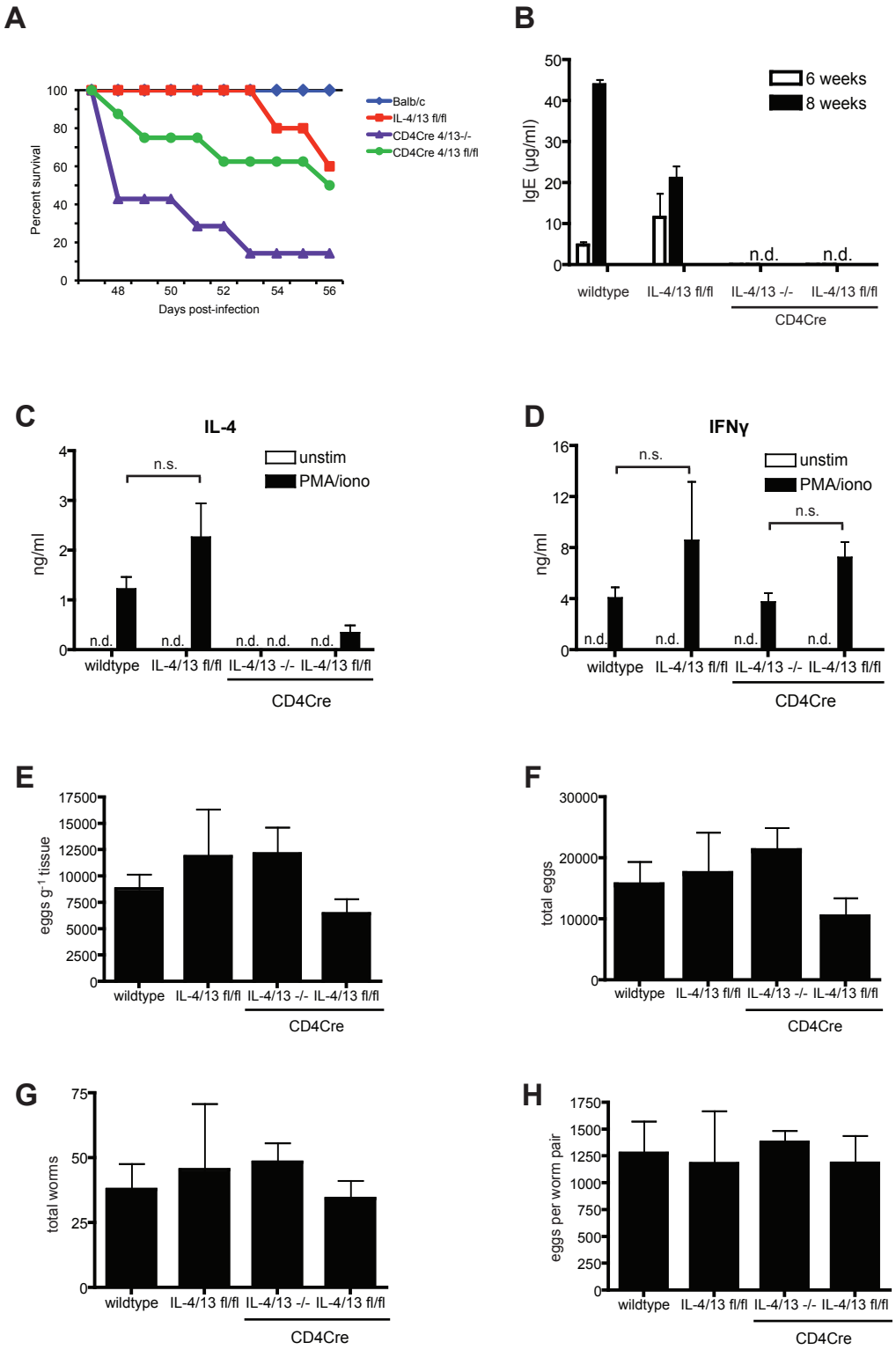
E. *S. mansoni* eggs per gram of liver tissue. Liver tissue was homogenized and digested with trypsin. Eggs collected by sedimentation and counted.

F. *S. mansoni* eggs in liver tissue based on total weight of livers.

G. Total worms recovered by perfusion of hepatic portal circulation. Worms fixed and counted under a dissecting microscope.

H. *S. mansoni* fecundity calculated as eggs produced per worm pair. 3-8 mice per group. Error bars represent SEM.

Figure 3


A
Percent survival
Days post-infection
Balb/c
IL-4/13 fl/fl
CD4Cre 4/13-/-
CD4Cre 4/13 fl/fl
B
IgE (µg/ml)
6 weeks
8 weeks
n.d.
wildtype
IL-4/13 fl/fl
IL-4/13 -/-
IL-4/13 fl/fl
CD4Cre
C
IL-4
ng/ml
unstim
PMA/iono
n.s.
D
IFNγ
E
eggs g-1 tissue
F
total eggs
G
total worms
H
eggs per worm pair

on the level of Type 2 cytokine production, we isolated whole splenocytes from infected mice and stimulated them *ex vivo* with PMA and ionomycin. While wildtype and IL-4/13 fl/fl splenocytes robustly produced IL-4 upon stimulation, IL-4 production from CD4Cre x IL-4/13 fl/fl was severely diminished with residual IL-4 likely from innate sources in the spleen (Fig. 3C). Unlike differences in IL-4 production, IFN-γ production upon cell stimulation was similar across all groups (Fig 3D). Despite differences in IgE and IL-4 production, there were no significant differences in *S. mansoni* parasite maturation, egg production in the liver, and overall worm fecundity (Fig. 3E-H). Thus, early events of schistosome infection are not impacted by the absence of IL-4 and IL-13 from CD4 T cells.

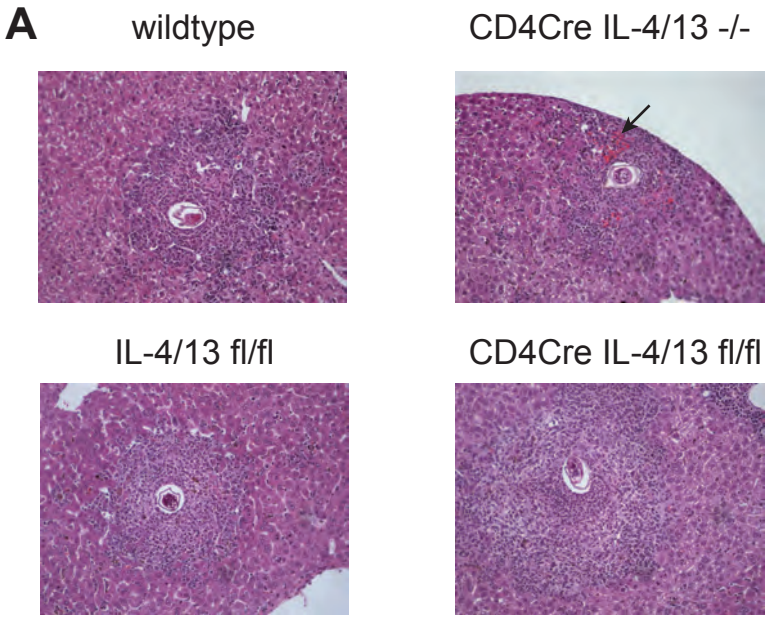
S. mansoni granulomas in the liver and small intestine are major sites of Th2-associated inflammation consisting of inflammatory cell infiltrates of macrophages, eosinophils, and CD4 T cells that sequester the parasite eggs (Anthony et al., 2007). Additionally, cytokines such as IL-4 and IL-13 have been demonstrated to play a role in the coordination of proper granuloma formation (Fallon et al., 2000; Herbert et al., 2008). Therefore, it is possible that IL-4 and IL-13 particularly from CD4 T cells could play a role in granuloma formation and the loss of such could account for the intermediate survival phenotype observed. We first assessed granuloma architecture in mice at an earlier timepoint (6 weeks) post-infection. Dense inflammatory cellular infiltrates were observed surrounding parasite eggs in the livers of both IL-4/13 fl/fl and CD4Cre x IL-4/13 fl/fl, much like the granulomas of wildtype control mice (Fig. 4A). These eggs were tightly walled off from the surrounding tissue, as hepatocytes with normal morphology were observed adjacent to the granulomas. Inflammatory cells also surrounded the eggs

Figure 4. Granuloma formation in *S. mansoni*-infected CD4Cre x IL-4/13 fl/fl mice

A. Granuloma formation in the livers of mice 6 weeks post-infection. Normal granuloma formation in CD4Cre x IL-4/13 fl/fl livers. Increased hemorrhage detected in CD4Cre x IL-4/13-/- granulomas (arrow). Liver sections stained with hematoxylin and eosin (H&E). Images shown at 20X magnification.

B. Decreased collagen deposition in CD4Cre x IL-4/13-/- and CD4Cre x IL-4/13 fl/fl livers compared to IL-4/13 fl/fl tissue. Masson trichrome stains of liver sections taken from tissue 8 weeks post-infection. Counterstain with Weigert's hematoxylin. Images shown at 20X magnification.

Figure 4


A
wildtype
CD4Cre IL-4/13 -/-
IL-4/13 fl/fl
CD4Cre IL-4/13 fl/fl
B
CD4Cre
wildtype
IL-4/13 fl/fl
IL-4/13 -/-

in CD4Cre x IL-4/13-/- livers; however, the granulomas were slightly more diffuse and less well organized, often lacking well-demarcated borders. Sites of hemorrhage were observed only associated with CD4Cre x IL-4/13-/- granulomas (Fig. 4A, top right panel), as well as apoptotic hepatocytes in the nearby tissue. With a decline in survival of the CD4Cre x IL-4/13 fl/fl cohort later in the course of infection, we assessed collagen deposition, as collagen forms a major structural component of granulomas. While intact granulomas rich in collagen were observed in infected wildtype livers, there appeared to be more diffuse granulomas with decreased collagen in the CD4Cre x IL-4/13 fl/fl resembling those seen in CD4Cre x IL-4/13-/- (Fig. 4B). However, a quantitative assessment of collagen content, such as an assay for hydroxyproline content, is needed to further define the qualitative difference observed by histology. Additionally, liver function tests will provide more definitive indicators of the extent of hepatic damage and systemic pathology in these groups.

OVA-induced airway inflammation

The elements of a stereotyped Type 2 immune response also constitute the major features of allergic airway inflammation. Using adoptive transfer models, Voehringer et al. (2006) demonstrated that while CD4 T cells were necessary for the recruitment of innate effector cells (eosinophils and basophils) to the lung in OVA-induced airway inflammation, IL-4/13 from the CD4 T cells was not. Additionally, Voehringer et al. (2006) also observed that these recruited innate effector cells, presumably via the production of IL-4/13, could mediate partial but significant pulmonary resistance upon increasing doses of acetylcholine.

We tested the conditional IL-4/13 knockout model by sensitizing CD4Cre x IL-4/13 fl/fl mice with i.p. OVA in alum and challenging them with repeat doses of intranasal OVA. We then assessed for airway hyperreactivity as measured by pulmonary resistance in response to increasing doses of acetylcholine, effector cell recruitment to the lung and in bronchoalveolar lavage (BAL) fluid, and serum levels of OVA-specific IgE. In CD4Cre x IL-4/13 fl/fl mice, we observed partial protection from pulmonary resistance in response to an intermediate dose acetylcholine, with significant protection at the highest dose (Fig. 5A). And while there was robust induction of IgE production in wildtype IL-4/13 fl/fl immunized with OVA, IgE induction was abrogated in CD4Cre x IL-4/13 fl/fl mice similar to CD4Cre x IL-4/13-/- mice (Fig. 5B). This is consistent with CD4 T cell-derived IL-4 playing an essential role in IgE production.

Pulmonary inflammation was visualized by hematoxylin and eosin (H and E) stain (Fig. 5C, top row). While no inflammation was observed in mice challenged with control phosphate buffered saline (PBS) (data not shown), inflammatory infiltrates could be observed for IL-4/13 fl/fl, CD4Cre x IL-4/13 fl/fl, and CD4Cre x IL-4/13-/- mice challenged with OVA. However, there were subtle differences in the composition of inflammatory infiltrates, as revealed by the eosin stain, where there appeared to be fewer eosinophils. Periodic acid-Schiff (PAS) stains were used to visualize mucus-secreting goblet cells (Fig. 5C, bottom row). While there was prominent goblet cell hyperplasia in IL-4/13 fl/fl lungs challenged with OVA, there was a complete absence of staining in CD4Cre x IL-4/13-/- lungs. The lungs of CD4Cre IL-4/13 fl/fl mice had intermediate staining for mucus.

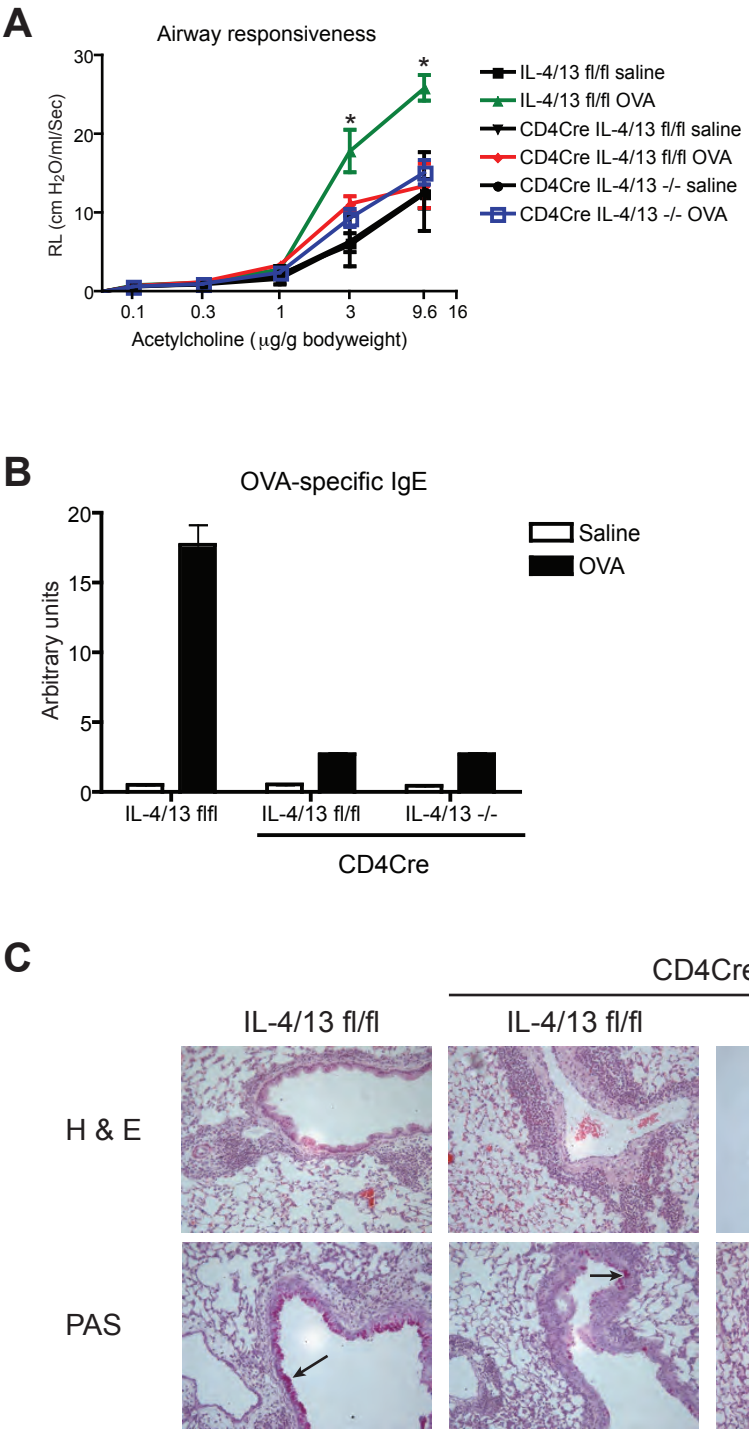
Figure 5. Partial protection from OVA-induced allergic airway inflammation in CD4Cre x IL-4/13 fl/fl mice.

