MECHANISMS OF TH2 IMMUNITY

IN PEANUT ALLERGIC SENSITIZATION

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By

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Abstract

Food allergies are immune system-driven diseases that lead to reproducible adverse reactions which can be fatal. These severe systemic reactions are primary mediated by immunoglobulin E (IgE) that is derived from B cells which have been activated by T helper type 2 (Th2) cells. While much work has advanced the clinical and pharmacological management of patients with allergic diseases, much remains to be elucidated about how individuals initially acquire allergy. This Thesis details a mechanism linking initial gastrointestinal exposure to peanut (PN) allergen, to the generation of Th2 cells: PN allergen activates epithelial cell secretion of interleukin (IL)-33 and eosinophil degranulation of eosinophil peroxidase, which causes $CD103^+$ dendritic cell (DC) activation and migration to mesenteric lymph nodes where DC OX40L engages naïve T cells to secrete IL-4 in an autocrine/paracrine manner to promote and consolidate Th2 cell differentiation. These events are followed by B cell activation and PN-specific IgE production, which sensitizes mast cells to be hypersensitive to PN re-exposure by causing immediate allergic reactions including anaphylaxis. This is later followed by eosinophilic inflammation that is partially mediated by innate lymphoid cells. As food allergy also serves as a unique model to better understand mechanisms of adaptive immunity, especially Th2 immunobiology, both basic science and clinical implications are discussed in this Thesis. Major themes include Th2 and disease heterogeneity, identification of 'the original source of IL-4', an unprecedented *in vivo* requirement for eosinophils in priming adaptive immune responses, and the need to weigh basic science findings against the human disease *in natura* litmus test. Looking forward, many questions remain to be answered in the field of food allergy research, but the findings of this Thesis may be one step towards the prevention, management or cure of a disease with growing public concern, potentially fatal consequences, and an unmet need in understanding its pathogenesis.

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Table of contents

List of figures and Tables

None

List of all Abbreviations and Symbols

Ab	Antibody
ACK	Ammonium Chloride Potassium lysis buffer
Ag	Antigen
Alum	Aluminum based adjuvant, often aluminium hydroxide
ANOVA	Analaysis of variance
APC	Antigen presenting cell, or allophycocyanin
APRIL	A proliferation inducing ligand
ASC	Apoptosis-associated speck-like protein containing a carboxy-terminal caspase activation and recruitment domains
BAFF	B cell activating factor
BCR	B cell receptor
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
BMDC	Bone marrow-derived DC
cAMP	Cyclic adenosine monophosphate
cDC	Conventional DC
cGAMP	Cyclic guanine MP-AMP
cGAS	cGAMP synthase
CIHR	Canadian Institutes of Health Research
CT	Cholera toxin
CTA	CT A subunits
CTB	CT B subunits
Cy	Cyanine
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DT	Diptheria toxin
DTR	Diptheria toxin receptor
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbent assay
EoE	Eosinophilic esophagitis
EPO	Eosinophil peroxidase
ER	Endoplasmic reticulum
FcεRI	Fc ε receptor I
FITC	Fluorescin isothiocyanate
FMO	Fluoresence minus one
FoxP3	Forkhead box P3
JAX	The Jackson Laboratories
KO	Knockout
L3	Third-stage larvae
LI	Large intestine
LP	Lamina propria
L-PAF	Lyso-PAF
LT	Heat-labile enterotoxin
LTi	Lymphoid-tissue inducer
MACS	Magnetic activated cell sorting
MAIT cell	Mucosal-associated invariant T cell
MC	Mast cell
MHC	Major histocompatibility
MHC II	MHC class II
MLN	Mesenteric lymph node
møs	macrophages
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene
NADPH	Nicotinamide adenine dinucleotide phosphate
Nb	*Nippostrongylus brasiliensis*
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK	Natural killer
NKT cell	Natural killer T cell
NOD	Nucleotide-binding oligomerization domain-containing protein
OIT	Oral immunotherapy
OVA	Ovalbumin
OVA-IgE	Ovalbumin-specific IgE
OX40L	OX40 ligand
p.r.	per rectum
PAF	Plaetet activating factor
pDC	Plasmacytoid DC
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI	Propidium iodide
pi	Post-infection
PN	Peanut

G protein	guanidine nucleotide binding protein
GATA	GATA binding protein
GI	gastrointestinal
GM1	ganglioside monosialiac 1
Gsα	G protein stimulatory α subunit
GWAS	Genome-wide association study
HDM	House dust mite
Hp	Heligmosomoides polygyrus
i.g.	intragastric(ally)
i.n.	intranasal(ly)
i.p.	intraperitoneal(ly)
i.v.	Intravenous(ly)
ICOS	Inducible costimulator
IEL	intra-epithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IgE	Immunoglobulin E
IL	Interleukin
IL-17RB	IL-17 receptor B
IL-5Tg	IL-5 Transgenic
ILC	Innate lymphoid cell
ILC2	Group 2 innate lymphoid cells
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
PN-IgE	PN-specific IgE
RIG-I	Rentoic acid-inducible gene 1
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
s.c.	subcutaneous(ly)
SEM	Standard error of the mean
SI	Small intestine
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
Th2	T helper type 2
TLR	Toll-like receptor
TNFRSF	Tumor necrosis factor receptor superfamily
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor
VDJ	Variable, diversity and joining (genes)
VSV	Vesicular stomatitis virus
WHO/IUIS	World Health Organization and International Union of Immunological Societies
WT	Wild-type
β2m	β2 microglobulin

Declaration of Academic Achievement

Chapters 2, 3 and 4 are three independent, but conceptually related bodies of work that, as of August 2013, have been or are being peer-reviewed prior to publication. The work conducted in each manuscript required a collaborative effort with several colleagues, resulting in multiple authors. My contributions to each manuscript are detailed below.

Chapter 2 **Chu DK***, Llop-Guevara A*, Walker TD, Flader K, Goncharova S, Boudreau JE, Moore CL, In TS, Waserman S, Coyle AJ, Kolbeck R, Humbles AA, Jordana M. IL-33, but not Thymic Stromal Lymphopoietin or IL-25, is Central to Mite and Peanut Allergic Sensitization. *J Allergy Clin Immunol.* 2013 Jan;131(1): 187-200.e8. doi:10.1016/j.jaci.2012.08.002. Epub 2012 Sep 21.

This work was conducted over the period of March 2009 – December 2011. This manuscript was an equal contribution (*). I, along with ALG (graduate student), designed and performed all experiments, analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. I led the work related to allergic responses to peanut, while ALG guided the work in house dust mite. Experimental assistance was provided by undergraduate students (CLM and TSI), graduate students (KF and JEB) and technicians (TDW and SG). RK and AAH (MedImmune LLC) provided reagents, knock-out mice and scientific input. SW (Professor, McMaster) and AJC (Pfizer) critically appraised the manuscript. MJ oversaw the project and edited the manuscript.

Chapter 3 **Chu DK**, Mohammed-Ali Z, Walker TD, Goncharova S, Llop-Guevara A, Barra NG, Gillgrass A, Jimenez-Saiz R, Ashkar AA, Bramson JL, Humbles AA, Kolbeck R, Waserman S, Jordana M. T helper cell-intrinsic IL-4 drives intestinal Th2 priming to oral peanut antigen, under the control of OX40L

and independent of innate-like lymphocytes. *Submitted to the Mucosal Immunology*.

This work was conducted from June 2010 – Feb 2013. I designed and performed all experiments, analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. Experimental assistance was provided by undergraduate students (ZM), graduate students (ALG, NGB, ALG) and technicians (TDW and SG) and a post-doctoral fellow (RJS). RK and AAH (MedImmune LLC) and AAA (Professor, McMaster) provided reagents, knock-out mice and scientific input. JLB (Professor, McMaster) and SW (Professor, McMaster) provided scientific input and appraised the manuscript. MJ oversaw the project and edited the manuscript.

Chapter 4 **Chu DK**, Walker TD, Goncharova S, Llop-Guevara A, Barra NG, Bassett JD, Jimenez-Saiz R, Fattouh R, Erjefält JS, Waserman S, McCoy KD, Humbles AA, Kolbeck R, Jordana M. Indigenous Enteric Eosinophils Are Essential to Initiate a Primary Th2 Immune Response *in vivo*. *Submitted to The Journal of Experimental Medicine.*

This work was conducted from Apr 2010 – May 2013. I designed and performed all experiments, analyzed and interpreted the data as well as wrote and prepared the manuscript for publication with one exception. JSE (Professor, University of Lund, Sweden) performed electron microscopy and analysis of samples that I generated and sent to him. Experimental assistance was provided by graduate students (ALG, NGB), technicians (TDW and SG) and post-doctoral fellows (JDB and RJS). RK and AAH (MedImmune LLC) provided reagents, mice and scientific input. RF (post-doctoral fellow, University of Toronto), SW (Professor, McMaster) and KDM (Assistant Professor, University of Bern) provided scientific input and appraised the manuscript. MJ oversaw the project and edited the manuscript.

Chapter 1.
Introduction

A Neologism from 1902, and its Relationship to Peanut Food Allergy

Anaphylaxis is a serious allergic reaction that is rapid in onset and may cause death. In 1913, Charles Richet was awarded the Nobel Prize for the discovery of anaphylaxis. In his acceptance speech he explained that 'anaphylaxis' was a neologism he created in 1902 to be the opposite of 'prophylaxis' (*1*). Whereas prophylaxis is characterized by protection from pathology due to prior exposure to a certain substance, such as in vaccination, anaphylaxis is the opposite: re-exposure to a certain substance leads to pathology such as acute, systemic sickness.

Anaphylaxis is a systemic allergic reaction. Substances that can generate allergic reactions are termed allergens, which are most often proteins. In individuals with allergy, allergen exposure by various routes can produce localized clinical manifestations: inhalation may result in allergic asthma, and skin exposure, atopic dermatitis. Food allergy manifestations include gastrointestinal (GI) discomfort, diarrhea, nausea and vomiting to cutaneous localized or generalized erythema (flushing), pruritus (itch) and urticaria (hives). Anaphylactic reactions may encompass the aforementioned and also include angioedema (deep swelling of lips, eyes, tongue, larynx) and/or cardiovascular collapse. Peanut (PN), milk, eggs, tree nuts, fish, shellfish, soy and wheat account for approximately 90% of all food allergic reactions in North America (*2-4*). PN allergy affects approximately 1-2% of the North American population. It is a severe food hypersensitivity in that, unlike most others, it is highly associated with anaphylaxis and typically not outgrown (*5*). Indeed, over 30,000 food-related anaphylactic reactions are treated in the United States each year, with PN implicated as the major culprit (*5*). The potential lethality of PN allergies is compounded by the fact that there are no available treatments for this disease. Consequently, therapeutic options are limited to promoting strict, life-long allergen avoidance. Indeed, clinical management of food allergies is instruction of allergen avoidance and administration of

intramuscular epinephrine in the event of an anaphylactic reaction. Despite these efforts, it has been reported that approximately 50% of PN allergic individuals will accidentally ingest PN every 3-4 years (*5*). Thus, PN allergy is in dire need of preventative and therapeutic strategies. Since food allergies have been recognized as immune system mediated diseases, most experimental and clinical studies on food allergy target established disease with allergy desensitization (termed immunotherapy) (*6*), herbal medicine treatment (*7*), or immune deviation (*8*). In this Thesis, we take the stance that prevention and treatment of food allergies may benefit from a better understanding of the immune processes that initiate PN allergy.

General Principles of the Immune System

Immunity is comprised of a layered, hierarchical participation of immune cells with the end goal of maintaining homeostasis (*9*). In this context, resident immune cells, such as macrophages (møs), remove threats to the health or function of the tissue and, therefore the organ and body. For example, pathogenic microbes may compete with host cells for resources (*e.g.* oxygen, iron, glucose) and in doing so, elaborate a number of endo- and exotoxins. Local tissue mechanisms attempt to spare resident cells from these threats by subclinical clearance; for example, mø and/or antimicrobial peptide-mediated killing of the microbes. When tissue-stressors cannot be controlled locally, adaptive immune responses are induced to orchestrate tissue inflammatory responses as well as promote the formation of immunological memory. Adaptive immune responses, particularly induced T helper (Th) types, are heterogeneous and (often) tailored for host-defense (*10*): Th1 responses, induced by interleukin (IL)-12 and effected by interferon (IFN)-γ, resist intracellular pathogens; Th2 responses, induced by IL-4 and classically effected by IL-4, IL-5 and IL-13, resist extracellular parasites/helminths; Th17 responses, induced by transforming growth factor

(TGF)-β, IL-6, IL-1, and IL-23 and effected by IL-17A and IL-17F, resist fungi and particular extracellular bacteria; T follicular helper (Tfh) cells promote adaptive humoral (antibody; Ab) responses to resist a wide variety of pathogens; T regulatory (Treg) cells promote resolution of inflammation and prevent excessive immune responses by multiple mechanisms, including production of IL-10 and TGF-β; Th22 and Th9 have been coined as additional distinct Th states but their presence and relevance to host defense, beyond defined experimental models, is not yet clear. A key feature of many of these Th types is that they home back to the site of stress (antigen (Ag) exposure) and instruct normally tissue-resident cells (*e.g.* møs) to perform specific functions aimed at restoring homeostasis. Th cells, which by definition bear cell surface CD4 ($CD4^+$), may also recruit additional cell types to tailor the type of inflammation to the type of pathogen. For most bacteria and fungi, neutrophils are recruited by Th1 or Th17 cells, while for most helminths, eosinophils are recruited by Th2 cells. A wealth of biomedical experience and research makes it clear that the aforementioned stressors often come in the form of pathogenic microbial and parasitic infection, of which humans, and other multicellular organisms, are under constant threat. Indeed, this is illustrated through the importance of vaccine-induced protection in global health and, similarly, increased probability of infection during immunocompromised or immunodeficient states. However, immunity without regulation has dire consequences; excessive or inappropriate immune responses often cause immunopathology, allergy, autoimmunity and malignancy. Hence, the maintenance of health requires a fine balance between too much, too little, and an appropriate type of immune response.

Intestinal Immunity Relevant to this Thesis

The GI tract plays a vital role in the digestion and absorption of macronutrients, including carbohydrates, proteins and fats. From proximal to distal, the small intestine (SI) is

divided into: duodenum, jejunum and ileum. The large intestine (LI): caecum, ascending colon, transverse colon, descending colon and rectum. In both mice and humans, the SI, primarily the duodenum and jejunum, is the site of digestion and absorption of nutrients. The main function of the ileum is the uptake of vitamin B12 and reuptake of bile acids. The LI's main role is to absorb water and store feces. Hence, the SI, especially the duodenum and ileum, may be the site of greatest food Ag exposure.