A. Mice sensitized with OVA and challenged with PBS or OVA. Airway hyperresponsiveness measured in response to increasing doses of acetylcholine. 3-6 mice per group. Error bars represent mean+/-SEM. *$p<0.05$ as determined by Student's t-test.

B. Serum IgE measured by ELISA. Lack of induction of OVA-specific IgE in sensitized CD4Cre x IL-4/13 fl/fl. 3-6 mice per group. Error bars represent SEM.

C. Hematoxylin and eosin (top row) stain and periodic acid-Schiff (PAS) (bottom row) stain of lung tissue from mice sensitized and challenged with OVA. Images shown at 20X magnification.

Figure 5


A
Airway responsiveness
RL (cm H2O/ml/Sec)
30
20
10
0
0.1
0.3
1
3
9.6
16
Acetylcholine (μg/g bodyweight)
*
*
IL-4/13 fl/fl saline
IL-4/13 fl/fl OVA
CD4Cre IL-4/13 fl/fl saline
CD4Cre IL-4/13 fl/fl OVA
CD4Cre IL-4/13 -/- saline
CD4Cre IL-4/13 -/- OVA
B
OVA-specific IgE
Arbitrary units
20
15
10
5
0
Saline
OVA
IL-4/13 flfl
IL-4/13 fl/fl
IL-4/13 -/-
CD4Cre
C
CD4Cre
IL-4/13 fl/fl
IL-4/13 fl/fl
IL-4/13-/-
H & E
PAS

Cell recruitment to the lung was also assessed as a parameter of pulmonary inflammation. In the BAL, total cell numbers were decreased in OVA-sensitized CD4Cre x IL-4/13-/- mice compared to IL-4/13 fl/fl mice (Fig. 6A), correlating with decreased severity of airway hyperresponsiveness compared to the wildtype group. The CD4Cre x IL-4/13 fl/fl group had slightly decreased overall numbers of cells in the BAL, but the number of macrophages, lymphocytes, and neutrophils (PMNs) were similar to that in the IL-4/13 fl/fl group. The slight decrease in total cell numbers in CD4Cre x IL-4/13 fl/fl BAL was due to a significant decrease in eosinophil recruitment (Fig.6A). These differences in the cell populations seen in the BAL was reflected in the number of cells in lung tissue as quantified by flow cytometry. CD4 T cells were increased across the three experimental groups with OVA challenge and the overall percent of CD4 T cells was equivalent across the groups (Fig. 6B, top panel). The number and percent of lung basophils also increased similarly across the three groups (Fig. 6B, bottom panel). Eosinophil recruitment to the lung was decreased in the CD4Cre x IL-4/13 fl/fl experimental group, nearly to the extent of impairment seen in CD4Cre x IL-4/13-/- mice (Fig. 6B, middle panel). This suggests that CD4 T cells that produce IL-4/13 largely play a role in eosinophil recruitment in this model.

Discussion

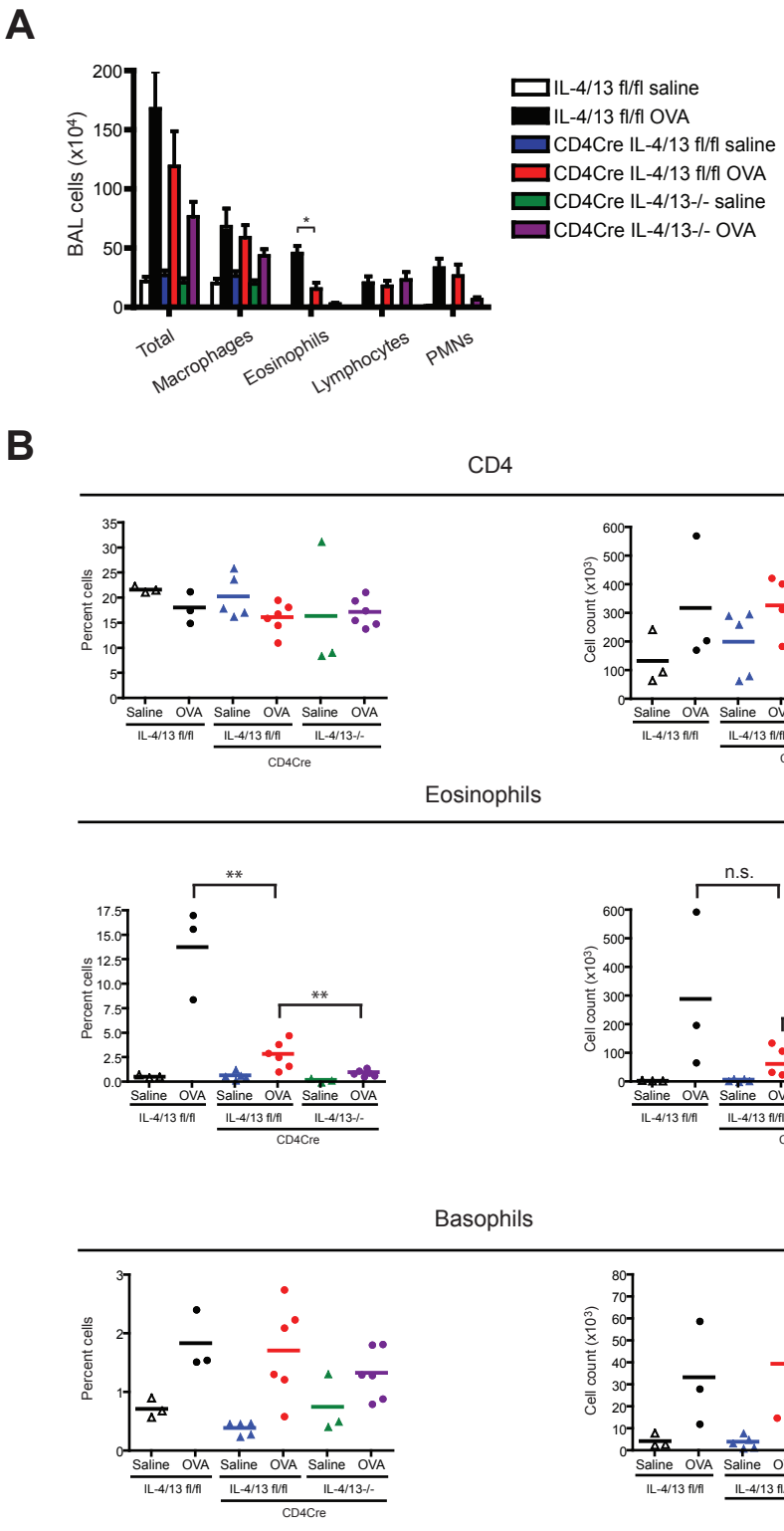
The conditional knockout model of IL-4/13 provides a powerful reagent in identifying the individual components of Type 2 responses and their relative contributions to acute and chronic inflammatory settings. Our finding, in which intestinal *N. brasiliensis* clearance in CD4Cre x IL-4/13 fl/fl mice was similar to wildtype mice, is

Figure 6. Decreased eosinophil recruitment to lungs in CD4Cre x IL-4/13 fl/fl mice with allergic airway inflammation

A. Numbers of total cells, macrophages, eosinophils, lymphocytes, and neutrophils (PMNs) in BAL fluid. Error bars represent SEM. *$p<0.05$ as determined using Student's t-test.

B. Percent and total CD4 T cells, eosinophils and basophils in left lobe of lung tissue in mice sensitized with OVA and challenged with PBS or OVA. Cells enumerated by flow cytometric analysis. Dots represent individual mice (3-6 mice/group). Bars represent the mean. *$p<0.05$, **$p<0.01$ as determined using Student's t-test. n.s.= not significant.

Figure 6


A
BAL cells (x10⁴)
200
150
100
50
0
*
Total
Macrophages
Eosinophils
Lymphocytes
PMNs
IL-4/13 fl/fl saline
IL-4/13 fl/fl OVA
CD4Cre IL-4/13 fl/fl saline
CD4Cre IL-4/13 fl/fl OVA
CD4Cre IL-4/13-/- saline
CD4Cre IL-4/13-/- OVA
B
CD4
Percent cells
Cell count (x10³)
Saline OVA Saline OVA Saline OVA
IL-4/13 fl/fl
IL-4/13 fl/fl
IL-4/13-/-
CD4Cre
Eosinophils
**
**
n.s.
*
Basophils

consistent with the IL-4/13-deficient CD4 T cell adoptive transfer used by Voehringer et al. (2006). Additionally, we observed cell recruitment patterns to the lung similar to the Voehringer et al. (2006) study (data not shown). The conditional knockout model confirms that in an acute infection setting, CD4 T cells (but not IL-4/13 derived from them) likely help recruit and organize innate effector cells that produce IL-4/13 sufficient to mediate worm clearance. Additionally, our results suggest that acute intestinal helminth clearance in this model does not require IgE.

This is in contrast to our findings in *S. mansoni* infection and OVA-induced airway hyperreactivity, where the conditional knockout mice had an intermediate phenotype, possibly due to the chronicity of infection or inflammation. *N. brasiliensis* infection is resolved within 10-12 days in healthy hosts, versus disease progression lasting at least 24 days (OVA-induced asthma model) or 56 days (*S. mansoni* infection). The intermediate phenotype observed in *S. mansoni* infection suggests that, in this setting of chronic inflammation, CD4 T cells play an important role in disease progression, possibly through: 1) inducing and sustaining IgE production, 2) maintaining the recruitment of IL-4/13-producing innate effector cells, or 3) promoting an unknown CD4 T cell-specific effector function. In the *S. mansoni* infection experiments, the hepatic granulomas in CD4Cre x IL-4/13 fl/fl mice by 8 weeks were more diffuse and had less collagen compared to those in wildtype or IL-4/13 fl/fl mice. Innate effector cell recruitment (such as eosinophils) or innate effector cell activation (such as basophils) may have been impaired, thus impacting the level of IL-4/13 produced. Previous studies have demonstrated that eosinophils are dispensible for *S. mansoni* infection (Swartz et al., 2006); therefore, basophils may be critical for IL-4 and/or IL-13 production in this

setting. Studies have demonstrated that IL-4-producing basophils are present during *S. mansoni* infection by using the KN2 IL-4 secretion reporter system (Mohrs et al., 2005; Sullivan et al., manuscript in preparation). Additionally, basophil production of IL-4 may be severely impacted by abrogated levels of antigen-specific IgE in the absence of IL-4 from CD4 T cells, as basophils express the high affinity FcεR and become activated upon crosslinking of IgE bound to the receptor. Also, we have not excluded the role if CD4 T cell-specific IL-4/13 on macrophage alternative activation. Therefore, further experiments with careful quantification of cell populations and collagen content in granulomas are warranted.

In the OVA-induced airway challenge experiments, we also observed an intermediate phenotype in the CD4Cre x IL-4/13 fl/fl mice. We observed partial protection from airway hyperreactivity, consistent with that observed by Voehringer et al. (2006). The decreased PAS staining in airway epithelia suggests that CD4 T cell-derived IL-13 may be partially responsible for goblet cell hyperplasia and mucous secretion. Blunting of innate cell recruitment was more dramatic in the CD4Cre x IL-4/13 fl/fl model compared to the model of IL-4/13-deficient CD4 T cell transfer. This difference may be due to homeostatic proliferation of T cells in the adoptive transfer model. Our data suggest a role for CD4 T cell-derived IL-4/13 in the recruitment of eosinophils but not basophils. The decrease in eosinophils could account for the protection in airway responsiveness. Taken together, these results suggest a role for CD4 T cell-derived IL-4 and IL-13 in specific aspects of airway inflammation. Our findings are consistent with previous studies that demonstrate that IL-4 from T cells is essential for IgE production.

While basophil recruitment to the lung was unaffected, they may rely on IgE for sustained activation to drive the host defense response.

In each of the three experimental models that we analyzed using the conditional IL-4/13 deficient mice, IgE production in Type 2 responses was dependent on IL-4 specifically from CD4 T cells. This is consistent with Type 2 cytokine production by T follicular helper cells in lymph nodes driving B cell immunoglobulin class switching and affinity maturation in germinal center reactions (Reinhardt et al., 2009). In the chronic disease models of *S. mansoni* infection and OVA-induced airway hyperresponsiveness, CD4 T cells may be important for early effector cell recruitment as well but also play a role later in the chronic phase by inducing and maintaining IgE production that will arm innate effector cells, particularly basophils to produce a high level of IL-4, since they express the high affinity FcεR.

These findings begin to highlight that although innate and adaptive components may have overlapping functions in acute responses, they may contribute nonredundant functions in the setting of chronic Type 2 inflammation. Additionally, we have not ruled out a role for IL-4 and IL-13 in secondary infections such as with *N. brasiliensis*; therefore, further analysis with our conditional knockout model is required. Extensions of the conditional knockout model can also be applied to studies in which IL-4/13 is deleted in specific innate cell types using different mouse models of Cre recombinase. Additionally, temporal regulation of IL-4/13 can also be analyzed using this system by crossing the conditional IL-4/13 mice with mice that express an inducible Cre recombinase. Temporal deletion of IL-4/13 would help differentiate their roles in early versus late phases of Type 2 responses, as well as in tissue repair. Regardless, as we

uncover new cells and cytokine mediators that interact with innate and adaptive immunity, we can start to appreciate the complex, yet elegant, orchestration of Type 2 responses.

MATERIALS AND METHODS

Mice

CD4Cre (Lee et al., 2001), IL-4/13-/- (McKenzie et al., 1999), and IL-4/13 fl/fl (Voehringer et al., 2009) were crossed onto the Balb/c background and were maintained in the UCSF specific pathogen-free barrier according to institutional guidelines and used between 6 and 8 weeks of age.