The structure of the SI supports its function (*11*). A single layer of nonciliated columnar epithelial cells (enterocytes) specialized in absorption of macronutrients and secretion of various digestive and communication molecules is in contact with the intestinal lumen. This layer also harbours a unique population of innate-like intra-epithelial lymphocytes (IELs) that often bear a γδ T cell receptor (TCR) or CD8. The basement membrane attaches the basolateral side of the epithelial layer to the lamina propria (LP), a network of loose connective tissue and tissue-resident immune cells. Like most other tissues, the SI LP contains møs, dendritic cells (DCs), mast cells (MCs), and B and T cells. More unique to the SI is a prominent population of eosinophils, but the biological significance of this is unknown (*12*). Common to these cells types is that their turnover within the mucosa is thought to be through repopulation from the mucosal vasculature, or drainage by afferent lymphatics to the mesenteric lymph node (MLN). The following sections describe these cells in more detail.

Lymphocytes

Lymphocytes are typically thought to be comprised of B cells, $CD4^+$ T cells and $CD8^+$ T cells. These cells are adaptive as opposed to innate because their Ag receptors are not germline encoded. T and B cells are unique somatic cells because they re-arrange their genome during development/maturation. The re-arrangement of multiple Variable (V),

Diversity (D) and Joining (J) genes results in each individual T or B cell bearing a unique TCR or B cell receptor (BCR). Natural killer (NK) cells are lymphocytes that bear only innate receptors and were previously considered the only exception to the idea that lymphocytes are adaptive immune cells. More recently, other innate-like lymphocytes have been identified, including γδ T cells, natural killer T (NKT) cells, and innate lymphoid cells (ILCs) (*13-15*). CD3 is a part of the signalling apparatus of the TCR and is common to all T cells. However, whereas conventional T cells bear a TCR composed of α and β heterodimers to recognize peptides in the context of major histocompatibility (MHC) molecules, γδ T cells utilize γ and δ chains. The nature of the molecule recognized by γδ TCRs is not definitively known, but is hypothesized to be phosphoAgs. It is also thought that γδ T cells may directly recognize Ag without any need for Ag presentation by MHC or MHC-like molecules. NKT cells share cell surface markers of NK cells and T cells, and uniquely recognize lipid Ags. ILCs are a novel cell type with no known TCR, but potent cytokine secretory capacity. Their functions in health and disease are an intense area of investigation. Whereas adaptive immune cells require several days to become activated and effect an immune response specific to a particular Ag, innate cells such as innate-like lymphocytes are able to respond rapidly because of their pre-formed receptors and preferential localization to mucosal surfaces. Indeed, innate-like lymphocytes are often cited as being important to a particular immune process because of their ability to rapidly produce cytokines typically produced by conventional, Ag-specific $CD4^+$ T cells, such as IFN-γ, IL-4, IL-5, and IL-13.

B cells are known for their relationship to Ab production and can also be divided into innate and more conventional, adaptive types. B-1 and marginal zone B cells innately secrete Ab. In contrast, B-2 B cells, which will be referred to from now on as B cells, capture Ag with their unique BCR, internalize this complex and present peptides within MHC II. B cells mostly reside within LN follicles nearby T cell zones, thereby facilitating interaction with

cognate $CD4^+$ Th cells at the interface of B and T cell zones. There, Th cells activate cognate B cells and cause affinity maturation and isotype class switching. Affinity maturation is an evolutionary process whereby point mutations are introduced into the VDJ genes, such that the highest affinity BCR can be selected for during B cell clonal expansion. During isotype switching, B cells switch from their mature, naïve state of expressing cell surface IgD and IgM to another immunoglobulin (Ig) isotype, *i.e.,* IgG3, IgG1, IgA1, IgG2, IgG4, IgE, or IgA2. In mice, the Ig heavy chain locus is instead arranged as IgG3, IgG1, IgG2b, IgG2a, IgE and IgA. Th2 responses are associated with IgE production, and in mice, also IgG1. Upon completion of affinity maturation and isotype switching, B cells differentiate into plasma cells, which are specialized in Ab production and secretion.

After being primed within LNs, Th and B lymphocytes emigrate via efferent lymphatics and return to the systemic circulation. There, they may recirculate through the blood, LNs, spleen and tissues as memory cells, or return to sites of continued Ag exposure to effect local tissue responses. Activated B cells also differentiate into Ab-secreting plasmablasts and home to the bone marrow to become highly secretory, non-proliferating, long-lived plasma cells. Although much has been characterized regarding innate and adaptive lymphocyte basic biology, the activity and contribution of these various cell types to food allergy remains to be determined.

Dendritic cells

DCs (*16, 17*) are a ubiquitous cell type that, along with møs and B cells, are termed professional Ag presenting cells (APCs). This is because APCs loaded with Ag are able to stimulate Ag-specific $CD4^+$ T cell activation *in vitro*. DCs are thought to be the dominant APC *in vivo*, because they are resident in tissues such as the skin, lung or gut, capture Ag and travel to draining LNs to interact with T cells. B cells typically reside within LNs and are

much less abundantly found in afferent lymph. Likewise, møs typically do not migrate to LNs but are abundant within non-lymphoid tissues. Further, compared to møs, DCs typically express higher levels of MHC II and are more efficient at stimulating T cell activation.

Multiple subtypes of DCs exist. A major division is between plasmacytoid DC (pDC) and conventional DC (cDC) types. pDCs can rapidly produce antiviral IFN-α and β. cDCs can be further subdivided into non-lymphoid and lymphoid subtypes, with all expressing high levels of CD11c and MHC II. Lymphoid DCs reside within lymphoid tissues such as the spleen and LNs. They additionally express either CD8α or CD4. Non-lymphoid DCs reside within non-lymphoid tissues such as the skin, lung and intestine. Each of these sites has its own unique set of DCs. In reference to the intestine (*18, 19*), the main non-lymphoid DC subtypes are $CD103^+CD11b^+$ and $CD103^+$, $CD11b^-$. Notably, although $CD11c^+$MHC $II^+CD103^-CX_3CR1^+$ cells were originally named DCs, they were later shown to be non-migratory, less efficient $CD4^+$ T cell activators, and have morphological features of møs (*20, 21*). In fact, these cells are now thought to be møs. How these cells function in health and disease continues to be explored. It is known that $CD11c^+CD103^+$ DC from the intestine carry ingested Ag to the MLN and preferentially induce Treg cell development (*22, 23*). Hence, they have been thought of as a cell type central to the development of tolerance to oral Ags (*24*). Notably, how each of these cell types contributes to the development of food allergy remains unknown.

Eosinophils

Most healthy tissues are typically devoid of eosinophils (*25-27*). These cells are thought of as specialized effector cells that are specifically recruited from the blood and bone marrow to tissues during adaptive Th2 responses. There, eosinophils are activated, produce a

number of different cytokines, and release their characteristic cationic granules such as eosinophil peroxidase (EPO) in a process termed degranulation. Such actions are thought to be critical for host defense against helminth infection, since there is evidence of attachment of eosinophils to helminths and degranulation. Further, eosinophil degranulation has been associated with the killing of helminths. Despite these associations, an indispensible role for eosinophils in host defense against helminths remains controversial (*28, 29*). In terms of allergic disease, eosinophils are thought to play a pathogenic role in chronic allergic inflammatory diseases such as allergic asthma, atopic dermatitis, or the non-classical food allergy, eosinophilic esophagitis. In these different tissues, recruited eosinophils typically have a short life span (days), and hence, they are thought of as a terminal effector leukocyte under the direction of adaptive immunity. The intestine is a unique site in terms of eosinophil biology because it natively houses the largest number of eosinophils in the body. A biologically significant role for them there has remained elusive for over 100 years (*12*). Hence, the contribution of indigenous enteric eosinophils, if any, to food allergy is unknown.

Mast cells

These are large cells that are densely packed with granules. Such granules are distinct from the ones within eosinophils. The relevance of MCs to this Thesis is not within the intestine, but rather as ubiquitous cell type that mediates anaphylaxis. Classically, allergic reactions, including anaphylaxis, are thought to be elicited by allergen-induced crosslinking of multiple monomeric allergen-specific IgE molecules that are bound to their high affinity receptor, FcεRI on MCs. This event causes MC degranulation, release of vacuole-stored preformed mediators (*e.g.,* histamine and β-hexosminidase) and rapid synthesis of other molecules (*e.g.,* prostaglandins, leukotrienes, and platelet activiating factor). Systemic

activation of MCs results in anaphylaxis: devastating vascular permeability, hypotension, underperfusion organ failure and, ultimately, death.

Although there is evidence for an immunomodulatory role for MCs, we have shown that these cells do not contribute significantly to SI Th2 priming to PN (*30, 31*). Therefore, in the context of this Thesis, their relevance is during the effector phase of Th2 responses.

General Principles of Th2 Immunity in Allergy

Allergic responses can be conceptualized within a timeline containing two distinct phases: sensitization (termed induction, development of allergy, or pathogenesis) and effector (termed elicitation, disease expression or pathophysiology, *e.g.* anaphylaxis).

Sensitization is the subclinical/asymptomatic immune response to primary allergen exposure that leads an individual to develop reproducible allergic reactions upon subsequent allergen exposures. Temporally, pro-allergic activation of SI epithelial cells during allergen ingestion leads to the elaboration of epithelial signals that influence nearby cellular behaviour. Conditioned cells within the LP, namely DCs, take up ingested allergen and go on to function as APCs by maturing (*e.g.,* increased MHC II, CD86, CD80 and CD40) and migrating to draining MLN. Mature APCs in draining LNs present degraded Ag peptides in the context of MHC II for recognition by $CD4^+$ T cells bearing cognate TCRs. During APC MHC II-Th cell TCR interactions, a number of cell-contact mechanisms are engaged, collectively termed costimulation. In the absence of costimulation, T cells do not become activated and are rendered unresponsive to future exposure to Ag. As a result of costimulation, T cells become activated, upregulate numerous cell surface molecules (*e.g.,* CD69 and CD25), proliferate and secrete cytokines. Activated Th cells undergo a process of polarization into various states, such as Th1, Th2, Th17, Treg and Tfh. Allergy is highly associated with Th2 cells, which are characterized by the high expression of IL-4, -5 and -13.

Although the cellular source is unknown, most *in vitro* and *in vivo* evidence supports a critical role for IL-4 in instructing Ag-specific $CD4^+$ T cells to differentiate into Th2 cells. These cells cause Ab/Ig isotype class switching of Ag-specific B cells via MHC II-TCR interactions, costimulation, and critically, IL-4. These events lead to the production of IgE and additionally in mice, IgG1. IgE produced by differentiated B cells, termed plasma cells, enters the circulation and is distributed to all tissues in the body. There, Ag-specific IgE binds to receptors such as FcεRI on the cell surface of MCs.

Re-exposure to allergens in individuals who have undergone allergic sensitization triggers an allergic reaction. Individual allergens bind to several allergen-specific IgE molecules that are bound to cell surface FcεRI, thereby clustering them together and causing MC activation. Such responses are rapid, manifest on the order of minutes and are termed immediate (or type I) hypersensitivity. Systemic MC activation in food allergy may cause anaphylaxis.

Concomitantly, allergen is taken up by local immune cells, such as DCs, which then activate memory Ag-specific $CD4^+$ Th2 cells within the tissue or the draining LN. These lymphocytes home to site of allergen exposure and cause inflammation rich in eosinophils. These events take place on the order of hours to days, and are termed delayed-type (or Type IV) hypersensitivity. In allergic asthma, this is the basis for chronic airway eosinophilic inflammation. In food allergy, this may cause GI symptoms, diarrhea and malabsorption. However, Th2-type responses are not always pathogenic. In fact, they are believed to be important for host defense against parasitic infection. Indeed, much understanding of GI Th2 immune responses stems from studying helminth parasitic infections of the GI tract. Regardless of whether one argues that parasitic infections may be fundamentally different than food allergy, what is evident is that much remains to be elucidated about the risk factors, disease pathogenesis, and the cellular/molecular processes that cause food allergy.

Insights into the Origins of Food Allergy in Humans

Unlike infectious diseases or autoimmunity (*32-34*), there are no known single gene deficiencies that clearly lead to the development of food allergies. However, it is known that food allergies tend to aggregate within families. Whether this is due to genetic or environmental causes is not clear. In terms of genetics, there are no reports of whole genome, whole exome, or genome-wide association studies (GWAS) for food allergy. Instead, there are multiple small candidate gene studies (*35-37*). Unfortunately, these types of studies are the most prone to bias, *i.e.*, false discovery rate. Nevertheless, small increases in relative risk ratios have been found in individuals carrying polymorphisms in genes coding for molecules such as fillagrin, CD14, FoxP3, IL-10, STAT6 and IL-13, but these associations have not always been reproducible (*35-37*).

In terms of environmental influences (*35, 37, 38*), a large amount of data has been generated in observational trials such as case-control or cohort designs. Again, these studies are thought to be susceptible to bias due to the lack of balance in prognostic factors that is controlled for in more rigorous trial designs such as randomized control trials. Within these limitations, two of the most prominent hypotheses that have been formulated are: 1) that the timing of allergenic food introduction is critical to the development of food allergy versus clinical tolerance, and 2) that the composition of the intestinal commensal bacteria may determine whether allergy or tolerance occurs. An illustrative example is that traditional clinical guidelines for patients with a history of allergy advised to delay introduction of allergic foods, such as PN, when feeding their infants. It was thought that early introduction of allergenic foods to an infant with a developing immune system would more likely result in an inappropriate immune response, and thus allergy. In 2008, a review of epidemiological evidence suggested that such delayed introduction practices did not decrease the incidence of food allergy, and hence, the guidelines were then changed to state that "there is no current

convincing evidence that delaying [PN and other highly allergenic food] introduction … has a significant protective effect on the development of atopic disease" (*36*). Some have argued that these delayed introduction practices actually led to increase in the incidence and prevalence of food allergy. Hence, the notion that early introduction of foods to a baby's diet may actually promote tolerance has recently surfaced.