T cell polarization- intracellular staining and cytokine production

24-well plates were pre-coated with 2 μg/ml each of anti-CD3 and anti-CD28 (BD Pharmingen) in PBS and incubated overnight at 4°C. Lymph nodes were harvested from wildtype Balb/c, IL-4/13 fl/-, and CD4Cre; IL-4/13 fl/- mice. Samples were homogenized and passed through 40 μm filters to generate single cell suspensions. CD4 T cells were isolated by positive selection using magnetic bead isolation (Miltenyi) according to manufacturer's instructions. 1 x 10^6 CD4 T cells in complete RPMI (10% FCS, pen/strep, L-glutamine) were added per well on the anti-CD3 and anti-CD28-coated plates and incubated with appropriate cytokines and antibodies for polarizing conditions for Th0 (50 U/ml IL-2), Th1 (50 U/ml IL-2, 10 ng/ml IL-12, 50 μg/ml anti-IL-4), and Th2 (50 U/ml IL-2, 50 ng/ml IL-4, 50 μg/ml anti-IFN-γ). All cultures were incubated at 37°C, 5% CO_2. On day 3 of culture, 800 μl of 1° supernatants were pipetted off and stored for analysis. Cells were then transferred to 6-well plates with 50 U/ml IL-2 to allow for expansion. Cells were split on days 5 and 7 incubated in 50 U/ml IL-2 to allow for further expansion. On day 8, cells were harvested and divided for intracellular cytokine staining and for supernatant harvest for ELISA. For ELISA, cells were re-plated

at 1 x 10^6 cells on a 24-well plate pre-coated with 2 μg/ml anti-CD3 for 2° stimulation. Culture supernatant (800 μl) was harvested on day 9 and stored for analysis. For intracellular cytokine analysis, cells were re-plated at 5 x 10^6 cells per well of a 6-well plate and incubated overnight in media with 50 U/ml IL-2.

For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 2 hours followed by secretion block with 3 μM monensin. Cells were fixed with 4% PFA, permeabilized with 0.1% saponin in PBS, and stained with antibodies to IFN-γ, IL-4, IL-13, and CD4. Isotype antibodies were used to control for staining (FITC-IgG1, PE-IgG1, APC-IgG1). Samples were analyzed on an LSRII (Becton Dickinson) and data analyzed on FloJo software (Treestar).

Semi-quantitative PCR

CD4 T cells were obtained from lymph nodes by magnetic bead isolation. Cells were lysed followed by DNA purification according to manufacturer's instructions (Gentra PureGene). 200 ng DNA with 1:3 serial dilutions were analyzed by PCR with primers to detect the presence of the FRT and loxP recombination sequences at the IL-4 locus. (Primer pair: 5'-ttaaaactgctaacacagtagaactac-3' and 5'-gaaacaggttctcattatgtag-3') PCR conditions were 35 cycles with 30s at 94°C, 30s at 58°C, 60s at 72°C followed by a final elongation for 10 min at 72°C. PCR products were visualized on a 1.5% agarose gel.

Helminth infection

L3 stage *Nippostrongylus brasiliensis* were harvested from fecal cultures of infected rats, washed extensively with 0.9% saline, and injected (500 organisms)

subcutaneously at the base of the tail. Mice were maintained for 5 days on antibiotic water (0.1 g/L polymixin B, 2 g/L neomycin). On day 12 of infection, blood was collected by cardiac puncture for serum IgE analysis. To quantify intestinal worm burden, small intestines were harvested, cut open longitudinally, and incubated in 15 ml Hanks' Balanced Salt Solution at 37°C for 3 hours. Worms were visualized under a dissecting microscope and enumerated.

Schistosoma mansoni was maintained in the laboratory using *Biomphalaria glabrata* snails. For *S. mansoni* infections, mice were injected subcutaneously with 200-280 cercariae and survival monitored. At 6 and 8 weeks post-infection, surviving mice were killed for tissue analysis. Blood for serum analysis was collected by cardiac puncture. At the time of euthanasia, the hepatic portal system was perfused for worm collection and count. To quantify hepatic egg burden, portions of livers were homogenized and digested in porcine trypsin in PBS at 37°C with shaking. Eggs were sedimented at 4°C and counted under a dissecting microscope. Portions of liver were also collected for fixation in formalin followed by paraffin embedding for histology. Splenocytes were collected and restimulated in culture with PMA and ionomycin to measure IL-4 and IFN-γ production. IgE, IL-4, and IFN-γ were measured by ELISA as described below.

Histology

Paraffin embedded liver sections stained with standard H&E. Collagen in liver tissue sections was visualized using Masson Trichrome Stain (Sigma-Aldrich) with

Weigert's Iron Hematoxylin (Sigma-Aldrich) counterstain according to manufacturer's instructions.

ELISA

For IgE ELISA, 96-well Immulon plates were coated overnight (4°C) with (concentration) anti-IgE capture antibody (BD). The plates were blocked with 3% BSA (USB Corp.) in PBS at room temperature for 2 hrs and washed off. IgE protein standards (0.5 μg/ml (BD) with 1:2 serial dilutions and serum samples (with 1:3 serial dilutions) were added to the plate with capture antibody and incubated overnight at 4°C. Plates were washed and incubated with biotinylated anti-IgE (BD) in 1% BSA in PBS and incubated at room temperature for 1 hr. After washing twice, plates were incubated with streptavidin alkaline-phosphatase (1:1000 dilution in 1% BSA in PBS) at room temperature for 1 hr. Plates were washed and developed with detection buffer (6.5 mM Na_2CO_3 , 18.5 mM $NaHCO_3$) with 5 mM NPP (Sigma) and 2 mM $MgCl_2$. Plates were read at 405 nm wavelength on plate reader (Spectramax; Molecular Devices).

For IL-4 ELISA, anti-IL-4 (11B11) capture antibody and biotinylated-anti-IL-4 (BVD6) antibody were used. For IFN-γ, anti-IFN-γ (R46A2) capture antibody and biotinylated anti-IFN-γ (XMG 1.2) antibody were used. ELISA analysis was performed as described above.

OVA-induced airway inflammation

Mice were given saline or 50 μg OVA emulsified in 1 mg alum i.p. on days 0, 7, and 14. On days 21, 22, and 23, mice were challenged by intranasal administration of 100

μg OVA. On day 24, one day after the last OVA challenge, mice were assessed for lung function with in response to acetylcholine. The tracheae of anesthetized (100 mg/kg Ketamine, 10 mg/kg xylazine, 2-3 mg/kg acepromazine) mice were cannulated with 20G adaptor tubes connected to a rodent ventilator and pulmonary mechanics analyzer (FlexiVent, SIRAQ Inc.). Mice were ventilated at a tidal volume of 9 ml/kg, a frequency of 150 breaths/minute, and 2 cm H_2O positive end-expiratory pressure. Mice were paralyzed with pancuronium (0.1 mg/kg intraperitoneally) and airway mechanics were measured by a linear single compartment model in response to increasing doses of acetylcholine (0.03, 0.1, 0.3, 1, and 3 μg/g body weight) administered through the tail vein.

Lungs were lavaged 3 times each with 1 ml PBS. After centrifugation (1000 rpm, 5 min.), the supernatant was removed and the cell pellet was resuspended in normal saline after RBC lysis. Total cells were counted with a hemacytometer. Cytospin preparations were prepared and stained with a HEMA 3 stain set (Fisher), and bronchoalveolar lavage (BAL) fluid cell differential percentages were determined based on light microscopic evaluation of >300 cells/slide.

After lavage, one lobe of the lung was collected for flow cytometric analysis. Lungs were homogenized and passed through 70 μm cell strainers to generate single cell suspensions. After red blood cell lysis with PharmLyse (BD Pharmingen), samples were washed with FACS wash buffer (PBS, 3% FCS, 0.05% NaN_3) and incubated with FcR block, and stained with the appropriate antibody mixtures. Antibodies used to stain samples include: APC-Alexa Fluor 750-anti-CD4 (RM4-5; CalTag), PE-anti-Siglec-F (E50-2440; BD Biosciences), PerCP Cy5.5-anti-CD11b (M1/70; BD Pharmingen), APC-

anti-pan-NK (CD49b) (DX5; eBioscience), FITC-anti-CD19 (1D3; BD Pharmingen), FITC-anti-IgE (R35-72; BD Pharmingen), PE-anti-CD131 ($\beta_{IL\text{-}3R}$, β_c) (JORO50; BD Pharmingen). Cells were incubated in 0.5 μg/ml DAPI nuclear stain to exclude dead cells. Cell counts were determined using CountBright Absolute Counting Beads (Invitrogen). Data was acquired on an LSRII (Becton Dickinson) and analyzed using FloJo software (Tree Star).

For histology, lungs were inflated with 10% buffered formalin to 25 cm H_2O of pressure and transferred into tubes containing 10% buffered formalin. Multiple paraffin-embedded 5-μm sections of the entire mouse lung were prepared and stained with hematoxylin and eosin (H&E) for regular morphology and with periodic acid-Schiff (PAS) for mucus production and evaluation.

Blood was also collected by cardiac puncture for serum IgE analysis. OVA-specific IgE levels were measured by ELISA using microplates coated with OVA. Diluted serum samples were added to each well, and the bound IgE was detected with biotinylated anti-mouse IgE (Pharmingen, R35-118). Colormetric assay was performed using streptavidin conjugated horseradish peroxidase (Pharmingen) followed by the addition of HRP substrate (TMB, BD Biosciences Pharmingen). OD readings of samples at 450 nm were obtained and the results are expressed as OD value (arbitrary units).

Acknowledgements

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CHAPTER III:

EOSINOPHILS SUSTAIN ALTERNATIVELY ACTIVATED MACROPHAGES IN ADIPOSE TISSUE

Abstract

Eosinophils are associated with immunity to helminthes and allergic disorders, often together with alternatively activated macrophages (AAM). AAM, which can be induced by the cytokines IL-4 or IL-13, are present in white adipose tissue and sustain normal glucose homeostasis. Here, we show that eosinophils constitute the major IL-4-expressing cells in white adipose tissues of mice. Eosinophils migrate into adipose when transferred into eosinophil-deficient animals and migration is impeded by integrin-blocking antibodies or after transfer into animals on high-fat diet. AAM are diminished in adipose tissues of eosinophil-deficient mice and are restored by reconstitution of the eosinophil lineage. In the absence of eosinophils, mice on high-fat diet develop increased body fat and impaired glucose tolerance, thus revealing an unexpected role for eosinophils in metabolic homeostasis through maintenance of AAM in adipose tissue.

Introduction

Adipose tissue macrophages have a central role in promoting chronic low-grade inflammation, which contributes to obesity and insulin resistance that characterize the metabolic syndrome (Hotamisligil, 2006). Although adipose macrophages from obese animals have a classically activated inflammatory phenotype, adipose macrophages from healthy lean mice have an alternatively activated phenotype (Lumeng et al., 2007). Impeding the ability of macrophages to become alternatively activated by disrupting the nuclear hormone superfamily receptor peroxisome proliferator-activated receptor (PPAR)-γ renders mice susceptible to diet-induced obesity and glucose intolerance (Odegaard et al., 2007; Bouhlel et al., 2007). Human PPARγ loss-of-function mutations are also associated with insulin resistance and type 2 diabetes mellitus (Jeninga et al., 2009). PPARγ is induced in macrophages by IL-4 or IL-13, and directly promotes arginase-1 expression, one of the signature genes in AAM (Martinez et al., 2009). Studies *in vitro* and with adipocyte cell lines suggest that adipocytes themselves can be sources of IL-4 and IL-13 (Kang et al., 2008), but analysis of adipose tissues *in vivo* are needed to ascertain more definitively the source of these cytokines.

Results

Alternatively activated macrophages in adipose tissue

Studies of AAM in adipose have relied on PCR-based detection of signature genes in order to confirm the presence of these cells (Weisberg et al., 2003), but single cell analysis has not been possible. With specific deletion of PPARγ in macrophages, (LysMCre), AAM development is impaired (Odegaard et al., 2007). Arginase-1 is a

signature gene in AAM that can be induced directly by IL-4 (Gordon, 2003). Using YARG arginase-1 reporter mice developed by Hong-Erh Liang in which YFP is expressed downstream of the last exon of arginase-1 (Reese et al., 2007), we detected *in vivo* the presence of AAM in adipose tissue. Initially, we detected AAM isolated from adipose tissue by a modified "walkout" cell dispersal protocol in YARG mice on an IL-4/13-sufficient background (Fig. 1, Bedford et al., 2006). These macrophages as analyzed by flow cytometry are $CD11b^{hi}$ $F4/80^{hi}$ and have the characteristic large cell size based on forward- and side-scatter characteristics. However, when YARG mice were crossed onto the IL-4/13-deficient background, alternative activation in macrophages isolated by walkout was dramatically diminished. Interestingly, the macrophages appear to be "starved" for IL-4/13 and are poised for alternative activation. When recombinant mIL-4 was added exogenously to the walkout cultures of YARG x IL-4/13-/-, alternative activation, as measured by YFP expression, increased and was detected at even higher levels when compared to alternative activation in the resting state in wildtype YARG adipose tissue macrophages (Fig. 1). This observation suggested that macrophage alternative activation can largely be driven by the presence of IL-4 in adipose tissue.

IL-4 competent cells in adipose tissue

The potential role of IL-4 in adipose tissue prompted us to probe further for IL-4-producing cells in adipose tissue. To begin an unbiased analysis of IL-4-expressing cells in perigonadal adipose tissue of mice on normal chow diet, we used 4get IL-4 reporter mice which contain a fluorescent GFP reporter downstream of an IRES element following the endogenous *il4* gene, thus facilitating recognition of IL-4-competent cells

Figure 1

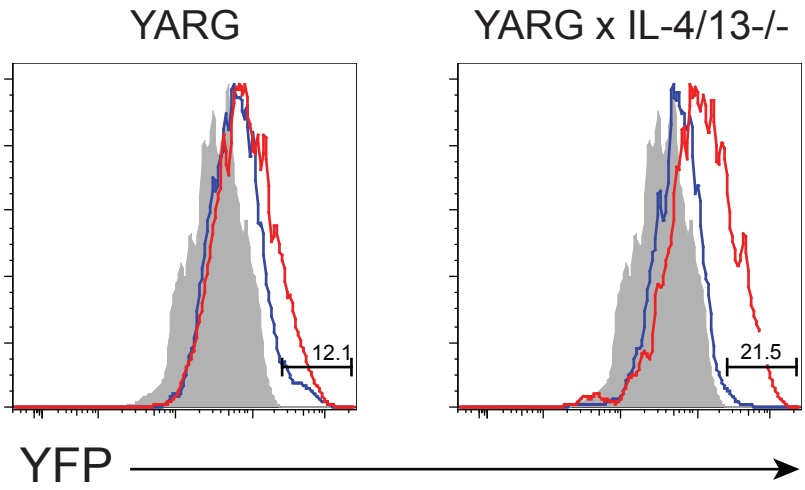


Figure 1. IL-4 induces adipose tissue macrophage alternative activation. Pergonadal white adipose tissue from YARG and YARG x IL-4/13-/- mice were minced in media and incubated overnight by the 'walkout' method. Tissue and cells were incubated untreated (blue histogram) or with 0.05 µg/ml recombinant IL-4 (red histogram). Gray histogram represents wildtype sample to control for autofluorescence. Live cells gated based on DAPI exclusion and forward- and side-scatter size characteristics. Macrophages gated as $CD11b^{hi}$ $F4/80^{hi}$. Values represent percent of IL-4-activated YFP+ macrophages (red histogram) as gated above background autofluorescence.

in vivo as revealed by their spontaneous fluorescence and without the need for re-stimulation *ex vivo* (Mohrs et al., 2001). We first analyzed cells spontaneously migrating out of minced adipose tissue during overnight incubation in media, thus enabling analysis of cells after minimal manipulation. Whereas small numbers of IL-4-expressing (GFP+) CD4 T cells could be recovered, the majority of emigrating GFP+ cells were eosinophils (Fig. 2A). After confirming that the conditions of tissue digestion did not alter the expression of surface molecules used for cell identification (data not shown), we enzymatically digested perigonadal adipose and recovered all IL-4-expressing cells for analysis. As assessed using standard surface markers and size characteristics (Fig. 3), 90% of the IL-4-competent cells recovered from perigonadal adipose of mice on normal chow diet were eosinophils, with the remainder comprising small numbers of basophils, CD4 T cells and innate lymphoid helper cells (Fig. 2B, Fig. 3). Like adipose macrophages, adipose eosinophils were CD11b+F4/80+, but were distinguished by expression of the sialic acid-binding immunoglobulin receptor superfamily member, Siglec-F (Fig. 2C; Fig. 3). Analysis of adipose tissues from 4get mice with a GATA1 promoter mutation that lack eosinophils (ΔdblGATA mice; Yu et al., 2002) confirmed that the isolated cells were eosinophils (Fig. 2C). Eosinophils upregulate the inhibitory Siglec-F receptor as they move from blood into tissues (Voehringer et al., 2007). In comparison to blood eosinophils from the same mice, adipose eosinophils had up-regulated Siglec-F expression, consistent with their residence in tissue (Fig. 2D). Examination of white adipose from non-reporter mice confirmed that eosinophil-like cells identified using standard histochemistry could be localized to crown-like structures with other hematopoietic cells in normal perigonadal fat (Fig. 2E). Flow cytometric and

Figure 2. Eosinophils are major IL-4-expressing cells in visceral adipose tissue.