Interestingly, infants delivered by Caesarean section have been reported to have an increased risk of developing food allergies (*39*). While the rationale behind why this might occur is not well understood, a possibility is that neonatal gut colonization by commensal bacteria can influence the development of food allergies and that Caesarean section births have delayed gut colonization as compared to vaginal births. In line with this is the hypothesis that the extensive and life-long communication between the immune system and more than 10^{14} resident gut bacteria (*40, 41*) critically influences whether tolerance or allergic disease develops upon Ag ingestion. Importantly, differences in the composition of gut flora between normal and atopic individuals have been reported (*42*). However, whether this relationship is causal and, if so, the direction of causality remains untested. In an attempt to mechanistically explain how microbes might impact the development of allergic disease, *the hygiene hypothesis* states that childhood microbial exposure protects from the development of allergic disease. More recently, this has been extended to be thought to be due to the development of a robust "regulatory network", made up of various regulatory immune cells (*43-45*). This regulatory network suppresses Th2 activity, thus preventing the development of allergic disease. Therefore, the hygiene hypothesis suggests that the disruption of childhood microbial exposures (for example, through the use of antibiotics, vaccines, or caesarean section) precludes the development of a regulatory network and predisposes individuals to develop allergy. While insights from clinical studies are interesting and may

help to explain why individuals develop food allergies, experimental approaches may also be beneficial.

Experimental Approaches to Investigate Food Allergy

Due to the difficulty identifying and examining young children undergoing the process of allergic sensitization, as well as the obvious ethical restrictions behind invasive immunological workup, most studies examining sensitization to allergens rely on animal experimentation.

Classical Mouse Models of Allergic Disease, Th2 immunity and Tolerance

Although rarely used to model food allergy, Th2 responses in mice have been classically investigated using intraperitoneal (i.p.) immunization of chicken egg ovalbumin (OVA) adsorbed to the adjuvant, aluminium hydroxide (alum). Grossly, this is the same adjuvant used in most human vaccines. Immunization in such a fashion results in the development of Th2 cells and high levels of OVA-specific IgE (OVA-IgE). This model has often been used to study allergic asthma, because alum/OVA sensitized mice challenged with OVA intranasally (i.n.) can develop features of human allergic asthma, such as eosinophilic inflammation and lung dysfunction upon OVA re-exposure. A major criticism of the alum/OVA model is that sensitization is induced by i.p. immunization, a route that bypasses how Ags, such as aeroallergens, normally enter the body, *i.e.,* through the respiratory mucosa. Hence, it has been argued that while alum/OVA models are an established way to induce Th2 responses, such responses may not be completely reflective of human Th2 immunobiology that is induced at mucosal surfaces.

More relevant to food allergy is the use of alum/OVA immunization for the investigation of oral tolerance (*46*). This is a phenomenon, mainly studied in mice, whereby feeding OVA before immunization with alum/OVA results in blunted OVA-IgE responses,

as compared to animals that were not fed before immunization. Feeding OVA, or other Ags, alone is associated with the generation of Treg cells, which are thought to dampen subsequent alum/OVA-induced Th2 and B cell activation, thereby resulting in the inhibited levels of IgE (*46*). The prevailing thought is that oral tolerance and food allergic sensitization are opposite outcomes: oral tolerance induces systemic hyporesponsiveness to food Ags, and a break or loss in oral tolerance leads to food allergy.

Modern/Conventional Models of Food Allergy

Almost all modern protocols of sensitization to foods such as PN are performed by intragastric (i.g.) gavage of mice with PN along with an adjuvant, cholera toxin (CT) (*47, 48*). This work was pioneered by Dr. Denis Snider at McMaster University (*49*). Gavage of PN without CT does not elicit food allergy, possibly owing to oral tolerance. To overcome this, most mouse models of PN allergy require weekly (or more frequent) gavage of PN+CT for four weeks. One to two weeks later, a challenge with PN is performed to induce anaphylaxis. For unclear reasons, i.g. PN challenge of sensitized mice does not robustly or reliably produce allergic reactions, including anaphylaxis. Therefore, PN is typically injected i.p., which does elicit anaphylaxis reliably. Our laboratory characterizes this response by a drop in rectal (core) body temperature of up to 10^{o}C over 40 minutes, a general indicator of disruption of homeostasis in mice (14), increased vascular permeability, which is read out as increased hematocrit; a method that I established during this Thesis work, and the release of plasma vasoactive mediators (*e.g.,* histamine and leukotrienes). Clinical signs, such as ear scratching, lethargy and sometimes seizures or death are observed. Late-phase eosinophilic inflammatory responses are observed 72 h after challenge in the peritoneal cavity.

Immunologically, PN sensitized mice show mixed Th1-Th2 polarization, exhibited by a mixed Ig profile (Th1-associated IgG2a and Th2-associated IgG1 and IgE), splenocyte

recall production of IL-4, IL-5, IL-13 (Th2 cytokines) and IFN-γ (a Th1 cytokine) and Th2-associated eosinophilic inflammation within the peritoneal cavity. Collectively, this model recapitulates many features of the human condition (*50, 51*), including GI sensitization, mixed Th1-Th2 polarization, severe allergic responses, including anaphylaxis, and Th2 polarized inflammatory responses. Thus, this PN allergy model also serves as a system to better understand Th2 immune biology. Throughout this Thesis, we used the severity of effector Th2 responses such as anaphylaxis (immediate hypersensitivity) and eosinophilic inflammation (delayed-type hypersensitivity) as measures of the degree of Th2 priming or sensitization.

PN Allergens

According to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee, allergens are named using the first 3 letters of the genus, followed by a single letter for the species and a number indicating the chronologic order of allergen purification (*52*). To be classified as an allergen, binding of IgE from at least 5, or 5% of sera of patients allergic to the respective allergen source must be shown by any of a number of methods (historically, western blot). To date, 11 PN (*Arachis hypogea*) allergens have been identified (*53-55*). The allergens that are recognized by >50% of a given patient population are designated as major allergens. For PN, these are considered to be *Ara h* 1, *Ara h* 2, *Ara h* 6 (60% homologous to *Ara h* 2), and *Ara h* 3. These proteins tend to be 15-60 kDa as monomers and heat and proteolytic-processing resistant eukaryotic plant seed storage proteins containing repetitive structures: *Ara h* 1 forms stable, allergic dimers, trimers and possibly, hexamers, while Ara h 2 may be able to partially inhibit trypsin activity due to its sequence homology to trypsin inhibitors (*53-55*).

The distinction between an allergen and immunogen, or allergenicity and immunogenicity is crucial, but often undefined and improperly taken as being interchangeable or synonymous. Allergens are molecules capable of eliciting an allergic reaction from a sensitized individual. This implies that the effector immune response of already-primed adaptive immunity is triggered upon allergen exposure. An immunogen is a molecule that triggers, at least, a primary immune response. That is, an immunogen elicits a primary adaptive immune response. The subtle distinction is then that the definition of allergens (IgE binding) does not necessarily imply that the allergen was the immunogen. This can, of course, be the case, but by strict definition, it need not be. Hence, while the proteins that may elicit PN-induced anaphylaxis (Ara h 1-11, with major allergens being Ara h 1, 2, 6, 3) have been identified, what remains to be determined is what protein and/or non-protein components act as immunogens to induce allergic sensitization. Given that the factors that induce PN allergy upon human exposure to PN are not known, and that gavage of PN by itself does not induce PN allergy in mice, an adjuvant is used to induce a food allergic Th2 response to PN. This serves to facilitate investigation of mechanisms of PN allergic sensitization.

CT Adjuvant as a Immunologic Tool to Induce Food Allergy

Vibrio cholera is a gram-negative bacterium that causes the disease, cholera (*56*). This disease is characterized by profuse, but non-inflammatory/bloody diarrhea and is primarily due to the action of the bacterium's secreted CT. This toxin is an archetypal member of the family of AB_5 bacterial toxins that are characterized by a homopentameric B subunit responsible for binding to host cell surfaces, and a toxigenic, enzymatically active A subunit (*57-59*). Members of this family include at least CT, *Escherichia coli* heat-labile enterotoxin (LT), *Campylobacter jejuni* enterotoxin, *Bordetella pertussis* pertussis toxin, *Shigella dysenteriae* shiga

toxin and *E. coli* shiga-like toxin. CT is best known to cause diarrhea and to act as a potent mucosal adjuvant. It is interesting to note that the gene encoding CT is actually encoded as a non-lytic bacteriophage, CTXϕ, which is capable of horizontal gene transfer through CT-encoded virions (*58*).

The mechanism of CT-induced diarrhea continues to be defined (*60, 61*). It is known that CT binds the ubiquitously expressed glycolipid, ganglioside monosialiac (GM) 1 via the action of CTB. Internalization then occurs with CT-containing endosomes trafficking to the Golgi and then, endoplasmic reticulum, where CTA is released from CTB and mimics endogenous misfolded proteins in order to be transported into the cytosol. There, CTA ribosylates cell membrane bound guanidine nucleotide binding protein (G protein) stimulatory α subunit (Gsα), leading to prolonged hyperactivation of adenylate cyclase and thus, production of cyclic AMP (cAMP). In small intestinal epithelial cells, this ultimately causes massive efflux of chloride through the ion channel, cystic fibrosis transmembrane conductance regulator, leading to secretory diarrhea. The mechanism of action of other AB_5 toxins continues to be investigated, but LT is thought to act similarly to CT. Interestingly, *Giardia lamblia* (also known as *G. intestinalis or G. duodenalis*) protozoa cause secretory diarrhea and have excretory-secretory products that act like CT, including GM1 binding and adjuvant activity (*62-64*). SV40 polyomavirus as also binds GM1 for internalization but no published studies have evaluated its adjuvant activity.

CT has long been recognized to be a potent mucosal adjuvant for IgA, IgG, IgE and Th2 responses (*47, 49, 65*). Hence, there is considerable interest in harnessing the adjuvant properties of CT for vaccine purposes (*66, 67*). However, the immune mechanisms of CT's adjuvant effect have remained largely enigmatic. A classical hypothesis is that the same pathway that induces diarrhea also induces immune responses. Whereas studies involving CTA mutants lacking ribosylation activity have reached a consensus that enzyme activity is

required to cause diarrhea, variable conclusions have been made regarding adjuvant activity (*53, 68*). In terms of Th2 responses, it is known that CTB+Ag does not induce IgG1/IgE production (*49*), but it remains unknown how CTA precisely induces Th2 responses. A second hypothesis is that CT's adjuvant ability results from increased intestinal permeability, and thus, access of ingested Ag into the blood. CT-induced increased intestinal permeability has been shown to be permissive for 3 kDa dextrans (*57*), but not 14.7 kDa proteins (*49*). Given that a multitude of ingested Ags are >15 kDa, including those from PN and CT, it remains unclear how important intestinal permeability is to CT's adjuvant activity.

Although interesting, these classical hypotheses do not directly aim to understand how CT interacts with the intestinal immune system. Studies investigating this have shown that oral exposure to CT can induce DC migration to the MLN (*69, 70*). A number of *in vitro* studies have shown that CT activates DCs for Th cell priming (*71*) and also downregulates DC IL-12 secreting potential (*55*), thus favouring Th2 induction. However, the cellular and molecular immune mechanisms that CT engages to produce Th2 responses to co-administered Ags *in vivo* are largely unknown.

Given CT's well known ability to induce Th2 responses to co-administered Ags via oral mucosal exposure, it has become the main method to model food allergic responses. Major advantages of this model include the induction of Th2 immunity through the oral mucosa, rather than a parenteral route, and for such responses to be able to cause anaphylaxis upon subsequent Ag challenge. Since the inciting events that cause human exposure to food allergens to develop into food allergy are unknown, the role of CT in animal models is best viewed as an immunologic tool that induces the clinical phenotype, food allergy. This may be due to the ability of CT to prevent the induction of oral tolerance (*72*). From this viewpoint, the immune pathways engaged by CT, other AB_5 toxins or CT-

like molecules (*e.g. G. lamblia* products), may also be operative during the pathogenesis of human food allergy.

Central Aims

Although it is known that the result of normal and pathological GI immune responses to ingested allergen is clinical tolerance and allergic sensitization respectively, it is not clear why or how allergy actually occurs. Thus, the principal goal of this Thesis was to investigate the immune mechanisms underlying allergic sensitization to PN by examining a murine model of PN allergy (*31*).

First, we hypothesized that intestinal epithelial exposure to ingested PN+CT would cause epithelial activation that would then result in the production of various immune mediators capable of activating nearby cells, such as DCs, to initiate a Th2 response. In 2001, a molecule named thymic stromal lymphopoietin (TSLP) was identified and first found to be able to induce B cell proliferation *in vitro (73)*. Further studies observed TSLP to be highly expressed in the epithelium. Studies involving alum/OVA mouse models of asthma resulted in the proposal that TSLP had a critical, indispensible role for inducing Th2 responses. The TSLP paradigm states that allergen exposure at barrier surfaces such as the lung or gut results in epithelial production of TSLP that, then acts on local DCs to: 1) downregulate Th1-inducing IL12p40, and 2) upregulate the costimulatory molecule OX40 ligand (OX40L). Naïve T cells interacting with TSLP-conditioned DCs that also bear Ag then preferentially differentiate into Th2 cells to cause allergic diseases. TSLP responses were also associated with the production of basophils, which had been reported to be critical for the induction of Th2 responses in asthma or helminth infection models (*74-77*). Activated basophils were also reported to be a source of TSLP (*75, 76*). Since 2001, other molecules associated with expression by epithelial cells and with pro-Th2 capacity were identified, namely IL-25 (IL-

17E) and IL-33 (*78, 79*). The contribution of these molecules to allergy was largely investigated using alum/OVA models of asthma or helminth infection. In some respects, this might be viewed as paradoxical because alum/OVA is administered i.p. (*i.e.* non mucosal) but the cytokines were thought to originate from mucosal epithelia (*80*). Hence, in **Chapter 2** we investigated whether and how epithelial-derived cytokines might initiate PN allergic Th2 responses. Our data show that the cytokine IL-33 induces intestinal DCs to express the costimulatory molecule OX40L, and that both IL-33 and OX40L are required to induce IL-4-dependent Th2 responses to PN independent of basophils. In contrast, TSLP, basophils, and IL-25 were not required to induce Th2 immunity.

In **Chapter 3**, we aimed to better understand the cellular source and regulation of IL-4 required to induce Th2 responses to PN. Although there is a large body of literature supporting a necessary role for IL-4 in inducing Th2 responses, precisely which cell provides IL-4 to instruct naïve T cells to differentiate into Th2 cells remains contentious and unresolved (*81, 82*). For example, various reports have suggested that innate cells capable of producing IL-4 early after activation are required for Th2 responses; these include MCs, basophils, γδ T cells and NKT cells and ILCs. More recently, Th2 responses pathways independent of IL-4 but, instead, dependent on costimulation have been proposed (*83*). Our data show that Th2 responses need not be reliant on accessory cell derived IL-4, or dichotomously costimulatory molecule-dependent versus IL-4 dependent. Instead, a $CD4^+$ T cell-intrinsic IL-4 program, under the control of DC OX40L, drives the development of intestinal Th2 immunity to food allergens.