A. Minced perigonadal adipose tissues from wildtype and IL-4 reporter (4get) mice were incubated in media overnight. 'Walkout' cells were collected and live cells were analyzed for expression of the GFP-marked IL-4 reporter allele. Top panels, CD4 T cells; bottom panels, side-scatter (SSC) high eosinophils. Outlines indicate GFP gates as set using cells from wildtype mice (left panels). Histograms show wildtype (gray) and 4get (red) cell populations analyzed for GFP expression (percentages indicated above bar for positive gate) among total recovered CD4 T cells (top) and eosinophils (bottom).

B. Analysis of total GFP-positive cells from enzymatically digested perigonadal adipose tissues from 4get mice on normal chow diet. Live cells gated based on DAPI exclusion and forward- and side-scatter size characteristics. Eosinophils are GFP+ $CD11b^{int}$ Siglec-F+. Other GFP+ cells in adipose tissues from 4get mice were identified using SSC/FSC profiles and the following markers: CD4 T cells: CD4+, CD3+; basophils: DX5+, FcεRI+; innate lymphoid helper cells: T/B/NK lineage-negative, c-Kit+, Sca-1+; Undefined: conjugates or other positive cells without the clear combination of markers noted.

C. Flow cytometric analysis of perigonadal white adipose tissue from wildtype and eosinophil-deficient (ΔdblGATA) mice with eosinophil gate outlined by Siglec-F and CD11b expression. CD11b+Siglec-F-negative cells are macrophages.

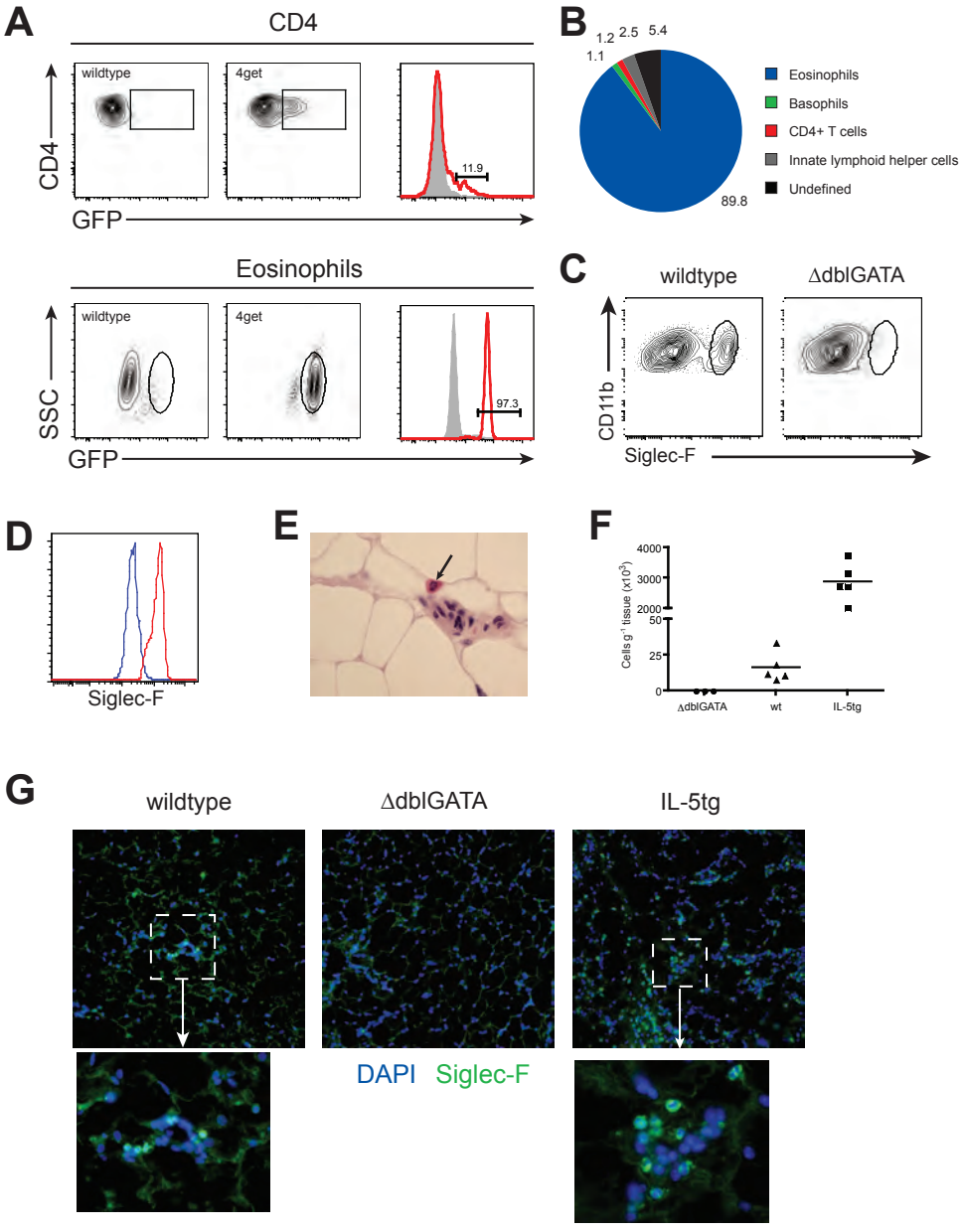
D. Surface Siglec-F expression levels on adipose (red) and blood eosinophils (blue) from the same mouse.

E. Hematoxylin and eosin stain of paraffin-embedded adipose with eosinophil adjacent to crown-like structure. Image shown at 400X magnification.

F. Eosinophils as quantitated by flow cytometry in perigonadal adipose from 8 wk-old mice from eosinophil-deficient (ΔdblGATA), wildtype (wt) and hypereosinophilic (IL-5tg) mice on normal chow diet.

G. Immunohistochemical stain for Siglec-F+ cells in adipose tissue from wildtype, eosinophil-deficient (ΔdblGATA) and hypereosinophilic (IL-5tg) mice. Siglec-F, green; nuclei counterstain with DAPI, blue. Images shown at 20X magnification

Figure 2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Eosinophils
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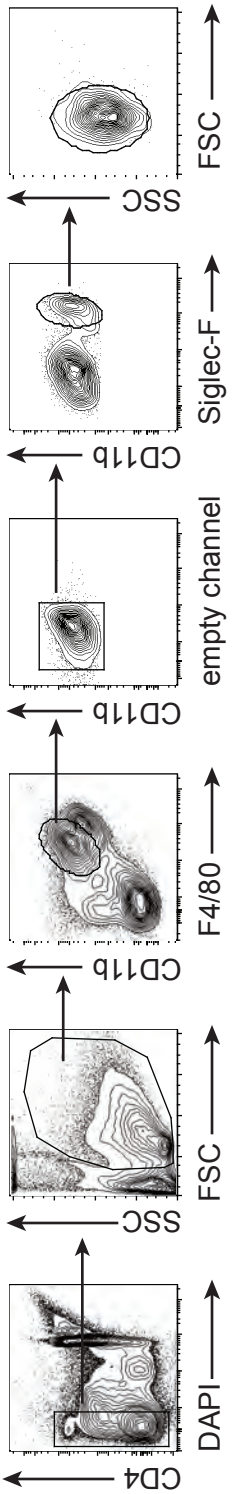
Siglec-F

Figure 3. Flow cytometric gating for adipose tissue eosinophils.

Cells were gated for live, DAPI-negative, and side- and forward-scatter (SSC/FSC) before analysis using CD11b and F4/80 antibodies. Eosinophils are in a $CD11b^{int}F4/80^{int}$ population and are additionally selected with an empty channel to gate out autofluorescent cells. Eosinophils are Siglec-F+ within the $CD11b^{int}$ population. Backgating on the Siglec-F+ cells confirms that the cells are eosinophils with a characteristic SSC/FSC profile.

Figure 3


CD4
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F4/80
CD11b
empty channel
CD11b
Siglec-F
SSC
FSC

immunohistochemical examination of adipose tissue from wild-type, eosinophil-deficient and hypereosinophilic IL-5 transgenic mice (Lee et al., 1997) confirmed that Siglec-F-positive cells with the appearance of eosinophils were present in adipose tissue in numbers that correlated with the eosinophil status of the mice (Fig. 2F, 2G).

Eosinophils produce IL-4 in adipose tissue

Using mice with a knockin human CD2 replacement gene at the IL-4 start site to mark cells that have secreted IL-4 protein (KN2; Mohrs et al., 2005), we could show that the majority of IL-4 secreting cells in adipose were eosinophils, although the number of IL-4-secreting cells was only a small population of the total IL-4-competent cells. We infected KN2 mice crossed onto the 4get background with *N. brasiliensis* larvae and analyzed lung and adipose tissue on day 10 of infection. In the lung, taken as a positive control, IL-4-competent cells that express surface hCD2 were eosinophils (SSC^{hi}), CD4 T cells, and CD4-negative SSC^{lo} cells (basophils and other cells). Lung eosinophils and CD4 T cells were in near equal proportion as a percent of hCD2-positive cells. In contrast, adipose tissue hCD2-positive cells were only eosinophils and CD4 T cells, with eosinophils composing the majority hCD2-positive cells (Fig. 4).

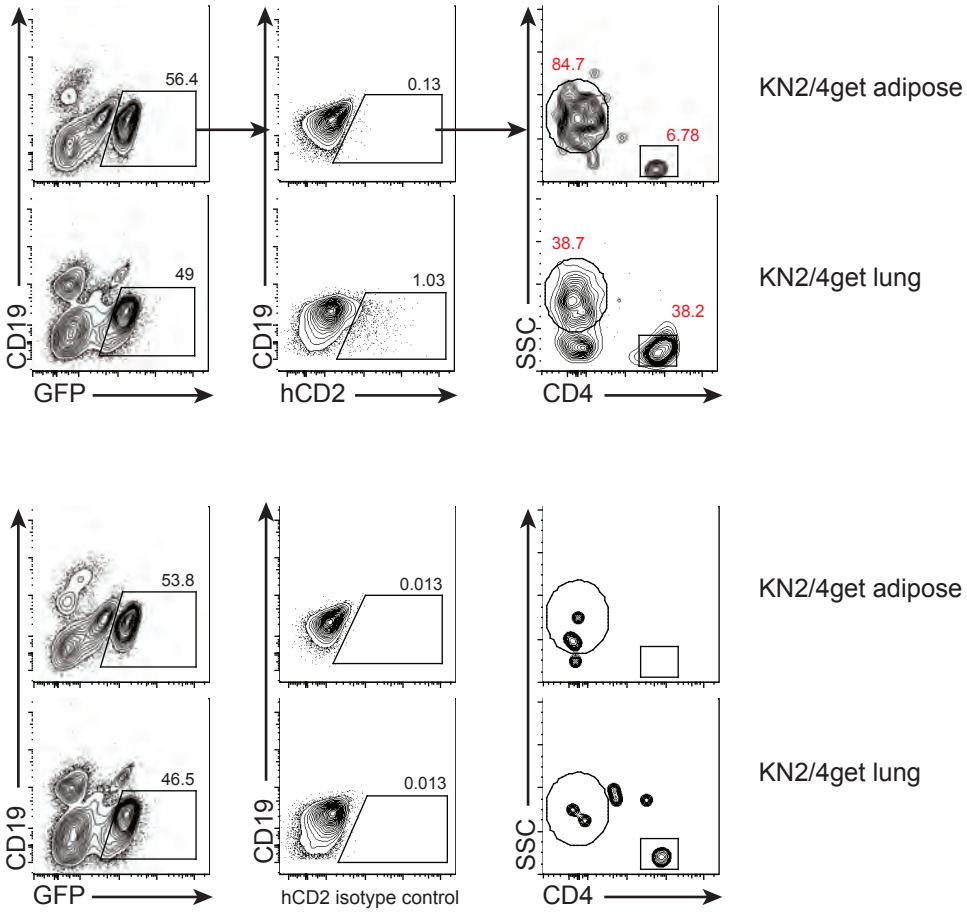
Eosinophil migration to adipose tissue is integrin-mediated and impaired by high fat diet.

To determine whether eosinophils can migrate into adipose tissues, we transferred eosinophils from the spleens of hypereosinophilic IL-5 transgenic x 4get mice to ΔdblGATA mice that lack eosinophils and harvested tissues for cell analysis. After three

Figure 4. Eosinophils are major IL-4 secreting cells in adipose tissue.

4get/KN2 mice were infected with the migratory helminth, *Nippostrongylus brasiliensis*, in order to induce IL-4-producing cells in the lung tissues for a positive control as previously described (Reinhardt et al., 2009). Ten days after infection, single-cell suspensions of lung and perigonadal adipose were prepared and analyzed for GFP fluorescence to assess IL-4 competence and for human CD2 expression to assess recent IL-4 secretion as described (Mohrs et al., 2005). Live cells were gated using DAPI exclusion and side- and forward-scatter (left panels; percentages of live cells shown). After gating for IL-4-competent cells based on expression of GFP, the positive cells were further gated based on expression of human CD2 (hCD2) as a marker for recent IL-4 secretion *in situ* (middle panels; percentages of live GFP+ cells shown). Isotype control antibody for hCD2 shown in lower panels. Gated values in right panels represent hCD2-positive cells as a percent of total hCD2-positive cells with the eosinophil and CD4 T cells gates circled. In adipose tissues, 85% of IL-4-secreting cells are eosinophils with the remainder CD4 T cells, whereas in lung, eosinophils, CD4 T cells and SSC^{lo}, CD4-negative cells comprise approximately equal sets of IL-4-secreting cells. Representative of 2 experiments shown.

Figure 4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hCD2 isotype control
SSC
CD4

days, eosinophils were readily recovered from lung, spleen and perigonadal fat. By seven days, however, when eosinophils had declined substantially in lung and spleen, eosinophils remained at stable levels in adipose (Fig. 5A). Intriguingly, eosinophils did not accumulate similarly in adipose tissues of mice that had been maintained on high-fat diet for an average of 15 weeks prior to adoptive transfer. Like eosinophils recovered from adipose tissues of wild-type mice, adoptively transferred eosinophils recovered from adipose had upregulated Siglec-F, consistent with their tissue residence, and distinct from the lower level of Siglec-F on spleen eosinophils harvested at the same time (Fig. 5B). In surveying a number of other body tissues, we also noted migration into and retention of eosinophils in the lamina propria of the small bowel, a site where eosinophils are constitutively present in wild-type mice (Fig. 5C). To confirm that eosinophil migration required transit from the blood to adipose tissues, we compared eosinophil accumulation in tissues of mice treated with antibodies that block VCAM-1- and ICAM-1-mediated transit on $\alpha 4$ and αL integrins expressed by eosinophils (Hogan et al., 2008). As compared with animals treated with isotype control antibodies, anti-integrin treatment completely blocked accumulation of eosinophils in adipose tissues (Fig. 5D). Conversely, in lung and spleen, where most eosinophils retain high Siglec-F expression and presumably remain within the abundant vasculature of these organs, eosinophils accumulated in animals treated with the anti-integrin antibodies. Anti-integrin antibodies similarly blocked eosinophil migration into adipose tissue after induction of endogenous eosinophilia with IL-25 (Fig. 6; Fort et al., 2001). Taken together, these data demonstrate that eosinophils can migrate via integrins and accumulate in adipose tissue, where they constitute the major IL-4-producing cells *in vivo*.

Figure 5. Eosinophil migration to adipose tissue is integrin-mediated and impaired by high-fat diet.

A. Eosinophils in the left lobe of the lung, spleen and perigonadal adipose tissue 3 and 7 days after adoptive transfer into eosinophil-deficient ΔdblGATA mice on normal chow (NC) or high-fat (HF) diet for approximately 15 wks.

B. Siglec-F expression levels on adoptively transferred eosinophils isolated from adipose (red) or spleen (blue) in the same animal.

C. 4get x IL-5tg eosinophils migrate to the small intestine lamina propria of ΔdblGATA mice and can be detected 7 days after adoptive transfer (right panel). 4get wildtype and eosinophil-deficient ΔdblGATA tissue sections included as controls. GFP, green; Siglec-F, red; DAPI, blue.

D. ΔdblGATA mice received antibodies to α4 and αL integrins (100 μg each) or control isotypes (IgG2a and IgG2b, 100 μg each) 2 hrs prior to adoptive transfer of eosinophils. Tissues were harvested 16 hrs later. Dots represent individual mice. Bars represent the mean. *$p<0.05$, **$p<0.01$ as determined using Student's t-test. n.s. = not significant.

Figure 5

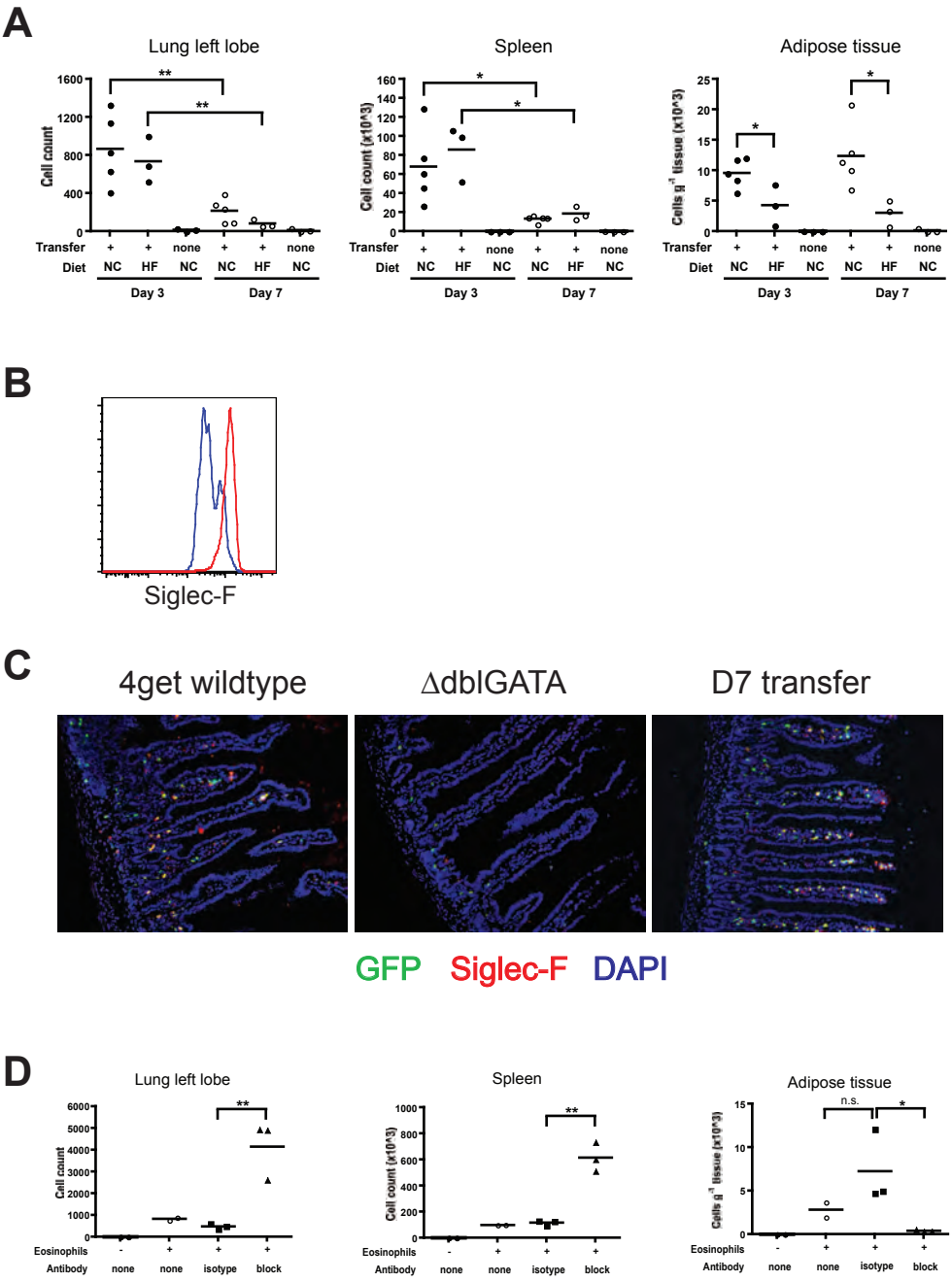
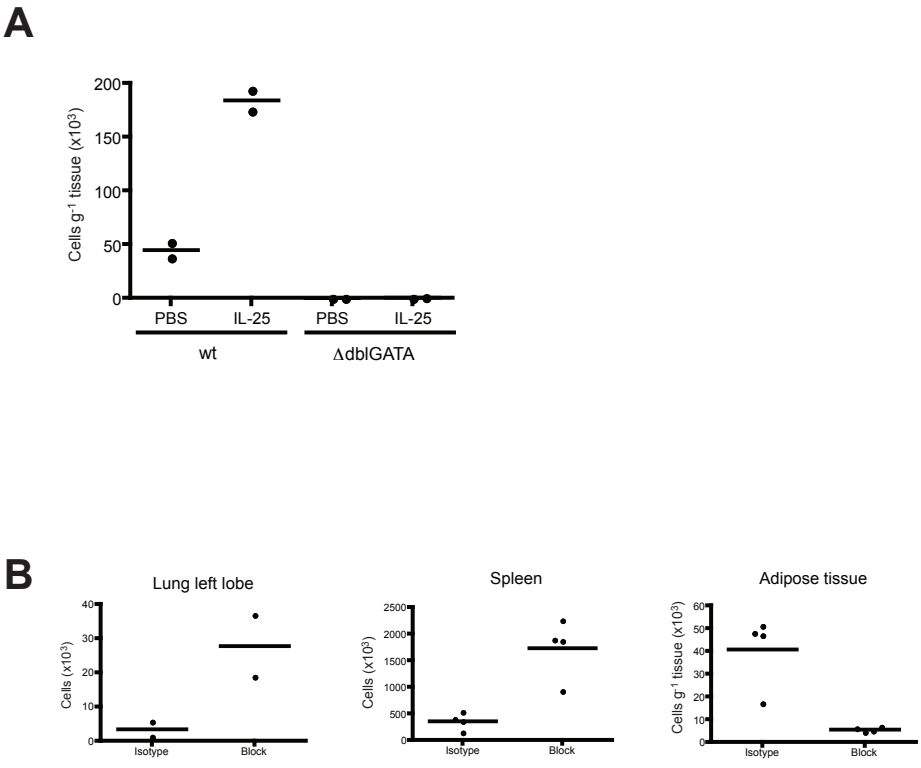

A
Lung left lobe
Spleen
Adipose tissue
Cell count
Cell count (x10^3)
Cells g^-1 tissue (x10^3)
Transfer
Diet
NC
HF
none
Day 3
Day 7
B
Siglec-F
C
4get wildtype
ΔdblGATA
D7 transfer
GFP
Siglec-F
DAPI
D
Lung left lobe
Spleen
Adipose tissue
n.s.
Eosinophils
Antibody
none
isotype
block

Figure 6. IL-25-induced eosinophil migration to adipose tissue is integrin-mediated.

A. 500 ng recombinant mIL-25 was administered intraperitoneally to 8 wk-old 4get or eosinophil-deficient (ΔdblGATA) x 4get mice for 4 consecutive days (day 0-3) and adipose tissue was analyzed for eosinophils on day 8.

B. 500 ng recombinant mIL-25 was administered intraperitoneally to 4get mice on 4 consecutive days. Mice received 100 μg each of antibodies against α4 and αL integrins or isotype control IgG2a and IgG2b antibodies on day 3 and 6. Tissues were analyzed for eosinophils by flow cytometry on day 8. Dots represent individual mice. Bars represent the mean.

Figure 6


A
Cells g-1 tissue (x103)
200
150
100
50
0
PBS
IL-25
PBS
IL-25
wt
ΔdblGATA
B
Lung left lobe
Cells (x103)
40
30
20
10
0
Isotype
Block
Spleen
Cells (x103)
2500
2000
1500
1000
500
0
Isotype
Block
Adipose tissue
Cells g-1 tissue (x103)
60
50
40
30
20
10
0
Isotype
Block

In vivo adipose tissue macrophage alternative activation is impaired in the absence of IL-4/IL-13 or eosinophils

Studies of AAM in adipose have relied on PCR-based detection of signature genes in order to confirm the presence of these cells, but single cell analysis has not been possible. Arginase-1 is a signature gene in AAM that can be induced directly by IL-4 (Martinez et al., 2009). We isolated cells in perigonadal visceral fat from YARG mice that have a fluorescent reporter introduced into the arginase-1 gene and that can used to identify alternatively activated macrophages *in vivo* (Reese et al., 2007). In both wild-type and YARG mice, similar numbers of CD11b+F4/80+ macrophages were present in adipose, and in YARG mice, fluorescent macrophages were readily detected without further manipulation, consistent with an alternatively activated phenotype as assessed directly *in vivo* (Fig. 7, Fig. 8A). We used size gating and Siglec-F expression to confirm that eosinophils do not express fluorescence in YARG mice (data not shown). In the absence of IL-4 and IL-13 (McKenzie et al., 1999), the numbers of total adipose macrophages remained similar, but the numbers of arginase-1-expressing macrophages diminished significantly, consistent with a role for these cytokines in sustaining the phenotype of adipose tissue macrophages and in agreement with prior studies using Stat6-deficient mice (Odegaard et al., 2007). Unexpectedly, adipose tissues from YARG mice that had been crossed onto the eosinophil-deficient ΔdblGATA background also demonstrated a significant reduction of AAM in adipose tissue, consistent with a role for eosinophils in sustaining this phenotype (Fig. 8A, 8B). To ascertain whether eosinophils are sufficient to sustain the AAM state in adipose tissue, we irradiated ΔdblGATA x YARG mice and reconstituted animals with bone marrow from 4get x IL-5 transgenic

Figure 7. Flow cytometric gating for YFP expression in adipose tissue macrophages from YARG mice.

Live cells were gated by DAPI exclusion and by side-/forward-scatter size (SSC/FSC). $CD11b^{hi}F4/80^{hi}$ cells were analyzed further to identify YFP+ macrophages in YARG mice (lower panels) that are absent in wildtype mice (upper panels). Backgating confirmed that the YFP+ cells are macrophages based on their large SSC/FSC size profile. Live YFP+ cells were also identified by gating with an empty channel to exclude autofluorescent cells falling along the horizontal axis to reveal cells absent from adipose tissues in wildtype mice but YFP+ in YARG mice (lower panels). Gated values represent percent of YFP+ cells as a percent of live cells.

Figure 7

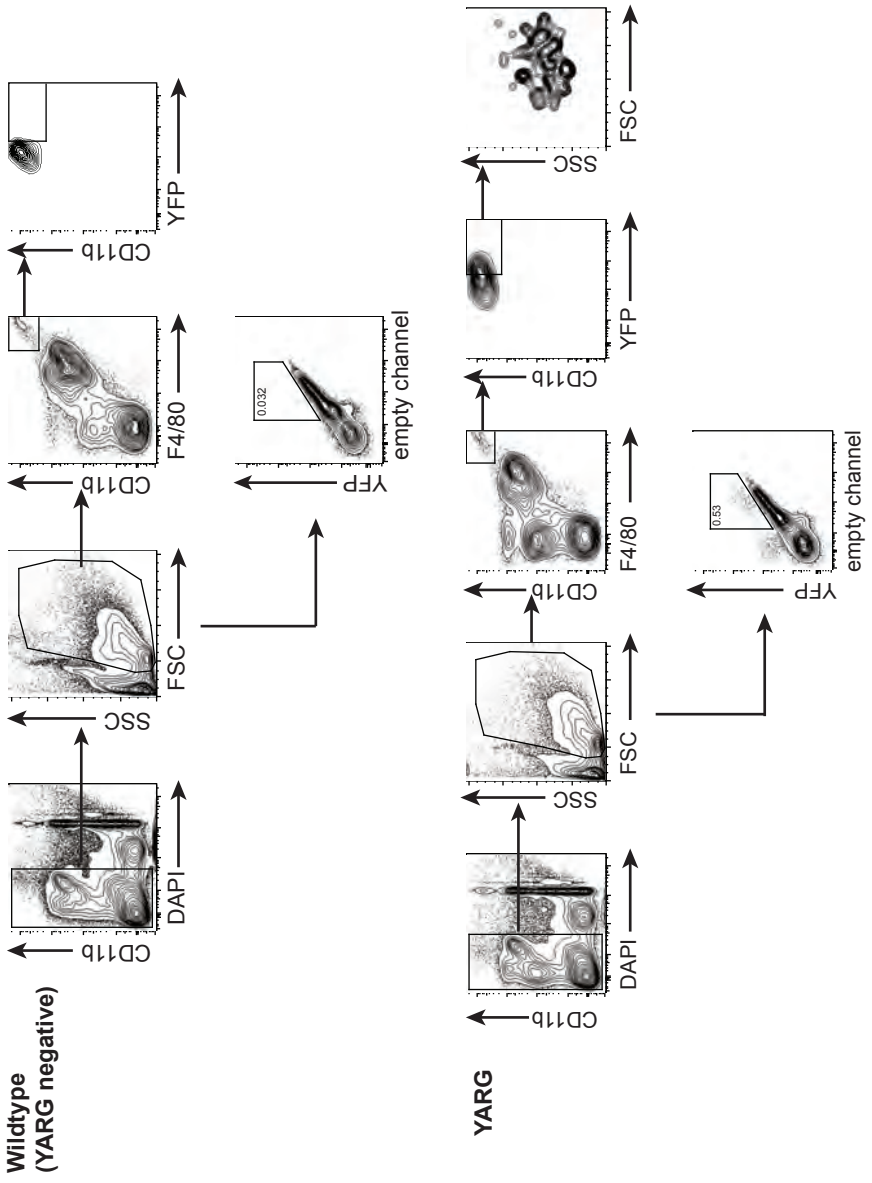

Wildtype
(YARG negative)
YARG
CD11b
DAPI
SSC
FSC
F4/80
YFP
empty channel
0.032
0.53

Figure 8. *In vivo* adipose tissue macrophage alternative activation is impaired in the absence of IL-4/13 or eosinophils.