The GI tract is a unique tissue in that it houses the largest number of eosinophils under normal, baseline conditions (*25, 26*). Eosinophils have long been hypothesized to play a role in anti-helminth host defense but definitive evidence for any immune function in the intestine has remained elusive for over 100 years (*12*). As the GI tract is a major site of food

allergen exposure, **Chapter 4** examines the role of GI indigenous eosinophils in intestinal immunity and food allergy. Our data show that small intestinal eosinophils regulate DC activation and migration to the draining LN through degranulation of the eosinophil granule, EPO, with critical relevance to the development of anaphylactic PN allergy.

Collectively, these three manuscripts examine conditions and mechanisms under which PN ingestion might produce an allergic-anaphylactic phenotype in humans. More specifically, this work shows how allergen-epithelial interactions activate the APC compartment (**Chapter 2**), how APCs are instructed to migrate to the draining LN (**Chapter 4**), and how APCs within the LN activate naïve T cells to become Th2 cells (**Chapter 3**).

Chapter 2.

IL-33, but not thymic stromal lymphopoietin or IL-25 is central to mite and peanut allergic sensitization

IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization.Chu DK, Llop-Guevara A, Walker TD, Flader K, Goncharova S, Boudreau JE, Moore CL, Seunghyun In T, Waserman S, Coyle AJ, Kolbeck R, Humbles AA, Jordana M. J Allergy Clin Immunol. 2013 Jan;131(1):187-200.e1-8.

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Mechanisms of allergy and clinical immunology

IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization

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Background: Allergen exposure at lung and gut mucosae can lead to aberrant T_H2 immunity and allergic disease. The epithelium-associated cytokines thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 are suggested to be important for the initiation of these responses.
Objective: We sought to investigate the contributions of TSLP, IL-25, and IL-33 in the development of allergic disease to the common allergens house dust mite (HDM) or peanut.
Methods: Neutralizing antibodies or mice deficient in TSLP, IL-25, or IL-33 signaling were exposed to HDM intranasally or peanut intragastrically, and immune inflammatory and physiologic responses were evaluated. *In vitro* assays were performed to examine specific dendritic cell (DC) functions.
Results: We showed that experimental HDM-induced allergic asthma and food allergy and anaphylaxis to peanut were associated with TSLP production but developed independently of TSLP, likely because these allergens functionally mimicked TSLP inhibition of IL-12 production and induction of OX40 ligand (OX40L) on DCs. Blockade of OX40L significantly lessened allergic responses to HDM or peanut. Although IL-25 and IL-33 induced OX40L on DCs *in vitro*, only IL-33 signaling was necessary for intact allergic immunity, likely because of its superior ability to induce DC OX40L and expand innate lymphoid cells *in vivo*.
Conclusion: These data identify a nonredundant, IL-33–driven mechanism initiating T_H2 responses to the clinically relevant allergens HDM and peanut. Our findings, along with those in infectious and transgenic/surrogate allergen systems, favor a paradigm whereby multiple molecular pathways can initiate T_H2 immunity, which has implications for the conceptualization and manipulation of these responses in health and disease. (J Allergy Clin Immunol 2013;131:187-200.)

Key words: *T_H2, allergy, asthma, house dust mite, peanut, thymic stromal lymphopoietin, IL-25, IL-33, OX40 ligand, innate lymphoid cells*

Allergies are immune-mediated hypersensitivity diseases mainly driven by T_H2 responses to normally tolerated environmental antigens. Allergic asthma is a chronic lung disease characterized by airway infiltration of T_H2 cells and eosinophils, increased mucus production, and reversible airway obstruction. It is estimated that 10% to 20% of the population is allergic to house dust mite (HDM), the most ubiquitous and pervasive aeroallergen worldwide.[1,2] Food allergy causes symptoms that range from mild erythema and pruritus to acute, life-threatening systemic reactions termed anaphylaxis. Peanut allergy affects approximately 1% to 2% of the general population[3,4] and is highly associated with anaphylaxis.[5]

Despite substantial advances in understanding the pathophysiology of these diseases, the initial cellular and molecular events that cause susceptible subjects to acquire allergic disease are not well understood. Research on the origin of allergic disease in human subjects is exceedingly difficult because sensitization is often clinically silent. Thus the immunologic basis of allergic sensitization is largely investigated in animal models.

Mechanistically, allergic responses have been proposed to be initiated after epithelial cell contact with antigens that leads to the production of a number of cytokines able to promote T_H2 immunity.[6] Paramount among these has been thymic stromal lymphopoietin (TSLP), which has been reported to condition dendritic cells (DCs) to favor T_H2 induction through 2 main actions[7]: (1) limitation of IL-12 production[8-10] and (2) upregulation of the costimulatory molecule OX40 ligand (OX40L).[11,12] Since the identification of TSLP, additional epithelium-associated cytokines also capable of inducing T_H2 immunity have been discovered, notably IL-25 (IL-17E), which signals through IL-17 receptor B

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Abbreviations used
BMDC: Bone marrow–derived dendritic cell
CT: Cholera toxin
DC: Dendritic cell
HDM: House dust mite
IL-17RB: IL-17 receptor B
ILC: Innate lymphoid cell
KO: Knockout
LN: Lymph node
OVA: Ovalbumin
OX40L: OX40 ligand
STAT6: Signal transducer and activator of transcription 6
TSLP: Thymic stromal lymphopoietin
TSLPR: Thymic stromal lymphopoietin receptor
WT: Wild-type
VSV: Vesicular stomatitis virus

(IL-17RB),[13,14] and IL-33, which signals through the ST2 receptor.[15] A majority of studies supporting a role for TSLP, IL-25, and/or IL-33 in the generation of allergic responses in the lung and gut have relied on conventional models of nonmucosal intraperitoneal sensitization to the surrogate allergen ovalbumin (OVA) adsorbed to aluminum-based adjuvants, such as aluminum hydroxide (alum). Thus how these cytokines initiate T_H2 sensitization to common allergens at mucosal sites remains to be elucidated.[16] Recent reports showed that intraperitoneal injection of recombinant IL-25, IL-33, or both can expand novel innate lymphoid cell (ILC) populations in the lung and gut that are able to produce T_H2 cytokines and promote host defense[17]; however, the presence and contribution of these cells during allergic sensitization remain unknown.

Here we sought to better understand how TSLP, IL-25, and IL-33 influence sensitization to the common allergens HDM and peanut in the lung and gut, respectively. To accomplish this, we used established models of HDM-induced allergic asthma[18,19] and peanut allergy and anaphylaxis.[20,21] In stark contrast to the increasingly prevalent opinion that TSLP is a master regulator of T_H2 responses and thus atopic disease,[22] we found that TSLP was dispensable for the generation of IL-4–dependent humoral and cellular immunity to HDM or peanut. Similar results were observed with respect to IL-25. In contrast, IL-33 played an essential role. In fact, our findings delineate an IL-33/OX40L/ILC pathway leading to T_H2 immunity in response to 2 clinically relevant allergens at 2 different mucosal sites.

METHODS

Supplemental information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Mice

Adult (6-8 weeks old) female BALB/c and C57BL/6 mice, thymic stromal lymphopoietin receptor (TSLPR) knockout (KO; B6 or C.129P2-*Crlf2*^{tm1Jni}) and IL-17RB KO (B6.129-*Il17rb*^{tm1Bud}; gifts from Amgen, Thousand Oaks, Calif) mice and ST2 KO mice (C.129P2-*Il1rl1*^{tm1Anjm}; gift from MedImmune, Gaithersburg, Md), were obtained from Charles River (San Diego, Wilmington, Saint-Constant, Quebec, Canada). IL-4 KO (B6.129P2-*Il4*^{tm1Cgn}) and wild-type (WT) control animals were from the Jackson Laboratory (Bar Harbor, Me). IL-13 KO (C.129P2-*Il13*^{tm2Anjm}) and signal transducer and activator of transcription 6 (STAT6) KO (B6.129S-*Stat6*^{tm1Jni}) mice were bred in house. All experiments were performed with age-, sex-, and strain-matched control animals and were approved by the Animal Research Ethics Board of McMaster University (Hamilton, Ontario, Canada).

Statistics

Data were analyzed and graphed with GraphPad Prism 5 software (GraphPad Software, San Diego, Calif). Continuous data are expressed as means ± SEMs and were analyzed by using the *t* test (unpaired, 2-tailed) or 1- or 2-way ANOVA with Dunnet or Bonferroni *post hoc* tests. Temperature data were analyzed with repeated measures. Ordinal data are shown as individual mice with medians and were analyzed with Mann-Whitney *U* tests. Differences were considered statistically significant at a *P* value of less than .05.

RESULTS

Airway sensitization to HDM does not require TSLP signaling

We sought to investigate the requirement of TSLP in experimental allergic asthma to the clinically relevant allergen HDM. This model[23] solely involves mucosal exposure, does not use any exogenous adjuvant, and critically requires the canonical T_H2-inducing molecule IL-4.[24] Consistent with a previous report,[25] TSLP was detected in the lungs of naive mice, and levels were increased upon 3-day HDM exposure (Fig 1, *A*), a protocol that induces sensitization without overt inflammation. TSLP upregulation was not detected in sera (data not shown), suggesting that cytokine signaling took place locally. Thus allergic sensitization to HDM is associated with TSLP production in the lung.

To test the functional role of TSLP signaling during sensitization, we used a 10-day intranasal HDM exposure protocol that recapitulates early features of the allergic diathesis.[18,19] TSLPR KO mice produced levels of T_H2-associated immunoglobulins (IgG_1 and IgE) comparable with those in WT HDM-exposed mice (Fig 1, *B* and *C*). Accordingly, the proportion of activated $CD69^+$ B cells was similar in both groups (Fig 1, *D*). Likewise, overall airway inflammation was comparable in HDM-exposed WT and TSLPR KO mice (Fig 1, *E*), with a similar degree of eosinophil influx (Fig 1, *F*). Histopathologic evaluation of lung sections showed comparable peribronchial and perivascular inflammatory responses and bronchial goblet cell hyperplasia/hypertrophy with mucus production (Fig 1, *G* and *H*). T-cell activation *in vivo* and *ex vivo* cytokine production were intact regardless of the presence or absence of TSLPR (Fig 1, *I* and *J*).

Similar results were observed in BALB/c mice treated before and throughout the HDM exposure protocol with a neutralizing anti-TSLP antibody (see Fig E1, *A-C*, in this article's Online Repository at www.jacionline.org), as well as in C57BL/6 TSLPR KO mice (see Fig E1, *D-F*). Collectively, these data show that allergic sensitization and airway inflammation in response to HDM occur independently of TSLP and its receptor.

These data, generated by using the common aeroallergen HDM, are in sharp contrast to the results published by 2 different groups reporting TSLP-dependent lung T_H2 responses to the surrogate allergen OVA in mouse models involving intraperitoneal alum-driven sensitization protocols.[26,27] This discrepancy was not due to colony differences because we observed similar TSLPR dependency in OVA plus alum–sensitized mice (see Fig E1, *A-H*). Thus although our data confirm a role for TSLP in this OVA model, they do not support the contention that TSLP is pervasively a key factor in the initiation of allergic asthma.

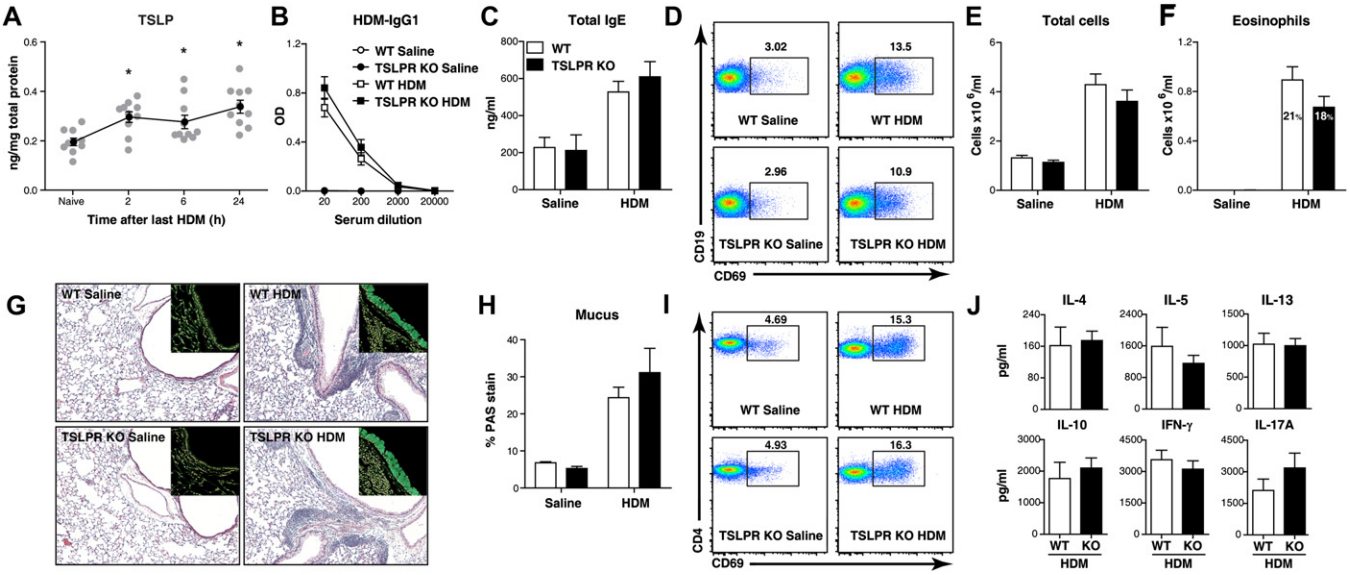

FIG 1. HDM-induced TSLP is not required for allergic sensitization. **A**, TSLP in lung homogenates from naive mice or after 3 days of HDM. **B-J**, BALB/c WT or TSLPR KO mice were exposed to saline or HDM for 10 days. HDM-specific IgG_1 (Fig 1, *B*) and total IgE (Fig 1, *C*) levels in serum. Representative lung $CD45^+CD3^-CD19^+$ B cells expressing CD69 (Fig 1, *D*). Total (Fig 1, *E*) and eosinophil (Fig 1, *F*) airway infiltrates in bronchoalveolar lavage fluid; mean percentage of eosinophils are shown in *bars*. Fig 1, *G*, Representative hematoxylin and eosin–stained lung sections (×100 magnification); *insets* show color-inverted periodic acid–Schiff *(PAS)*–stained sections (×400 magnification). Fig 1, *H*, Morphometric quantification of periodic acid–Schiff staining. Fig 1, *I*, Representative CD69 expression by lung $CD45^+CD3^+CD4^+$ T cells. Fig 1, *J*, Cytokine levels in supernatants of splenocytes stimulated with HDM. Shown are 2 to 3 experiments with 3 to 6 mice per group per experiment. $*P < .05$ vs naive.

Gastrointestinal sensitization to peanut is IL-4 and STAT6 dependent, but does not require TSLP signaling or basophils

To investigate whether the previous results were idiosyncratic of the site and allergen used, we analyzed the role of TSLP in the induction of T_H2 immunity in the gastrointestinal tract. We have previously shown that intragastric exposure of mice to peanut along with cholera toxin (CT) induces strong gastrointestinal T_H2 responses, such that subsequent peanut challenge elicits systemic anaphylaxis.[20,28] Previously reported mRNA analysis of steady-state intestinal *tslp* expression showed the highest levels in the caecum and large intestine.[10] After ingestion of peanut plus CT for 3 days, we observed enhanced TSLP protein levels in the duodenum but not in other intestinal segments (Fig 2, *A*). TSLP was not detected in intestinal washes or sera (data not shown). Thus the initiation of peanut allergy is associated with TSLP upregulation in the small intestine.

We next attempted to induce gastrointestinal T_H2 immunity to peanut in the absence of TSLP signaling. Both WT and TSLPR KO mice sensitized to peanut produced similarly increased levels of peanut-specific IgG_1 and IgE (Fig 2, *B* and *C*). Functionally, both groups of mice experienced similar anaphylactic responses upon peanut challenge, as measured by comparable decreases in core body temperature, increases in hematocrit levels (a physiologic measure of systemic vascular leakage), and development of clinical signs ranging from pruritus to seizure or death (Fig 2, *D-F*). Using B cell–independent but $CD4^+$ T cell–dependent peritoneal eosinophilic inflammation 72 hours after intraperitoneal challenge as an *in vivo* measure of T-cell function (data not shown),[20] we found robust responses in both the peanut-sensitized and challenged groups (Fig 2, *G* and *H*). Furthermore, *ex vivo* splenocyte cytokine production was also comparable in both groups (Fig 2, *I*).

Anti-TSLP antibody administration throughout sensitization generated similar results (see Fig E2, *A-C*, in this article's Online Repository at www.jacionline.org and data not shown). We also considered that the type of antigen, adjuvant, or route of administration might have affected TSLP dependency, but WT and TSLPR KO mice sensitized with OVA plus CT intragastrically, peanut plus alum intraperitoneally, peanut plus CT intraperitoneally, or the conventional peanut plus CT intragastrically produced similarly increased levels of antigen-specific IgG_1 and IgE and, upon challenge, comparable anaphylactic responses (see Fig E2, *D-O*, and data not shown). Collectively, these results show that intact TSLP signaling is not required for gastrointestinal T_H2 priming and subsequent humoral or cellular food-induced allergic responses.

Basophils, which can respond to and produce TSLP, have been reported to be critical for T_H2 priming in some experimental systems.[29,30] Anti-CD200R3 antibody (clone Ba103) depletion of basophils during HDM exposure was recently reported to partially decrease eosinophilic airway inflammation but not alter T_H2 priming and cytokine production.[31] In the case of peanut, depletion of basophils using anti-asialo GM1[32] or Ba103[21] did not impair peanut-specific IgG_1 or IgE production and subsequent anaphylactic responses to peanut (see Fig E2, *P-R*, and data not shown). Hence basophils are not required for HDM or peanut sensitization.

TSLP has been proposed to be a master regulator of T_H2 immunity and reported to require IL-4.[33,34] Indeed, canonical T_H2 differentiation requires IL-4–mediated activation of STAT6. We have shown that HDM-induced allergic asthma requires IL-4.[24]

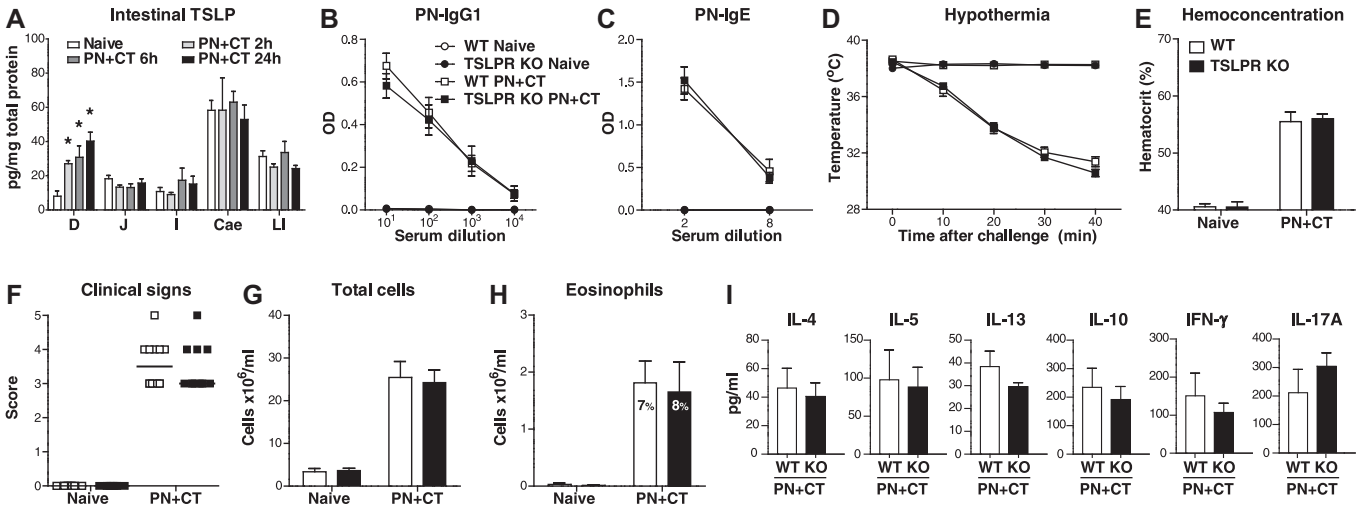

FIG 2. Peanut *(PN)*–induced TSLP is not required for allergic sensitization. **A**, TSLP in tissues from naive mice or after 3 days of peanut CT. *Cae*, Caecum; *D*, duodenum; *I*, ileum; *J*, jejunum; *LI*, large intestine. **B-I**, C57BL/6 WT or TSLPR KO mice were sensitized to peanut and, 1 week later, challenged intraperitoneally. Serum levels of peanut-specific IgG_1 (Fig 2, *B*) and IgE (Fig 2, *C*) 1 day before challenge. Anaphylactic assessment by core body temperature (Fig 2, *D*), hematocrit levels (Fig 2, *E*), and clinical signs (Fig 2, *F*). Total (Fig 2, *G*) and eosinophil (Fig 2, *H*) infiltrates in peritoneal lavage; mean percentages of eosinophils are shown in *bars*. Fig 2, *I*, Cytokine production by peanut-stimulated splenocytes. Shown are 2 to 3 experiments with 3 to 8 mice per group per experiment. $*P < .05$ versus naive.

We considered that the peanut allergy model did not induce canonical T_H2 induction and hence did not require TSLP. STAT6 or IL-4 KO mice exposed to peanut plus CT did not produce any detectable levels of peanut-specific IgG_1 or IgE (data not shown) or show any clinical or physiologic indication of anaphylaxis upon challenge (see Fig E3, *A-F*, in this article's Online Repository at www.jacionline.org). In contrast, IL-13 KO mice generated robust IgG_1 and anaphylactic responses (see Fig E3, *G-I*). Additionally, peanut-stimulated splenocytes from peanut-sensitized WT and IL-13 KO, but not IL-4 and STAT6 KO, mice produced significantly increased amounts of IL-5 compared with levels seen in naive mice (not shown). Therefore this peanut allergy model is dependent on the canonical T_H2-inducing molecules IL-4 and STAT6, yet functions independently of TSLP.

Overall, data from 2 distinct models of allergic disease lead us to conclude, at variance with the recent proposition,[7,22,35,36] that TSLP does not play a universal role in initiating T_H2 allergic immunity at mucosal surfaces.

HDM and peanut functionally replace TSLP action on DCs

TSLP has been reported to instruct DCs to facilitate T_H2 responses through limiting the production of IL-12p40[8-10] and inducing OX40L.[11,12] To better understand why the development of HDM-induced allergic asthma and peanut allergy did not require TSLP, we investigated whether these allergens could influence DC behavior. To achieve this, bone marrow–derived dendritic cells (BMDCs) were conditioned with complete medium, TSLP, HDM, or peanut and subsequently stimulated with medium or LPS. Few (<5%) DCs conditioned with medium alone spontaneously produced IL-12p40, whereas more than 50% of DCs were IL-12p40$^+$ upon LPS stimulation (Fig 3, *A* and *B*). As expected,[9,10] TSLP conditioning significantly inhibited this effect. Conditioning with peanut or HDM also limited DC IL-12p40 production to the same extent as TSLP (Fig 3, *A-C*). These effects were not due to allergen-elicited TSLP production from DCs[37] because culture supernatants did not have detectable levels of TSLP (data not shown). These data indicate that promotion of T_H2 responses by IL-12p40 inhibition can take place after DC contact with either TSLP or the allergens HDM or peanut.

We next investigated whether common allergens could also functionally mimic TSLP induction of OX40L on DCs *in vitro*. We found that vesicular stomatitis virus (VSV) infection of DCs strongly induced OX40L; thus we used this as a positive control. Compared with unstimulated DCs, incubation with HDM or peanut allergens significantly increased the proportion of $CD11c^+OX40L^+$ cells and the median fluorescence intensity of OX40L in a manner comparable with TSLP (Fig 3, *D-F*). Again, levels of TSLP upon HDM or peanut stimulation were undetectable (data not shown), implying that OX40L induction on DCs was not due to autocrine TSLP signaling. These data indicate that the common allergens HDM and peanut functionally mimic TSLP action on DCs, therefore rendering it redundant during DC conditioning for T_H2 induction.

OX40L plays an important role in allergic responses to HDM and peanut

We next asked whether OX40L might be important for T_H2 induction to HDM or peanut *in vivo*. First, we evaluated OX40L protein expression after 3 days of allergen exposure. Lung OX40L expression was increased upon HDM exposure, with induction on $CD11c^+CD11b^{hi}MHCII^{hi}$ myeloid DCs (Fig 4, *A-D*). Peanut plus CT administration induced OX40L in the duodenum (Fig 4, *E*). Accordingly, the proportion of mesenteric lymph node (LN) $CD11c^+CD11b^+MHCII^+$ DCs expressing OX40L and their median fluorescence intensities were also significantly increased (Fig 4, *F-H*). The frequency of myeloid DCs in the lung and mesenteric LNs after acute allergen exposure increased 2- to 5-fold (see Fig E4 in this article's Online Repository

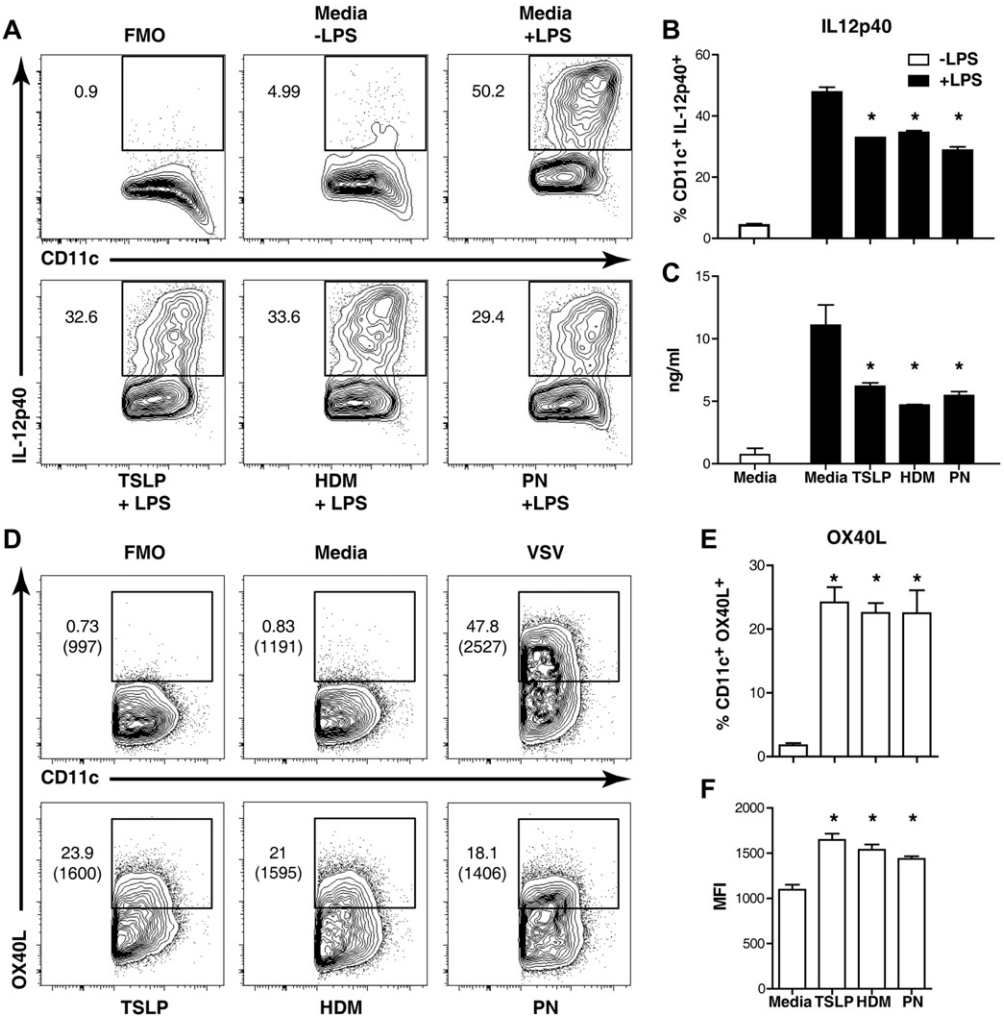

FIG 3. HDM and peanut *(PN)* mimic TSLP in downregulating IL-12p40 and upregulating OX40L. **A-C**, BMDCs were conditioned with media, TSLP, HDM, or peanut and then stimulated with media or LPS. Representative plots (Fig 3, *A*) and quantification (Fig 3, *B*) of $CD11c^+IL\text{-}12p40^+$ cells. Fig 3, *C*, IL-12p40 in culture supernatants. **D-F**, BMDCs were stimulated with media, VSV positive control, TSLP, HDM, or peanut. Representative plots (median fluorescence intensity in brackets; Fig 3, *D*), quantification of $CD11c^+OX40L^+$ cells (Fig 3, *E*), and OX40L median fluorescence intensity (Fig 3, *F*). Shown are 2 to 3 experiments performed in triplicate. $^*P < .05$ versus media plus LPS (Fig 3, *B* and *C*) or media only (Fig 3, *E* and *F*). *FMO*, Fluorescence minus one.

at www.jacionline.org). Thus sensitization to HDM or peanut plus CT is associated with OX40L upregulation, especially on DCs.