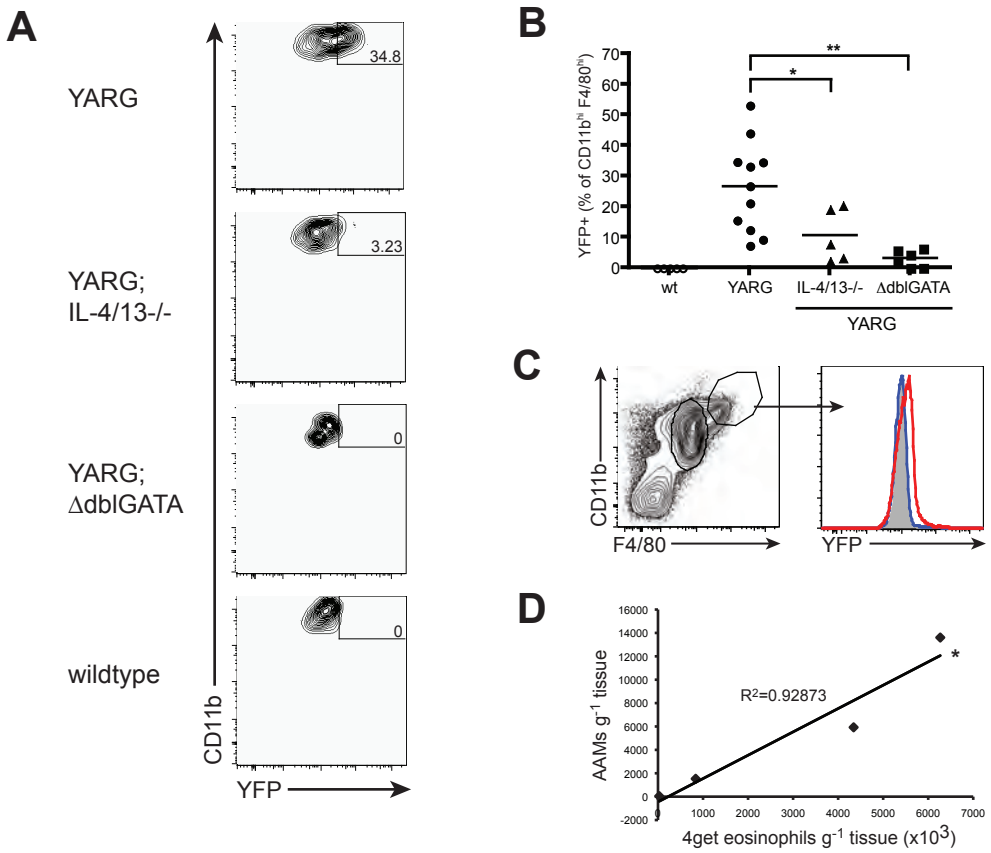
A. Flow cytometric analysis of adipose tissue cells from indicated mice on normal chow diet. Gates outline YFP-positive cells as a percent of total $CD11b^{hi}F4/80^{hi}$ macrophages.

B. Quantification of alternatively activated macrophages in perigonadal adipose as assessed by spontaneous YFP fluorescence in wildtype (no YFP allele); YARG; IL-4/IL-13-deficient x YARG; eosinophil-deficient ΔdblGATA x YARG. *$p<0.05$, **$p<0.01$ as determined using Student's t-test.

C. ΔdblGATA x YARG mice were irradiated and reconstituted with bone marrow cells from 4get x IL-5 transgenic mice. After 19-28 days, perigonadal adipose tissues were harvested and analyzed for eosinophils ($CD11b^{int}$ $F4/80^{int}$ left outlined gate; eosinophils were GFP-positive, not shown) and macrophages ($CD11b^{hi}$ $F4/80^{hi}$ right outlined upper gate). Further gating reveals YFP+ AAM in eosinophil-reconstituted mice (red histogram), but not in non-reconstituted mice (blue gate). Gray area indicates adipose macrophages purified from wildtype (non-reporter) mice to control for autofluorescence in the macrophage gate. Representative analysis of results from 5 animals.

D. The numbers of eosinophils reconstituting perigonadal adipose tissues in eosinophil-deficient mice correlates with numbers of AAM expressing the marker arginase-1 allele. *$p<0.05$, Pearson correlation coefficient $r = 0.96$ (GraphPad Prism, GraphPad Software, Inc.).

Figure 8


A
YARG
YARG;
IL-4/13-/-
YARG;
ΔdblGATA
wildtype
34.8
3.23
0
0
CD11b
YFP
B
YFP+ (% of CD11b^hi F4/80^hi)
70
60
50
40
30
20
10
0
**
*
wt
YARG
IL-4/13-/-
ΔdblGATA
YARG
C
CD11b
F4/80
YFP
D
AAMs g-1 tissue
16000
14000
12000
10000
8000
6000
4000
2000
0
-2000
R2=0.92873
*
1000
2000
3000
4000
5000
6000
7000
4get eosinophils g-1 tissue (x10^3)

mice. After 3-4 weeks, the perigonadal adipose tissues from reconstituted mice became populated with GFP+ eosinophils and YFP+ AAMs that were not present in non-reconstituted ΔdblGATA mice (Fig. 8C). Additionally, the numbers of eosinophils recovered within the perigonadal adipose tissue of the reconstituted mice correlated statistically with the numbers of arginase-1-expressing macrophages in the same tissue (Fig. 8D).

Metabolic analysis of eosinophil-deficient and hypereosinophilic mice

During these experiments, it became apparent that the visceral adipose of hypereosinophilic IL-5 transgenic mice was visually smaller than the same tissues from wild-type mice when both were fed normal chow diets (Fig. 9A). In addition to eosinophilia, these mice had marked increases in the numbers of eosinophils in adipose (Fig. 2F). By dual energy X-ray absorptiometry (DEXA) scan, IL-5 transgenic mice had a trend toward decreased body fat content (but not statistically significant) and decreased adiposity (Figure 9B, 9C). As compared to wildtype mice, IL-5 transgenic mice had a significantly improved response to a fasting glucose challenge when analyzed on normal chow diets (Fig. 9D). Although differences in adipose were less apparent in eosinophil-deficient ΔdblGATA mice maintained on normal chow diet (not shown), alterations in adiposity were greatly augmented when the mice were put on high fat diet. Under these conditions, analysis using DEXA demonstrated significant increases in total body fat and percentage fat content in eosinophil-deficient mice as compared to normal mice maintained on high fat diet, suggesting a role for eosinophils in protecting against diet-induced obesity (Fig. 9E, 9F). This was confirmed following glucose challenge after

Figure 9. Metabolic analysis of eosinophil-deficient and hypereosinophilic mice.

A. Perigonadal fat tissues (testis attached) from hypereosinophilic IL-5 transgenic (IL5tg) and littermate controls (wildtype).

B. DEXA analysis of total, lean, and fat tissue composition of 8 wk-old IL-5 transgenic (IL-5tg) and wildtype littermate mice maintained on normal chow diet. error bars = SEM; n.s.= not significant.

C. DEXA analysis of adiposity as assessed by percent body fat in IL-5tg and wildtype littermate mice. 8 wk-old mice on normal chow diet. Dots represent individual mice. Bars represent mean. *$p<0.05$ as determined using Student's t-test.

D. Fasting male 8 wk-old wildtype (wt) or IL5 transgenic (IL-5tg) littermates maintained on normal chow (NC) diet were challenged with intraperitoneal glucose and blood was sampled for glucose at times indicated. Data compiled from two independent experiments with 6-7 mice in each group. *$p<0.05$, **$p<0.01$ as determined using Student's t-test; error bars = SEM.

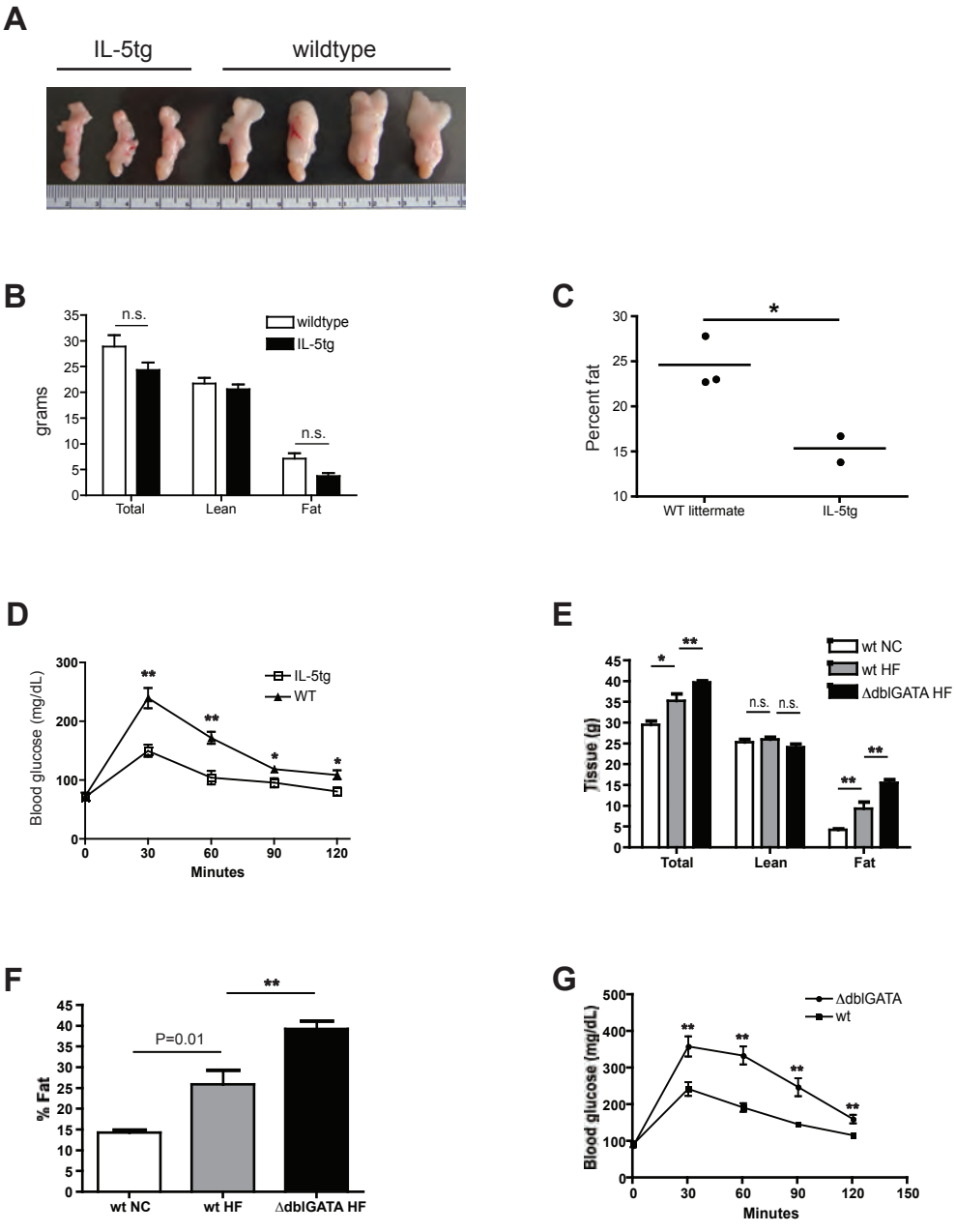
E. DEXA analysis of total, lean and fat tissue composition in eosinophil-deficient (ΔdblGATA) and wildtype (wt) mice on normal chow (NC) or high-fat (HF) diet for 15 wks. *$p<0.05$, **$p<0.01$ as determined using Student's t-test; error bars = SEM; n.s. = not significant.

F. DEXA analysis of adiposity as assessed by percentage body fat in eosinophil-deficient and wildtype mice on HF diet for 15 wks. **$p<0.01$ as determined using Student's t-test; error bars = SEM.

G. Intraperitoneal glucose tolerance test in fasting male eosinophil-deficient and wildtype mice maintained on HF diet for 15 wks. Data compiled from 3 independent experiments

with 5-8 mice in each group. **$p<0.01$ as determined using Student's t-test; error bars = SEM.

Figure 9


A
IL-5tg
wildtype
B
grams
n.s.
wildtype
IL-5tg
n.s.
Total
Lean
Fat
C
Percent fat
*
WT littermate
IL-5tg
D
Blood glucose (mg/dL)
IL-5tg
WT
Minutes
E
Tissue (g)
wt NC
wt HF
ΔdblGATA HF
n.s.
Total
Lean
Fat
F
% Fat
P=0.01
wt NC
wt HF
ΔdblGATA HF
G
Blood glucose (mg/dL)
ΔdblGATA
wt
Minutes

fasting, which revealed significantly impaired glucose tolerance in eosinophil-deficient mice as compared to wildtype animals maintained on high-fat diet (Fig. 9G).