Mice were treated with blocking anti-OX40L antibody or control immunoglobulin throughout HDM administration to test the importance of OX40L on HDM sensitization *in vivo*. Compared with control immunoglobulin-treated and HDM-exposed mice, serum IgG_1 and IgE levels and B-cell activation were significantly attenuated in mice treated with anti-OX40L antibody (Fig 5, *A-C*). The overall inflammatory response was decreased by about 70%, with eosinophil counts being reduced approximately 90% (Fig 5, *D* and *E*). Lung tissue histology confirmed these findings and, in addition, demonstrated reduced goblet cell responses (Fig 5, *F*). $CD4^+$ T-cell activation and the expression of IL-4 and IL-5, but not IFN-γ or IL-17A, were significantly decreased in the absence of OX40L signaling, suggesting a selective impairment of T_H2 immune responses (Fig 5, *G* and *H*).

Blockade of OX40L during peanut sensitization attenuated peanut-specific IgG_1 and IgE levels (Fig 5, *I* and *J*). Upon peanut challenge, anti-OX40L antibody–treated mice experienced approximately 50% less severe anaphylactic hypothermia and hemoconcentration and had less severe clinical signs compared with control immunoglobulin-treated mice (Fig 5, *K-M*). In addition to impaired B-cell responses, T-cell immunity was also hindered, with less than half as many inflammatory cells and approximately 90% less eosinophils in anti-OX40L antibody–treated mice (Fig 5, *N* and *O*). Production of T_H2, but not T_H1 or T_H17, cytokines was impaired (Fig 5, *P*). Collectively, these results show that HDM and peanut allergen–driven humoral and cellular responses operate in a TSLP-independent but OX40L-dependent manner.

IL-25 and IL-33 induce OX40L on DCs *in vitro*

Because mucosal allergen exposure results in TSLP production, we next asked whether other epithelium-associated cytokines with T_H2 induction capacity analogous to TSLP, namely IL-25 and IL-33, were produced during HDM or peanut sensitization. After 3

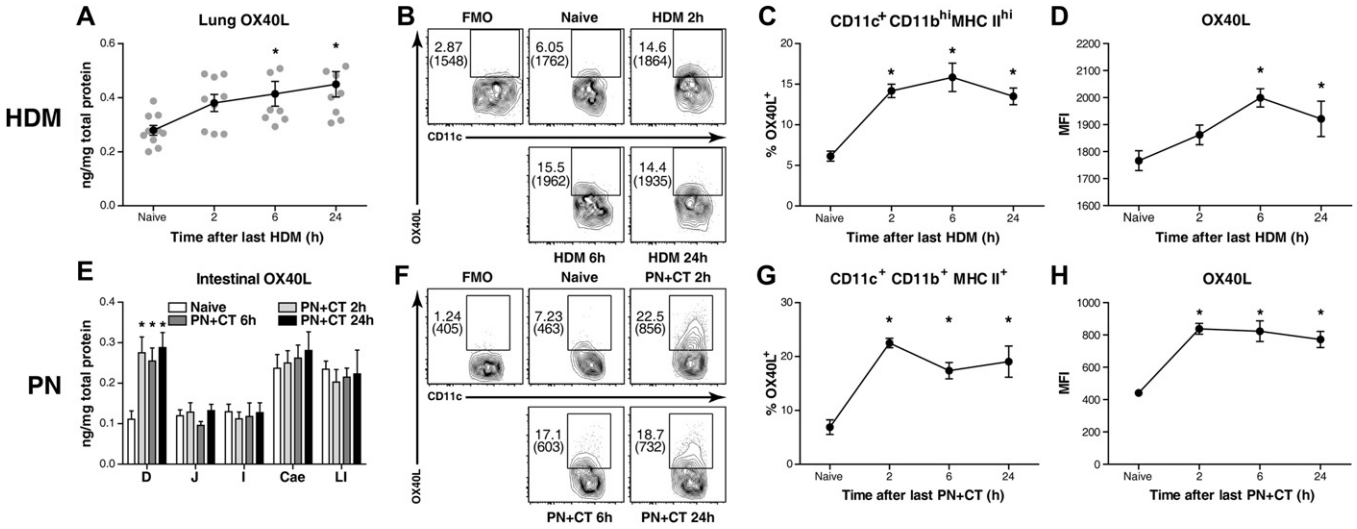

FIG 4. HDM and peanut *(PN)* sensitization induce OX40L on DCs *in vivo*. Mice were exposed to HDM intranasally (**A-D**) or to peanut plus CT intragastrically (**E-H**) for 3 days. Fig 4, *A* and *E*, Kinetics of OX40L induction. Representative plots (median fluorescence intensity in brackets) of OX40L on lung $CD11c^{+}CD11b^{hi}MHCII^{hi}$ DCs (Fig 4, *B*) or mesenteric LN $CD11c^{+}CD11b^{+}MHCII^{+}$ DCs (Fig 4, *F*). Fig 4, *C, D, G,* and *H,* Quantification of OX40L induction on DCs. Shown are 2 to 3 experiments with 5 to 6 mice per group per experiment. $*P < .05$ versus naive. *Cae,* Caecum; *D,* duodenum; *FMO,* fluorescence minus one; *I,* ileum; *J,* jejunum; *LI,* large intestine.

days of HDM exposure, lung IL-25 and IL-33 were induced with slightly different kinetics (Fig 6, *A* and *C*). Similarly, acute peanut plus CT exposure increased duodenal levels of IL-25 and IL-33 (Fig 6, *B* and *D*). These cytokines were not detected in the serum (data not shown), suggesting local rather than systemic influence. They were also not increased in the bronchoalveolar lavage fluid or intestinal washes of allergen-exposed mice (not shown), suggesting that basolateral rather than apical secretion of these cytokines was associated with allergic sensitization.

Because upregulation of IL-25 and IL-33 in allergen-exposed tissues (Fig 6, *A-D*) preceded or was coincident with increased DC OX40L expression (Fig 4), we next evaluated whether IL-25 and IL-33, like TSLP, also induced DC OX40L *in vitro*. DCs have been previously shown to be responsive to IL-33[38] and IL-25.[39,40] Here we show that these cytokines were equally capable of inducing OX40L on DCs *in vitro* (Fig 6, *E* and *F*). TSLP levels were undetectable in all cultures that did not receive TSLP; likewise, IL-25 and IL-33 were undetectable in all cultures that did not receive IL-25 or IL-33 (data not shown). These data suggest that allergen exposure results in the elaboration of multiple epithelium-associated cytokines that converge on DC maturation, including OX40L upregulation. Consequently, we considered whether IL-25, IL-33, or both were important for the induction of allergic disease *in vivo*.

IL-25 and its receptor are dispensable for pulmonary and gastrointestinal allergic sensitization

IL-25 signaling requires the presence of its cytokine-binding receptor subunit, IL-17RB,[14] and exogenous recombinant IL-25 results in severe T_H2 pathology in the lung and gut.[13] Hence to investigate the requirement of IL-25 signaling on the induction of T_H2 responses to HDM and peanut, we attempted to induce allergic asthma or peanut allergy in IL-17RB KO mice.

Compared with saline-exposed mice, HDM-exposed WT or IL-17RB KO mice had similarly increased HDM-specific IgG_1 and total IgE levels and, accordingly, B-cell activation, suggesting equivalent sensitization (Fig 7, *A-C*). Total inflammatory cell numbers, proportions and numbers of eosinophils, and goblet cell responses were comparable between the HDM-exposed WT and IL-17RB KO groups (Fig 7, *D-F*). In regard to T-cell immunity, $CD69^{+}CD4^{+}$ T cells and *ex vivo* HDM-specific cytokine production were equally increased to greater than that seen with saline controls in both WT and IL-17RB KO mice (Fig 7, *G* and *H*). Thus T_H2 responses to HDM can fully develop in the absence of IL-25 signaling.

Similar to pulmonary T_H2 responses to HDM, gastrointestinal sensitization of WT and IL-17RB KO mice to peanut resulted in indistinguishably increased peanut-specific IgG_1 and IgE levels above those seen in corresponding naive mice (Fig 7, *I* and *J*). Upon peanut challenge, both groups of peanut-sensitized mice had similar anaphylactic responses (Fig 7, *K-M*). Deficiency of IL-25 signaling also did not impair T-cell priming, as evidenced by peritoneal inflammation and cytokine production (Fig 7, *N-P*).

Treatment of mice with an anti–IL-25 antibody before and throughout sensitization to HDM or peanut resulted in data similar to those generated by using IL-17RB KO mice (see Fig E5 in this article's Online Repository at www.jacionline.org). Therefore although IL-25 is capable of inducing OX40L *in vitro*, these data comprehensively show that IL-25 signaling is not required to generate B- and T-cell allergic immunity to HDM or peanut *in vivo*.

IL-33 signaling mediates allergy to peanut and HDM

Because neither TSLP nor IL-25 played a significant role in initiating HDM or peanut allergic phenotypes, we investigated the relevance of IL-33, which binds the ST2 receptor.[15] Compared with HDM-exposed WT mice, serum HDM-specific IgG_1 levels were significantly decreased and IgE levels were diminished by more than 50% in ST2 KO mice (Fig 8, *A* and *B*). Similarly, the extent of airway and lung inflammation and mucus production

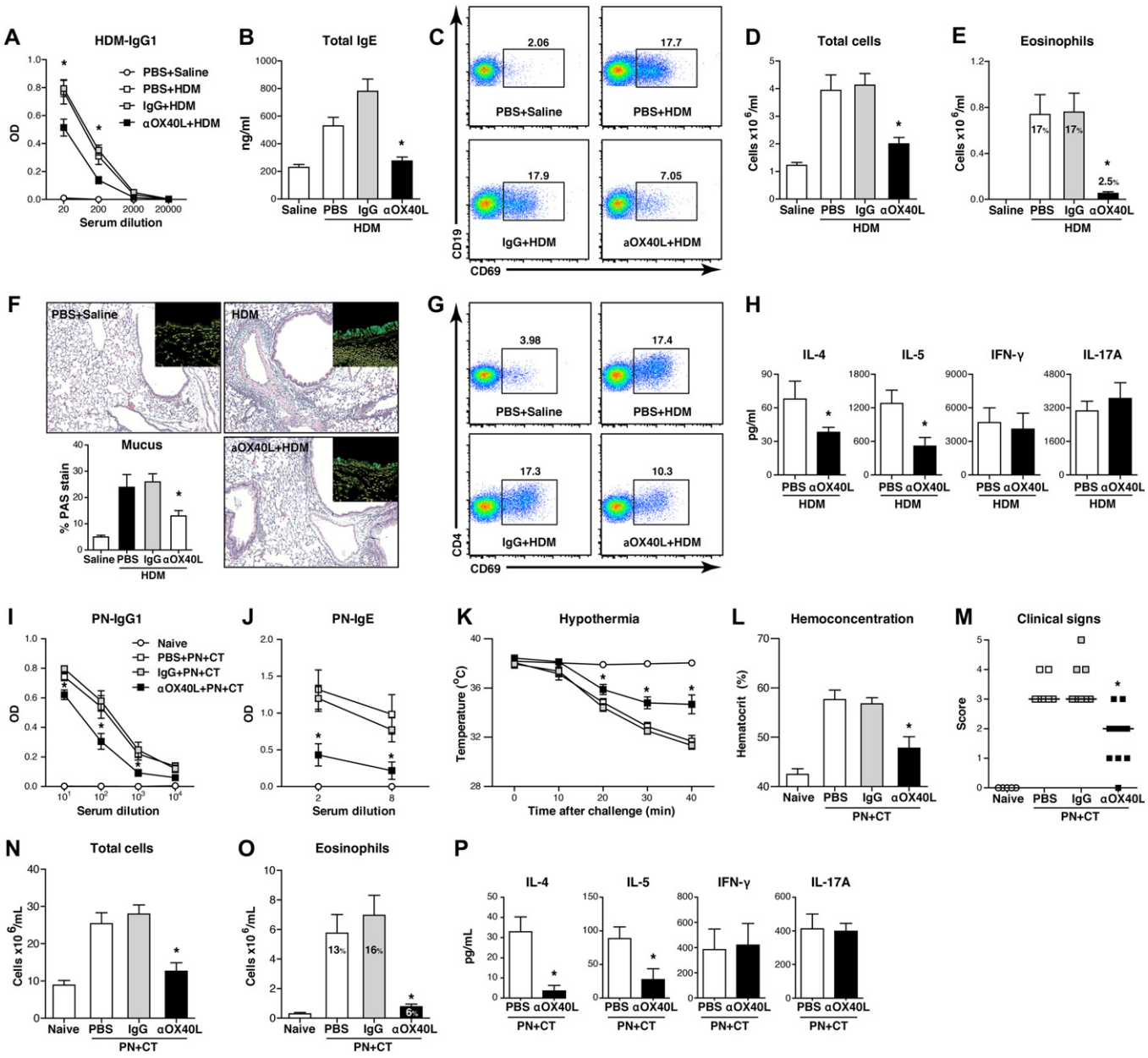

FIG 5. OX40L is required for allergic sensitization to HDM and peanut *(PN)*. **A-H**, Mice received PBS vehicle, IgG, or anti-OX40L before and throughout 10 days of saline or HDM exposure. HDM-specific IgG_1 (Fig 5, *A*) and total IgE (Fig 5, *B*) levels in serum. Fig 5, *C*, Representative CD69 expression by lung $CD45^+CD3^-CD19^+$ B cells. Total cell (Fig 5, *D*) and eosinophil (Fig 5, *E*) counts in bronchoalveolar lavage fluid; mean percentages of eosinophils are shown in *bars*. Fig 5, *F*, Representative lung histology stained with hematoxylin and eosin (×100 magnification) or periodic acid–Schiff *(PAS)* insets (×400 magnification). HDM exemplifies similar sections from PBS plus HDM and IgG plus HDM groups. Periodic acid–Schiff quantification is shown at *bottom left*. Fig 5, *G*, Representative CD69 expression by lung $CD45^+CD3^+CD4^+$ T cells. Fig 5, *H*, Cytokine production by HDM-stimulated splenocytes. **I-P**, Mice received PBS, IgG, or anti-OX40L during peanut sensitization and were challenged 1 week later. Serum peanut-specific IgG_1 (Fig 5, *I*) and IgE (Fig 5, *J*) levels 1 day before challenge. Fig 5, *K-M*, Anaphylactic assessment. Total (Fig 5, *N*) and eosinophil (Fig 5, *O*) cell counts in peritoneal lavage; mean percentages of eosinophils are shown in *bars*. Fig 5, *P*, Cytokine production by peanut-stimulated splenocytes. Shown are 3 to 8 mice per group per experiment. $*P < .05$ versus PBS or IgG mice exposed to HDM or peanut plus CT.

was lower in HDM-exposed ST2 KO mice, with the infiltration of eosinophils decreased by more than 80% (Fig 8, *C-E*). The proportion of activated $CD4^+$ T cells in the ST2 KO mice was dramatically decreased, as was the *ex vivo* production of IL-4, IL-5, IFN-γ, and IL-17A (Fig 8, *F* and *G*), suggesting an overall impairment in adaptive immune priming.

In response to the peanut sensitization regimen, peanut-specific IgG_1 and IgE levels were diminished by more than 80% in ST2

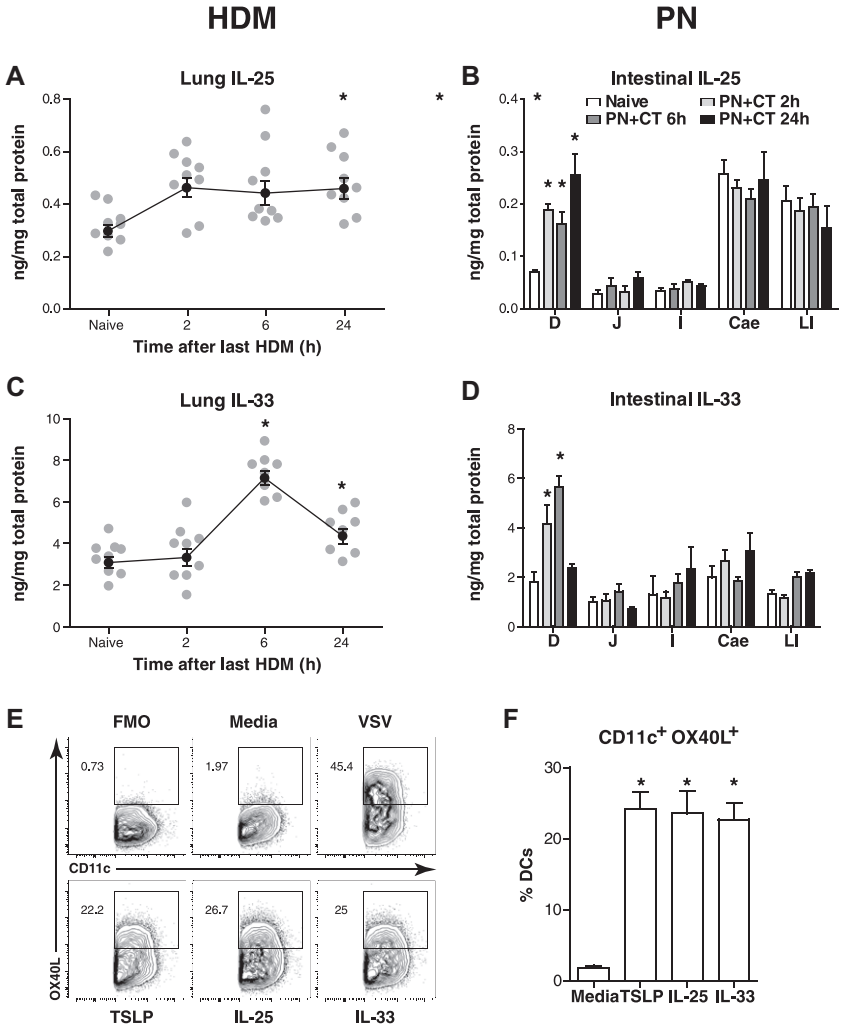

FIG 6. IL-25 and IL-33 induce OX40L on DCs *in vitro*. Kinetics of IL-25 (**A** and **B**) and IL-33 (**C** and **D**) induction after 3 days of HDM or peanut *(PN)* plus CT. Representative plots **(E)** and quantification **(F)** of $CD11c^+OX40L^+$ BMDCs stimulated with media, VSV, recombinant TSLP, IL-25, or IL-33. Two experiments were performed with 5 to 6 mice per group per experiment (Fig 6, *A-D*) or replicates of 2 to 4 (Fig 6, *E* and *F*). $*P < .05$ versus naive or media. *Cae*, Caecum; *D*, duodenum; *FMO*, fluorescence minus one; *I*, ileum; *J*, jejunum; *LI*, large intestine.

KO mice compared with those seen in WT control animals (Fig 8, *H* and *I*). This translated into protection from anaphylactic shock on peanut challenge because these mice did not exhibit hypothermia and had 50% less hemoconcentration and minor clinical signs of anaphylaxis compared with those seen in sensitized and challenged WT mice (Fig 8, *J-L*). Eosinophilic inflammation in peanut plus CT–exposed ST2 KO mice was markedly reduced by about 95%, suggesting severely inhibited T_H2 priming (Fig 8, *M* and *N*). In agreement, T_H2 cytokine production *ex vivo* was also significantly decreased in ST2 KO mice (Fig 8, *O*).

Although IL-33 has been reported to play a role during the effector phase of passive anaphylaxis to 2,4-dinitrophenol,[41] we did not find IL-33 to drive the anaphylactic event in our model of active anaphylaxis to peanut. ST2-Fc treatment only during the sensitization phase ameliorated anaphylaxis because of reduced peanut-specific IgE production (see Fig E6, *A-D*, in this article's Online Repository at www.jacionline.org); treatment before induction of anaphylaxis[41] had no effect on hypothermia or hemoconcetration (see Fig E6, *E-H*). These data point toward a central role for IL-33 during sensitization, rather than the effector phase of peanut allergy. Collectively, these data show that unlike TSLP and IL-25, IL-33 plays a nonredundant role in the initiation of respiratory (HDM) and food (peanut) allergy.

Allergen-elicited IL-33 potently induces DC OX40L and ILCs *in vivo*

We observed that TSLP, IL-25, and IL-33 could induce OX40L *in vitro*, yet only IL-33 was required for the generation of T_H2 immunity to HDM or peanut *in vivo*. To better understand how DCs responded to these cytokines *in vivo*, mice received 0.5 μg of recombinant TSLP, IL-25, or IL-33 intranasally for 3 days, and 24 hours later, tissues were analyzed.[12,14,42] In the lung the levels of total and DC OX40L were increased in response to IL-33 but not TSLP or IL-25 (Fig 9, *A* and *B*), suggesting that OX40L induction *in vivo* is exquisitely sensitive to IL-33.

Correlating with OX40L induction, we also observed the expansion of a lineage-negative population ($CD45^+Lin^-$;

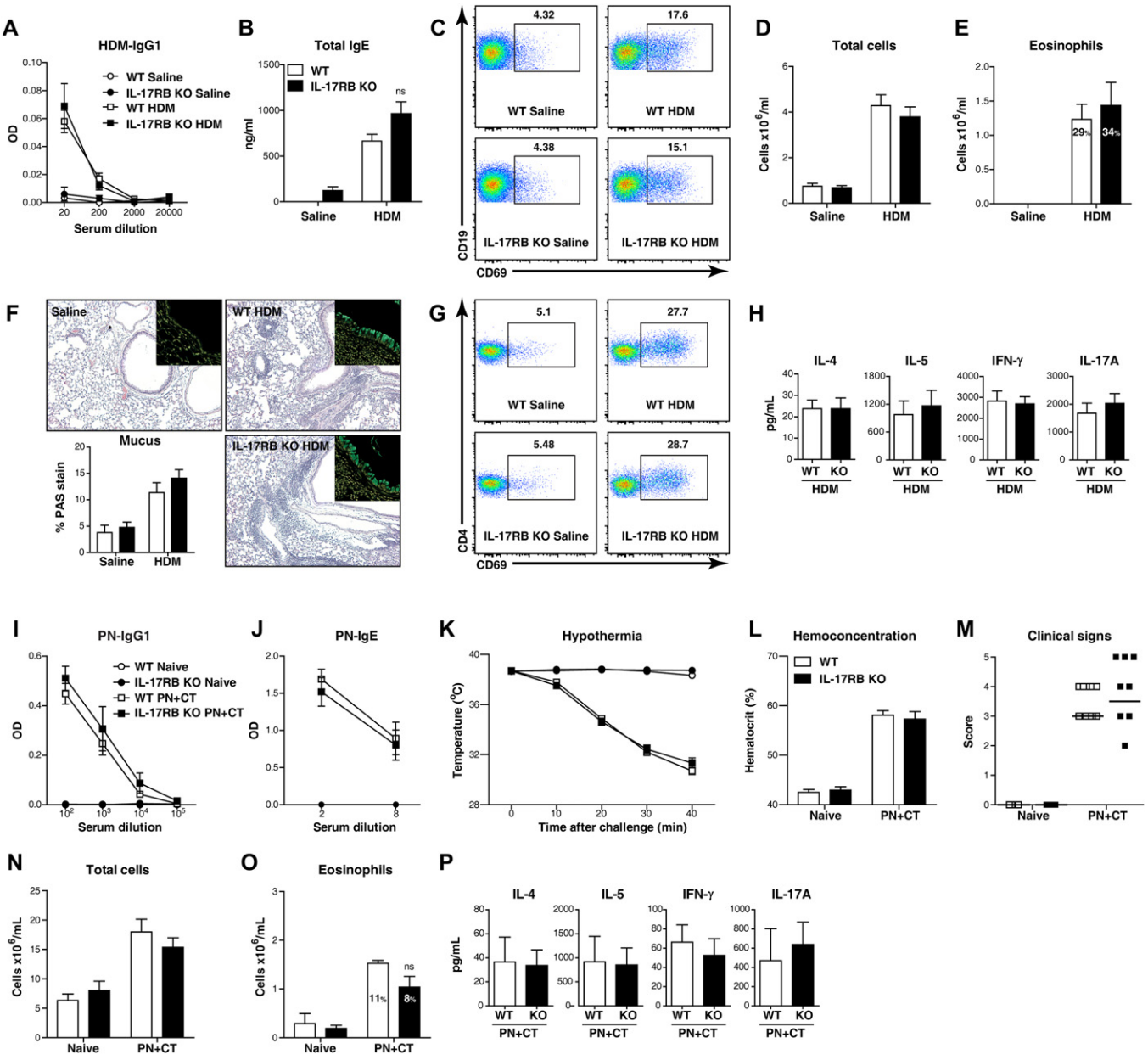

FIG 7. IL-17RB is dispensable for allergic sensitization to HDM and peanut *(PN)*. **A-H**, WT and IL-17RB KO mice were exposed to 10 days of HDM. Serum HDM-specific IgG_1 (Fig 7, *A*) and total IgE (Fig 7, *B*) levels. Fig 7, *C*, Representative lung $CD45^+CD3^-CD19^+$ B-cell expression of CD69. Total (Fig 7, *D*) and eosinophil (Fig 7, E) counts in bronchoalveolar lavage fluid; mean percentages are shown in *bars*. Fig 7, *F*, Representative lung histology stained with hematoxylin and eosin (×100 magnification) or periodic acid–Schiff (PAS; ×400 magnification) insets. Saline exemplifies similar sections from the WT saline and IL-17RB KO saline groups. Periodic acid–Schiff analysis is shown at *bottom left*. Fig 7, *G*, Expression of CD69 by lung $CD45^+CD3^+CD4^+$ T cells. Fig 7, *H*, Cytokine production by HDM-stimulated splenocytes. **I-P**, WT or IL-17RB KO mice were sensitized to peanut and challenged intraperitoneally 1 week later. Serum peanut-specific IgG_1 (Fig 7, *I*) and IgE (Fig 7, *J*) levels 1 day before challenge. Fig 7, *K-M*, Anaphylactic assessment. Total (Fig 7, *N*) and eosinophil (Fig 7, *O*) cell counts in peritoneal lavage fluid; mean percentages are shown in *bars*. Fig 7, *P*, Cytokine production by peanut-stimulated splenocytes. Shown are 2 experiments with 2 to 8 mice per group per experiment.