IL-13-expressing cells in adipose tissue

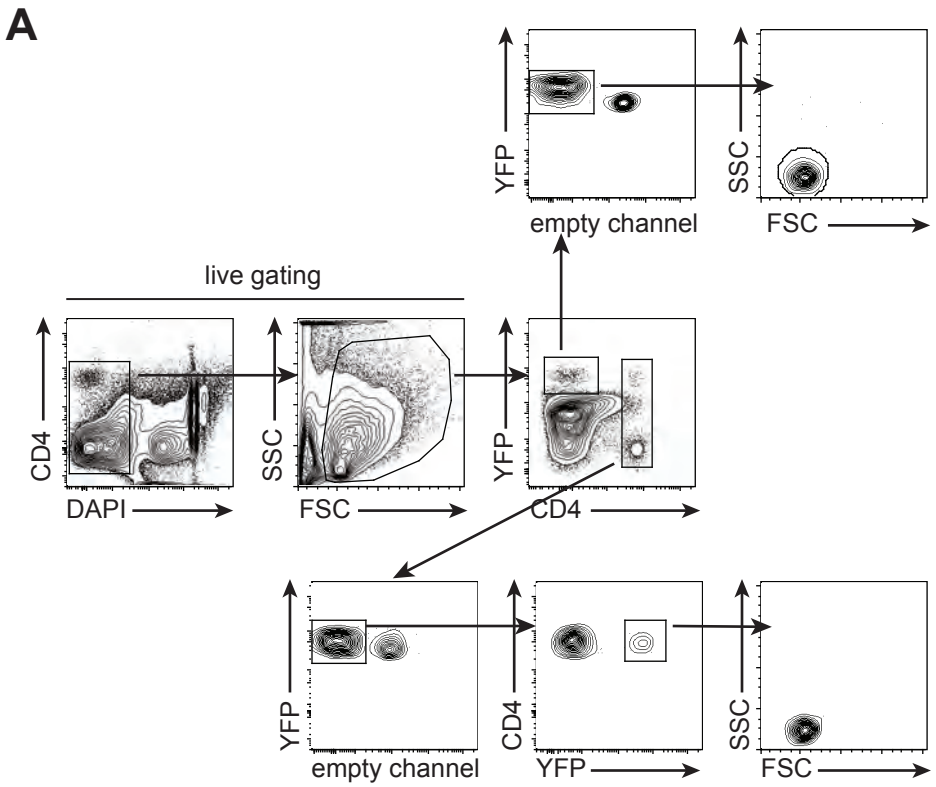
We also investigated sources of IL-13 in adipose using lineage marked mice generated by Hong-Erh Liang in which Cre is expressed downstream of IL-13 (YetCre13) are crossed with fluorescent reporter mice in which a transcriptional stop signal flanked by two loxP sites under the ROSA26 promoter is upstream of a YFP expression cassette (R26R-EYFP; Srinivas et al., 2001). Therefore, any cells that express IL-13 are marked by YFP. After gating on live cells by DAPI exclusion and by side-/forward-scatter (SSC/FSC) size characteristics, we detected two populations of YFP-positive cells: a CD4-positive population and a CD4-negative population (Fig. 10A). We confirmed that the CD4-positive cells are T cells based on SSC/FSC size profile. The CD4-negative YFP+ population upon further gating were also small by SSC/FSC gating and were negative for eosinophil (CD11b, Siglec-F) and basophil (DX5, common beta chain) markers (data not shown). Thus, this CD4-negative population is so far undefined by specific cell surface markers and warrants further study. We quantified the CD4-positive and CD4-negative IL-13-expressing populations in adipose tissue of YetCre13 x R26R-EYFP mice. Cells were isolated from adipose by the enzymatic digestion cell dispersal technique described above. The CD4-negative population far outnumbered the CD4 T cell population as cells per gram adipose tissue (Fig. 10B). However, the IL-13-expressing CD4-negative population is still far smaller compared to the number of IL-4-expressing cells seen in 4get adipose tissue (20,000 IL-4-expressing eosinophils versus

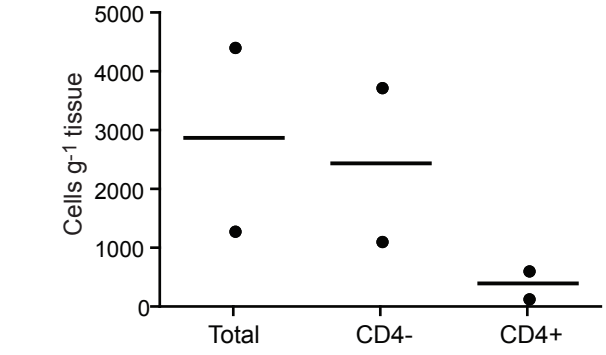
Figure 10. IL-13-expressing cells in adipose tissue.

A. Flow cytometric analysis of perigonadal adipose tissue from YetCre13 x R26R-EYFP mice. Live cells gated based on DAPI exclusion and forward- and side-scatter size characteristics. YFP+ cells further selected with an empty channel to gate out autofluorescent cells. YFP+ CD4+ T cells are further gated to confirm their small forward- and side-scatter size characteristics. YFP+ $CD4^-$ cells also had small forward- and side-scatter characteristics.

B. Quantification of IL-13-expressing cells in perigonadal adipose tissue. Total live, $CD4^-$ and CD4+ cells shown gated according to the scheme as described above. Dots represent individual mice. Bars represent the mean.

Figure 10


A
YFP
empty channel
SSC
FSC
live gating
CD4
DAPI
SSC
FSC
YFP
CD4
YFP
empty channel
CD4
YFP
SSC
FSC


B
Cells g-1 tissue
5000
4000
3000
2000
1000
0
Total
CD4-
CD4+

2,500 CD4-negative IL-13-expressing cells per gram adipose tissue). The sample sizes of the YetCre13 x R26R-EYFP were small in these preliminary experiments; therefore, further study is needed.

Discussion

Despite the emergence of much literature defining a role for AAM in sustaining insulin sensitivity and glucose homeostasis, the mechanisms responsible for maintaining these cells in healthy adipose tissues are not known. Here, we show that eosinophils are the predominant IL-4-expressing cells recovered from healthy lean adipose tissues, where these cells organized at crown-like structures with other hematopoietic cells. Recently, an F4/80^{lo} population of cells in lean adipose tissue was observed to express transcripts for IL-4 (Bassaganya-Riera et al., 2009). While this was hypothesized to be a subset of the AAM population, we believe that these are likely eosiniophils instead based on our studies with cytokine reporter mice and flow cytometric analyses. And although others have reported IL-4/IL-13 production from adipocytes themselves (Kang et al., 2008), we did not see fluorescence in adipocytes taken from 4get mice (data not shown) despite our ability to demonstrate fluorescence in multiple hematopoietic cell types (Fig. 2B). Rather, as shown here using loss-of-function and gain-of-function experiments, our data reveal an unexpected role for eosinophils in regulating the status of AAM in visceral adipose tissues.

Despite their appearance in allergy and states of parasitism, particularly by intestinal helminthes, the biologic role of eosinophils remains incompletely defined. Eosinophils likely play a role in tempering secondary larval parasite migrations (Rothenberg and

Hogan, 2006), but a role in limiting primary parasitism is unclear, with some studies demonstrating a role for eosinophils in sustaining parasitism, thus hinting at an ancient symbiosis (Fabre et al., 2009). Although sparse in blood of persons in developed countries, eosinophils are often elevated in individuals in rural developing countries where intestinal parasitism is prevalent and metabolic syndrome rare (Karita et al., 2009). We speculate that eosinophils may have evolved to play a role in optimizing metabolic homeostasis during nutrient competition with ubiquitous intestinal parasites (Hotamisligili and Erbay, 2008). Our findings add further support for the intertwined relationship between metabolism and immunity (Hotamisligil, 2006), and are consistent with the enrichment of inflammatory and immune response genes associated with obesity in humans (Emilsson et al., 2008; Chen et al., 2008).

MATERIALS AND METHODS

Mice

BALB/c 4get (Mohrs et al., 2001), 4get/KN2 (Mohrs et al., 2005), YARG (Reese et al., 2007), IL-5 transgenic (Dent et al., 1990), IL-4/IL-13-deficient (McKenzie et al., 1999) ΔdblGATA mice (Yu et al., 2002), R26R-EYFP (Srinivas et al., 2001), and YetCre13 (Liang et al., manuscript in preparation) were maintained in the UCSF specific pathogen-free barrier according to institutional guidelines and used between 8 and 23 wks of age. Where indicated, mice were fed high-fat diet (D124551; Research Diets, Inc.) for 15 wks.

Cells and flow cytometry

For "walkout" isolation of adipose tissue stromal vascular fraction cells, perigonadal adipose tissue was minced in media (RPMI, 10% FCS, 0.02 M HEPES, L-glutamine, penicillin, streptomycin) and incubated overnight at 37°C, 5% CO_2. Where indicated, rmIL-4 was added to a final concentration of 0.05 μg/ml. For direct isolation of the stromal vascular fraction (SVF) with enzymatic digestion, perigonadal adipose tissues were minced, dispersed by shaking in medium (low-glucose DMEM, 0.2 M HEPES, 10 mg/ml fatty acid-poor BSA (USB Corp.)) containing 1.2 U Liberase Blendzyme 3 (Roche) and 25 μg/ml DNase I (Roche) and passed through 100 μm filters to generate single-cell suspensions. Two rounds of incubations were done, each with shaking at 215 rpm at 37°C, for 20 minutes. Collected samples were centrifuged at 1000 x g for 10 minutes to pellet the SVF; adipocytes that floated to the top during centrifugation were discarded. Red blood cells in the SVF were lysed using PharmLyse (BD Pharmingen).

Cell suspensions were washed with FACS buffer (PBS, 3% FCS, 0.05% NaN_3), incubated with FcBlock, and stained with the indicated antibodies. Antibodies used to stain samples include: APC-eFluor 780-anti-CD4 (RM4-5; eBiosciences), PE-anti-Siglec-F (E50-2440; BD Biosciences), APC-anti-CD11b (M1/70; BioLegend), APC-eFluor 780-anti-CD11b (M1/70; eBiosciences) and PE-Cy7-anti-F4/80 (BM8; eBiosciences), Biotin-anti-pan-NK (CD49b) (DX5; eBioscience), Biotin-anti-FcεRIα (MAR-1; eBioscience). For biotin-conjugated antibodies, secondary reagent PE-Cy7-Streptavidin (BD Pharmingen) was used. Cells were incubated in 0.5 μg/ml DAPI nuclear stain to exclude dead cells. Cell counts were determined using CountBright Absolute Counting Beads (Invitrogen). Data was acquired on an LSRII (Becton Dickinson) and analyzed using FloJo software (Tree Star).

***In vivo* metabolic analyses**

DEXA scanning was performed using a PIXImus instrument according to the manufacturer's instructions (GE Healthcare). Glucose tolerance testing was performed after fasting mice for 14 hrs and challenging with 2 gm glucose per kg body weight intraperitoneally. Blood glucose was quantitated at indicated times using a glucose meter (Bayer).

Bone marrow reconstitution and adoptive transfers

Bone marrow cells were collected from femurs and tibias of 4get x IL-5 transgenic mice, and, after lysis of red blood cells, were transferred intravenously (15 x 10^6 cells/recipient) into irradiated (400 rad) ΔdblGATA x YARG recipient mice. Mice

were maintained on antibiotic water (0.12 g/L 7-chortetracycline monohydrochloride; Acros Organics). Bone marrow reconstitution was monitored by blood collections on 7 and 18 days post-transfer, and mice were killed for analysis of perigonadal fat 20-28 days after transfer.

For adoptive transfer, spleens from 4get x IL-5 transgenic mice were homogenized and passed through a 70 μm filter to generate a single-cell suspension. After lysing red cells, the samples were incubated with FcBlock and stained with the indicated antibodies. Siglec-F-positive eosinophils were quantified by flow cytometry using CountBright Absolute Counting Beads (Invitrogen). Eosinophils (50×10^6) were transferred to recipient mice by intravenous injection. Mice were analyzed 3 and 7 days post-transfer. For integrin blockade, recipient mice were pre-treated with intravenous injection of 100 μg each of anti-α4 (PS/2, kindly provided by D. Erle, UCSF) and anti-αL antibodies (M17/4, Bio X Cell), followed by cell transfer 2 hrs later as described above. Tissues were analyzed 24 hours later.

IL-25-induced eosinophilia

500 ng of recombinant mIL-17E (IL-25; R&D Systems) in 1% BSA/PBS was administered by i.p. injection for 4 consecutive days (days 0 to 3). For IL-25-induced eosinophilia with integrin blockade, IL-25 was administered to wildtype mice as described above. Mice were treated with intravenous injection of 100 μg each of anti-α4 and anti-αL antibodies or isotype antibodies IgG2a and IgG2b on days 3 and 6. Tissues were analyzed on day 8 by flow cytometry as described above.

Immunohistochemistry

Perigonadal white adipose tissue was fixed in formalin and embedded in paraffin prior to sectioning. Tissue sections were stained with hematoxylin and eosin. For immunofluorescence, tissue sections were stained with an anti-Siglec-F antibody (BD Pharmingen) followed biotin-goat anti-rat Ig (BD Pharmingen) secondary antibody with tyramide amplification (PerkinElmer). Nuclei in tissue sections were visualized with DAPI.

For immunofluorescence of the small intestine, whole tissue was fixed in 4% paraformaldehyde (Sigma) in PBS, saturated in 30% sucrose/PBS and frozen in OCT embedding medium (Sakura) prior to sectioning. Tissue sections were stained with antibodies against GFP (4get) using rabbit anti-GFP polyclonal antibody (ab 6556; abcam) and Siglec-F (BD Pharmingen), followed by tyramide amplification. Nuclei were counterstained with 1 μg/ml DAPI.

Acknowledgements

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CHAPTER IV:

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The data that we have presented here demonstrate that innate effector cells contribute unique functions in the setting of chronic conditions, not just in responses to parasites and allergens, but in the regulation of metabolic homeostasis as well. To our knowledge, this is the first report of eosinophils in adipose tissue, and thus our initial findings raise many more questions about the biology of the eosinophil and its role in metabolism. It is not known exactly how eosinophils interact with alternatively activated macrophages, nor do we know with what other cells in adipose tissue may interact. Still undetermined are the signals that regulate recruitment and retention of eosinophils in metabolically active tissues. Asking such questions will help us better understand more broadly how the immune system has evolved over time to develop unique functions to allow organisms to adapt and respond to their environments.

How do eosinophils actually function in metabolism?

Eosinophils have long been assumed to solely function in the context of immunity. With their specialized intracellular granule components, eosinophils have been documented for their antiviral (Phipps et al., 2007) and anti-helminth functions (Daly et al., 1999). Eosinophil recruitment to tissues is a prominent feature of Type 2 responses such as helminth infection, allergy, and asthma. However, the functions of eosinophils remain incompletely defined. While eosinophils have been reported to be dispensable for some of the major features of primary Type 2 responses, their role in secondary infections has been proposed (Knott et al., 2007). Therefore, it is possible that eosinophils may instead be playing a role in maintaining tissue homeostasis and metabolism. Here we report an unexpected role of eosinophils in the regulation of metabolic homeostasis by interacting with other components of the immune system that influence the inflammatory status of metabolically active tissues that control energy balance. Our observations prompt us to re-examine and extend our understanding of eosinophil function.

Further definitive metabolic assessments of hypereosinophilic IL-5 transgenic and eosinophil-deficient ΔdblGATA mice are needed to complement our observations made by DEXA scans and glucose tolerance tests. The next steps to understand the effects of eosinophils on metabolism is to assess the level at which they may be affecting glucose homeostasis. Fasting insulin levels and insulin tolerance tests are currently ongoing to determine whether eosinophils directly affect pancreatic function. Insulin signaling, via phosphorylation of Akt downstream of the insulin receptor, is also currently being analyzed to determine if eosinophils affect glucose uptake in other metabolically active tissues such as liver and muscle. To assess more global effects of the presence and

absence of eosinophils on metabolism, analysis of hypereosinophilic IL-5 transgenic and eosinophil-deficient ΔdblGATA mice using metabolic cages is needed. Metabolic cages will provide information regarding any changes in food intake, oxygen consumption, CO_2 output, and physical activity.

What is the mechanism by which eosinophils induce macrophage alternative activation?

We find that macrophage alternative activation in adipose tissue depends on the presence of eosinophils. Indeed we find that when eosinophil-deficient mice are reconstituted with IL-5 transgenic bone marrow to induce endogenous eosinophilia, macrophage alternative activation is restored with the extent of activation correlating with the level or eosinophilia. To extend our findings, we can next ask whether the interaction between eosinophils and macrophage alternative activation in adipose tissue is direct or indirect.

This can first be assessed *in vitro* by co-culturing adipose tissue macrophages from our YARG reporter mice directly with eosinophils to determine if alternative activation can be induced. We can also incubate eosinophils sequestered in transwells with adipose tissue to determine if secreted factors from eosinophils can induce macrophage alternative activation without cell-cell contact. We can also incubate adipose tissue macrophages with supernatants from activated eosinophils cultured *in vitro* to test if a soluble factor is responsible for inducing alternative activation. The supernatants from the cultured eosinophils can be analyzed for IL-4 content or other factors that activate macrophages.

The eosinophil thus far appears to be the strongest candidate as the cellular source of IL-4 for adipose tissue macrophage alternative activation. This is based on four observations that we have made: 1) adipose tissue macrophage alternative activation is diminished *in vivo* in the absence of IL-4/13; 2) exogenous IL-4 can upregulate alternative activation *in vitro*; 3) eosinophils comprise the major IL-competent and IL-4-producing cell population in adipose tissue as evidenced by reporter mouse models; and 4) macrophage alternative activation is correlated with the level of eosinophilia induced by bone marrow reconstitution. To further assess the role of eosinophil-derived IL-4, we can use *in vitro* co-culture and transwell experiments as described above using IL-4-sufficient and IL-4-deficient eosinophils to determine if adipose tissue macrophage alternative activation can be modulated. We can also extend our observations from the bone marrow reconstitution studies by comparing ΔdblGATA x YARG mice reconstituted with IL-4/13-sufficient IL-5 transgenic bone marrow versus those reconstituted with IL-4/13-deficient IL-5 transgenic bone marrow. Assessing the level of induction of macrophage alternative activation in this model will aid in further defining the role of eosinophil-derived IL-4 in adipose tissue.

How are eosinophils recruited to adipose tissue?

In addition to further defining the role of eosinophils in macrophage alternative activation, we can explore the effect of AAM and adipose tissue in general on eosinophil recruitment and activation. The surprising finding of eosinophils resident in adipose tissue *in vivo* suggests that signals or chemotactic factors recruit them from the circulation to the tissue. Eosinophils constitutively express the chemokine receptors

CCR3 and CCR1 (Ponath et al., 1996; Phillips et al., 2003), as well as receptors to prostaglandins and leukotrienes (Bandeira-Melo et al., 2000; Shiraishi et al., 2005). Additionally, leptin has been demonstrated to have a chemokinetic effect on eosinophils (Wong et al., 2007). Further analysis of adipose tissue will be required to determine if known cytokines such as IL-5 and chemotactic factors such as the eotaxins are present in adipose tissue. Assays such as RT-PCR can be done to determine levels of expression of these recruitment factors. Further analysis can help determine if adipocytes or cells of the stromal vascular fraction such as macrophages are the source of chemokines, and if levels of these factors are altered in the setting of diet-induced obesity. Additionally, the role of AAM in eosinophil recruitment to adipose tissue also has not yet been determined. We are generating AAM-deficient (LysMCre x PPARγ fl/fl) mice on the 4get background in order to enumerate eosinophils in adipose tissue.

We were intrigued by the observation that fewer adoptively transferred eosinophils could be recovered from the adipose tissue of mice maintained on high fat diet. One possibility is that the eosinophils failed to enter the adipose tissue, a milieu of inflamed fat skewed away from a protective Type 2 immune-like environment towards a Type-1-like setting. Combined with our understanding that eosinophil entry into adipose tissue is integrin-mediated, one possibility is that the molecular signature of blood vessels in adipose tissue is regulated by diet and inflammatory status. Therefore, further experiments are needed to verify this, such as analysis by RT-PCR to assess the expression levels of ICAM and VCAM in the stromal vascular fraction. Additionally, eosinophil migration can also be tested *in vitro* using transwell experiments by incubating eosinophils in the presence of lean or obese adipose tissue.

The decreased number of eosinophils we observed could also be due to impaired cell survival in obese adipose tissue. One mechanism proposed in published literature is that it may be due to TNF receptor activation. TNF receptors have been identified on human eosinophils, and upon activation, induce apoptosis (Zeck-Kapp et al., 1994; Zeck-Kapp and Kapp, 1995). Though this needs to be verified in animal models, one can speculate that with the increased levels of TNF-α in obese adipose tissue, eosinophil activation and apoptosis could be induced at a higher rate.

What promotes eosinophil survival in adipose tissue?

In our experiments, eosinophils adoptively transferred into eosinophil-deficient mice preferentially survived in lean adipose tissue compared to spleen and lung. This suggests that specific factors may be present in adipose tissue to confer selective survival of eosinophils. One study suggests that leptin, an adipokine that regulates food intake, may have an anti-apoptotic effect on eosinophils by causing delayed cleavage of Bax, a pro-apoptotic molecule (Conus et al., 2005). More definitive experiments such as BrDU labeling are needed to quantify the extent of survival and turnover of eosinophils in lean versus obese fat. While adoptively transferred eosinophils had impaired entry or survival in obese, eosinophil-deficient adipose tissue, the effect of diet-induced obesity upon resident eosinophils initially present in adipose tissue has not been addressed. We hypothesize that with induction of tissue inflammation as a result of increased fat storage, the influx of inflammatory cells and the upregulation of pro-inflammatory cytokines will skew away from or overwhelm the protective function of eosinophils and AAM as well as impair further recruitment and survival of eosinophils to adipose.

Other studies have begun to offer insight into the regulation of eosinophil survival in other peripheral tissues. For example, based on BrdU labeling, eosinophil numbers that accumulate such as during infection is mainly due to increased survival of already existing eosinophils rather than eosinophils newly mobilized from the bone marrow (Ohnmacht et al., 2007). This suggests that signals are produced to induce prolonged survival in specific tissue sites. An analysis of the small intestine, thymus, and uterus demonstrated that eosinophils in those tissues had longer lifespans compared to eosinophils in the blood and lung (Carlens et al., 2009). Decreased numbers of eosinophils were observed in the small intestines in common γ-chain deficient mice, suggesting that tissue-specific common γ-chain-dependent signals are produced to maintain homeostatic numbers of eosinophils. Similar signals may also be important for maintaining eosinophil numbers in adipose tissue and, therefore, further exploration is needed.

Eosinophils as a link between immunity and metabolism

If we specifically consider eosinophils, is there a link between metabolism and eosinophil-related immunologic disorders such as allergy and asthma? With the parallel rise in the incidences of obesity and asthma, links between the two have been proposed. Indeed, body mass index (BMI) has been found to have a positive correlation with the prevalence and clinical severity of asthma (Ford et al., 2004). In a number of cases, obesity often precedes the development of asthma (Ford, 2005). Changes in cytokine and adipokine production correlated with the increased fat accumulation in obesity is proposed as the pathological process underlying the two conditions. For example, the

level of adiponectin, an anti-inflammatory adipokine is negatively correlated with the level of obesity. Furthermore, it has been suggested that decreased adiponectin leads to decreased IL-10 production by adipocytes and macrophages (Hersoug and Linneberg, 2007), thereby dramatically affecting immunosuppressive functions and immunological tolerance. In support of this, adiponectin-deficient (ADN-/-) mice are predisposed to diabetes (Maeda et al., 2002). And in a model of chronic allergic airway inflammation, adiponectin-deficient mice developed decreased lung compliance, exacerbated pulmonary hypertension, and increased pulmonary vascular remodeling (Medoff et al., 2009). However, the relationship of eosinophils to these pulmonary changes is unclear, as conflicting results have been reported. Medoff et al. (2009) observed elevated levels of CCL11 (eotaxin-1) correlating with increased eosinophil and macrophage recruitment to the lungs of ADN-/- mice. In contrast, a study of diet-induced obesity in wildtype mice demonstrated decreased lung eosinophilia and decreased IL-5 in BAL fluid (de Vries et al., 2008). The findings in mice support a study where asthmatic obese patients had decreased numbers of sputum eosinophils compared to an asthmatic nonobese patient cohort (van Veen et al., 2008). These studies suggest that eosinophil mobilization is affected in conjunction with obesity. Whether this is positively or negatively correlated with lung function, further study is needed to clarify these discrepancies. At this point we can only speculate that there might be some redistribution of eosinophils from one tissue to another, possibly affecting disease progression.

Broad functions of eosinophils beyond immunity

Our findings suggest that eosinophils play a broader role beyond that associated with immune function. While we report the presence of eosinophils in white adipose tissue, other anatomically distinct types of adipose tissue remain unexplored, as subcutaneous and brown fat have been suggested to have different metabolic functions (Tran et al., 2008; van Marken Lichtenbelt et al., 2009). Ultimately, it will be interesting to determine if our finding of eosinophils in murine adipose tissue can be extended to human adipose tissue, and if we can apply our understanding of Type 1 versus Type 2 immunobiology to metabolic diseases. Cases of lipodystrophy have been reported to be associated with human PPAR dysfunction (Jeninga et al., 2009), thus providing further evidence that macrophage alternative activation is critical for metabolic homeostasis.

Eosinophils may also be functioning in the regulation of other metabolically active tissues such as the liver and small intestine. Eosinophils are a major IL-4-competent population in the lamina propria of the small intestine, yet their role in that tissue remains undefined. We can speculate that eosinophils may not only function in maintaining integrity of the mucosal barrier, but also by influencing metabolism by affecting nutrient uptake. Eosinophils also homeostatically migrate to other tissue sites such as the thymus, uterus, and mammary gland, suggesting that they may also function in tissue maintenance and repair. At many of these sites, macrophages are found alongside eosinophils, suggesting a close functional relationship. Eosinophil numbers and eotaxin levels in the uterus correlate with estrogen levels during the estrus cycle, suggesting that eosinophil control is closely intertwined with the endocrine system (Gouon-Evans and Pollard et al., 2001). In the mammary gland, macrophages and

eosinophils are important for mammary gland development and remodeling (Gouon-Evans et al., 2000). Interestingly, IL-4 and IL-13 have been implicated in this process (Khaled et al., 2007).

More broadly, how are eosinophils homeostatically maintained with respect to numbers and tissue distribution? Eosinophil numbers are normally kept low in the lung to avoid inducing airway hyperresponsiveness, yet need to be present in sufficient numbers in helminth infection (especially secondary infection) and in adipose tissue for metabolic homeostasis. Additionally, circulating eosinophil numbers have been demonstrated to vary throughout a 24-hour cycle with some periodicity observed in humans and mice (Halberg and Visscher, 1950; Singh et al., 1987). This periodic flux in blood eosinophil numbers has been suggested to correlate with the severity of asthma symptoms. Interestingly, it has been reported that circulating eosinophil numbers can synchronize with food intake schedules (Pauly et al., 1975). This suggests that homeostatic maintenance of eosinophils can be influenced by energy levels.

Are there other aspects of Type 2 immunity involved in metabolic regulation?

Although eosinophils comprised the vast majority of IL-4-competent cells in adipose tissue, the other IL-4-competent (4get-positive) immune cell components of adipose tissue stromal vascular fraction, and their role in metabolism, have yet to be explored. For example, although CD4 T cells were only a small 4get-positive population, we observed a deficit in macrophage alternative activation in Rag2-deficient mice (data not shown). Also, basophils were a very small 4get-positive population, yet they can produce high levels of IL-4 on a per cell basis (Khodoun et al., 2004), and therefore may

still provide a functional role in macrophage alternative activation. Additionally, observations in our lab support the hypothesis that IL-4 and IL-13 may be expressed by different subsets of innate and adaptive immunity (H.-E. Liang, manuscript in preparation). Our preliminary data demonstrate that the major IL-13-competent cell populations in adipose tissue are CD4 T cells and an unidentified population consisting of small cells. Although these IL-13-competent cell populations are much smaller in number compared to that of IL-4-competent eosinophils in adipose tissue, the role of IL-13 in adipose tissue macrophage activation and in metabolism remains unexplored.

Other recently described cytokines involved in Type 2 immunity may also play a role in metabolic homeostasis. For example, we have demonstrated that eosinophils in adipose tissue increase in response to IL-25. Additionally, it has been recently reported that a population of 'natural helper cells' can be found in adipose tissue (Moro et al., 2010). These cells produce Th2-associated cytokines IL-5 and IL-13. While the Moro et al. study examined mesenteric adipose tissue, visceral perigonadal white adipose tissue needs to be analyzed for this population of innate lymphoid helper cells. However, it is interesting to speculate that there may be some interaction of these cells with eosinophils.

Inflammation, metabolism, and tissue repair are closely intertwined.

Studies that reveal the presence of eosinophils in lymphoid and non-lymphoid tissues suggest that we re-evaluate and expand our understanding of the immune system in a larger context. With the immune system at the core of inflammation, along with mechanisms of inflammation implicated in metabolic dysregulation, it is enticing to speculate that there is crosstalk between components of immunity and metabolism.

Recently, analyses of molecular networks as a way to comprehensively associate variations in gene expression patterns with complex disease traits have implicated a network of macrophage and inflammatory genes in adipose tissue. This "macrophage-enriched metabolic network" (MEMN) enriched for genes that are conserved in mouse adipose tissue suggests that "immune pathways" may play a role in adipose tissue and are causally linked to obesity and metabolic syndrome (Emilsson et al., 2008; Chen et al., 2008).

Increasing evidence suggests that components of the immune system link the intertwined relationships involving inflammation, tissue repair, and metabolism. We can begin to ponder the evolution of physiologic mechanisms and metabolism in the context of an organism's interaction with its environment, along with how it has shaped the immune repertoire. It is tempting to re-examine both the hygiene and the thrifty gene hypotheses in relation to one another. The hygiene hypothesis posits that the dramatic rise in the prevalence of allergic disorders, particularly in urbanized regions of industrialized countries, can be attributed to limited exposure early in childhood to Th1-biased bacterial and viral pathogens (Yazdanbakhsh et al., 2002). This is complemented by the observation that the prevalence of allergic disorders is low in developing countries. It has been hypothesized that, without early stimulation of a Type 1 response, the developing immune system is altered in such a way (possibly at mucosal sites) that a person could become hyperresponsive to Type 2-associated stimuli. But if humans living in industrialized environments have limited exposure to Th1-associated stimuli, how does this explain the dramatic rise in the cases of obesity if, as the data suggests, metabolic dysregulation is skewed toward Type 1 inflammation? One provocative theory is that

some Type 1-associated genes have been selected not only for host defense mechanisms, but also as thrifty genes. The thrifty gene hypothesis proposes "mutations [were] incorporated into the genome during millennia of lean times to protect against starvation" (Roth, 2009). As a result of infectious diseases, such as tuberculosis in the early 19th and 20th centuries, proinflammatory defenses conferred a selective immunological advantage. The ability to mount a strong immune response as well as the ability to induce robust tissue repair mechanisms would thus help ensure survival from injury and infection. From a metabolic perspective, this retention of pro-inflammatory genes in adipose tissue would likely help to maintain high circulating levels of lipids and sugars, thus providing energy to support the immune system during infection. But in the modern era, cleaner environments with reduced exposure to bacteria and viruses, accompanied by a calorie-rich Western diet and sedentary lifestyle, the retained proinflammatory genes may now drive the propensity towards obesity and metabolic syndrome. This is purely speculation at this point, but it raises the idea of the importance of maintaining balance within the immune system as an age-old evolutionary strategy for controlling energy stores for survival. This may possibly be one of the roles of innate immunity spanning from simple organisms to humans.

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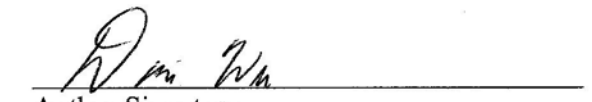

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