$CD3^-CD19/B220^-CD11c^-CD11b^-$) expressing c-kit, Sca-1, and Thy1.2 (CD90.2) after administration of IL-33 but not TSLP or IL-25 (Fig 9, *C* and *D*). Expression of ST2 and inducible costimulator was present on 30% to 50% of these cells (data not shown). Recent reports describe these novel cells as ILCs, which can respond to IL-25, IL-33, or both and expand on airway viral or gastrointestinal helminth infection.[17] Increased OX40L expression and expansion of ILCs was observed not only in the lung but also in the thoracic LNs, small intestine, and mesenteric LNs upon intraperitoneal IL-33 (data not shown). These data suggest that T_H2 induction to HDM or peanut relies on the ability of IL-33 to upregulate OX40L and expand ILCs. Accordingly, the

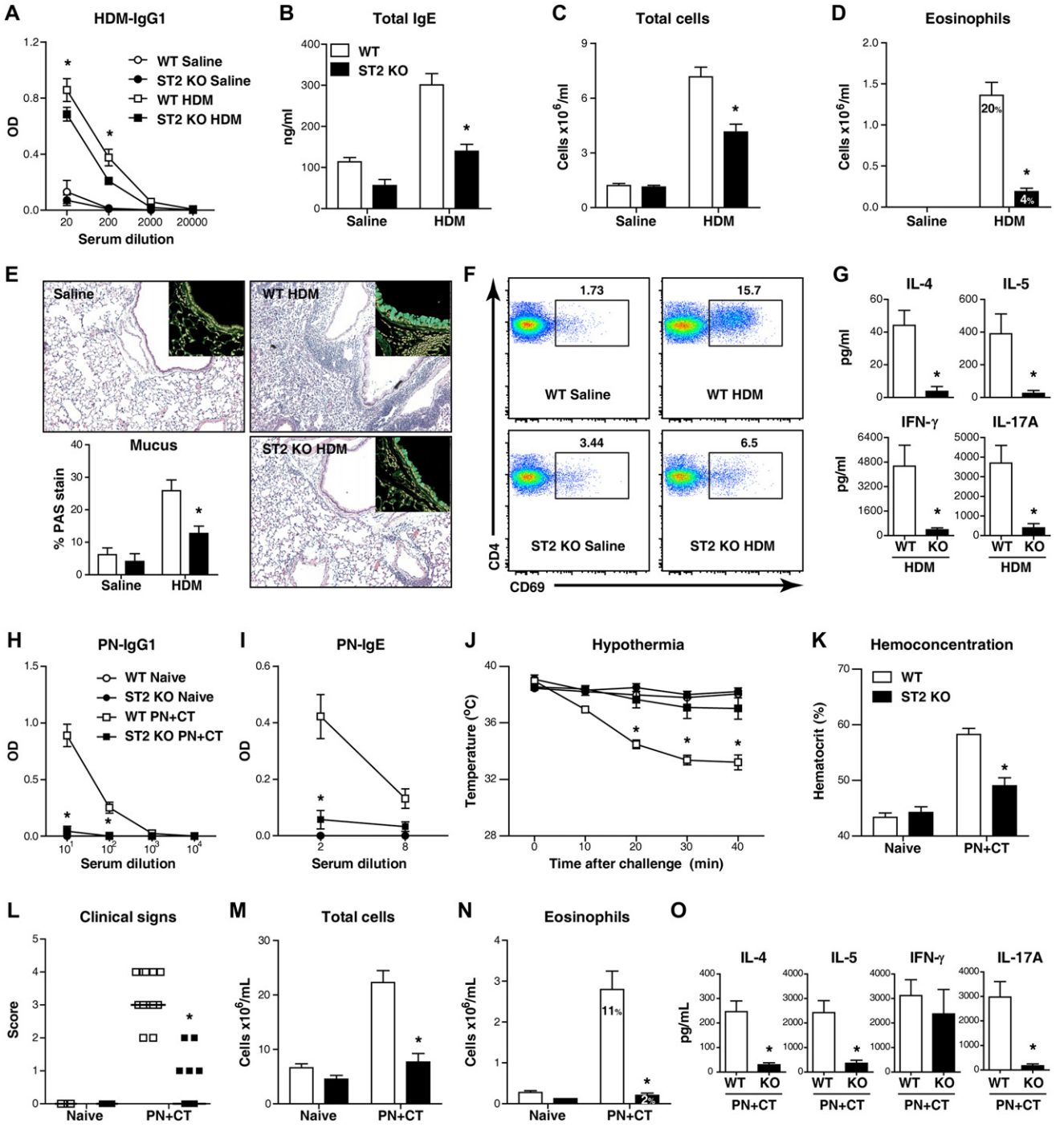

FIG 8. The IL-33 receptor ST2 is required for allergic sensitization to HDM and peanut *(PN)*. **A-G**, WT and ST2 KO mice were exposed to 10 days of HDM. Serum HDM-specific IgG_1 (Fig 8, *A*) and total IgE (Fig 8, *B*) levels. Total (Fig 8, *C*) and eosinophil (Fig 8, *D*) counts in bronchoalveolar lavage fluid; mean percentages are shown in *bars*. Fig 8, *E*, Representative lung histology stained with hematoxylin and eosin (×10 magnification) or periodic acid–Schiff (*PAS*; ×40 magnification) insets. Saline exemplifies similar sections from the WT saline and ST2 KO saline groups. Periodic acid–Schiff analysis is shown at *bottom left*. Fig 8, *F*, Expression of CD69 by lung $CD45^+CD3^+CD4^+$ T cells. Fig 8, *G*, Cytokine production from HDM-stimulated splenocytes. **H-O**, WT or ST2 KO mice were sensitized to peanut and then challenged intraperitoneally 1 week later. Serum peanut-specific IgG_1 (Fig 8, *H*) and IgE (Fig 8, *I*) levels 1 day before challenge. Fig 8, *J-L*, Anaphylactic assessment. Total (Fig 8, *M*) and eosinophil (Fig 8, *N*) cell counts in peritoneal lavage fluid; mean percentages are shown in *bars*. Fig 9, *O*, Cytokine production by peanut-stimulated splenocytes. Shown are 2 experiments with 2 to 8 mice per group per experiment. *$P < .05$ versus WT mice exposed to HDM or peanut plus CT.

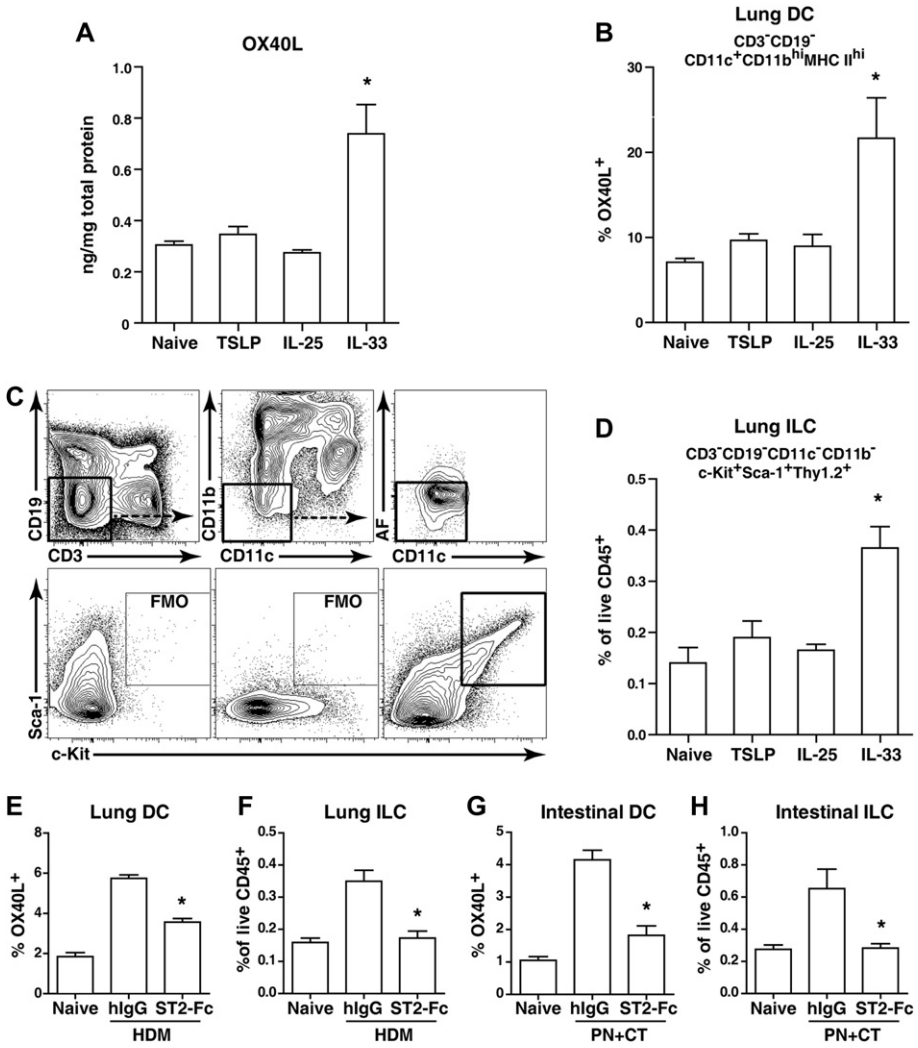

FIG 9. HDM and peanut *(PN)* induce DC OX40L and ILCs *in vivo* through IL-33. Recombinant TSLP, IL-25, or IL-33 (**A-D**) or HDM or peanut plus CT (**E-H**) was administered intranasally or intragastrically for 3 days. Fig 9, *A*, OX40L normalized to total protein in lung homogenates. Fig 9, *B*, Quantification of DC OX40L induction. Fig 9, *C*, Gating strategy for ILCs. Fig 9, *D*, Quantification of ILC expansion. Fig 9, *E-H*, Effect of ST2-Fc or IgG on DC OX40L and ILC induction. Shown are 2 to 3 experiments with 2 to 5 mice per group per experiment. $*P < .05$ versus naive, TSLP, or IL-25 groups (Fig 9, *A-D*) or allergen-exposed immunoglobulin-treated mice (Fig 9, *E-H*). *AF*, Autofluorescence; *FMO*, fluorescence minus one.

proportion of lung and small intestinal OX40L$^+$ DCs and ILCs was abrogated in allergen-exposed mice treated with ST2-Fc compared with those treated with control immunoglobulin (Fig 9, *E-H*). IL-33, more so than TSLP or IL-25, is therefore central to HDM- or peanut-mediated upregulation of OX40L and expansion of ILCs during allergic sensitization.

DISCUSSION

It has been increasingly recognized that T_H2 immunity results from adaptive immune responses that are shaped by initial innate signals. Among these, epithelium-associated cytokines, such as TSLP, IL-25, and IL-33, have been the subject of intense investigation. Here we investigated the initiation of allergic asthma and food allergy using models involving 2 clinically relevant allergens and different mucosal sites and sensitization strategies. We show that the development of HDM and peanut allergy, both of which evolve through canonical T_H2 pathways (see Fig E3),[24] is TSLP and IL-25 independent and, conversely, requires IL-33 for OX40L induction, ILC expansion, and, ultimately, disease manifestation. We used established KO mice, with their proper strain-matched control animals, as well as neutralizing antibody strategies to validate our conclusions.

Studies in models of allergic inflammation[26,27] have led to the proposition that TSLP is a master regulator of allergic responses.[22,35] Notably, sensitization in these studies was achieved through exposure of a nonmucosal site (eg, peritoneal cavity) to the model antigen OVA adsorbed to aluminum-based adjuvants. At variance, our data in the HDM model demonstrate that the extent of allergic sensitization, airway eosinophilic inflammation, and lung pathology was comparable in the presence or absence of TSLP signaling. Furthermore, peanut sensitization, systemic anaphylactic responses, and eosinophilic inflammation were intact in the absence of TSLP, its receptor or basophils. Similarly, anaphylaxis was not prevented when using OVA instead of peanut, which is in accordance with a recent report showing intact

OVA plus CT–induced anaphylaxis in TSLPR-deficient mice.[43] Thus the requirement for TSLP in generating T_H2 immunity to allergens is not as pervasive as originally proposed. To our knowledge, TSLP-independent T_H2 responses have only been reported in helminth infection systems.[8,44]

The ability of TSLP to induce T_H2 responses has been mainly attributed to inhibition of IL-12 production and upregulation of OX40L on DCs.[8,10,11] Here we show that HDM and peanut inhibited DC IL-12 production similarly to TSLP, thus making it redundant. This concept is in agreement with data suggesting that TSLP-independent antihelminth responses are due to helminth excretory-secretory products that supplant TSLP inhibition of DC IL-12.[8] Furthermore, HDM and peanut also mimicked TSLP induction of OX40L on DCs. Thus our data identify common allergens (HDM and peanut) that mimic TSLP actions on DCs, resulting in the induction of T_H2 responses independently of TSLP.

In OVA peptide–pulsed DC/T-cell cocultures, Blázquez and Berin[45] suggested a critical role for OX40L in T_H2 cytokine production. Here we found that allergen exposure induced OX40L on DCs *in vitro*, as well as *in vivo*, in the lung and duodenum. Neutralization of OX40L significantly impaired HDM or peanut sensitization, with effects similar to those reported recently in HDM-exposed OX40 KO mice.[46] Thus our work shows that these models of HDM and peanut allergy originate from a TSLP-independent but OX40L-dependent pathway.

We (Fig 6) and others[25,47] have shown increased levels of IL-25 and IL-33 in the lung after HDM exposure. Here we also report that these cytokines are produced in the duodenum after peanut plus CT exposure. TSLP and IL-33 have been shown to induce OX40L on DCs *in vitro*.[11,48] We confirm these data and further demonstrate that IL-25 can upregulate OX40L to a similar extent as TSLP and IL-33. Consequently, these data indicate that multiple epithelium-associated cytokines are elaborated upon allergen exposure to the respiratory and gastrointestinal mucosae, all converging on, at least, OX40L upregulation on DCs for T_H2 induction.

Although exogenous IL-25 is capable of inducing T_H2 responses,[13] we found no significant effect on immunoglobulin, cytokine, and inflammatory parameters when we blocked IL-25 during HDM or peanut sensitization or examined IL-17RB KO mice. These findings are at variance with previous reports using experimental models of intraperitoneal OVA plus alum–induced asthma in which blockade of IL-25 significantly reduced T_H2 cytokine production and eosinophilic inflammation.[49] IL-25 has also been implicated in promoting T_H2 immunity in host defense against some helminths that infect the gut.[50,51] Thus although IL-25 is increasingly considered key in the regulation of T_H2 immunity,[52] our data clearly indicate that IL-25 is not necessary for T_H2 priming and subsequent effector responses to allergens, such as HDM and peanut.

With respect to IL-33, Coyle et al[53] initially showed a critical role for ST2, which was later identified as the IL-33 receptor, in both T_H2 cellular and humoral responses in an OVA model.[53] However, subsequent studies yielded variable conclusions on the importance of IL-33 for T_H2 responses.[54,55] Our data demonstrate that IL-33 signaling is required for T_H2 priming to both HDM and peanut. These effects translated into significantly less lung pathology and anaphylaxis and were associated with a superior ability of IL-33, over TSLP or IL-25, to increase OX40L levels, particularly on DCs, *in vivo*. In addition, IL-33 markedly expanded a population of ILCs, which have recently been identified and shown to be capable of producing T_H2 cytokines.[17] Although ILCs were reported to be expanded during secondary responses to OVA,[56] we report, for the first time, that allergic sensitization to HDM and peanut are associated with ILC expansion and that this is IL-33 dependent.

TSLP, IL-25, and IL-33 are typically recognized as epithelium-derived cytokines, but other cell types are able to produce these cytokines, particularly in chronic inflammatory states. The specific cellular sources, targets, and functional effects of these cytokines on chronic allergic disease severity, progression, and/or exacerbation are only beginning to be investigated. For example, IL-25 has recently been shown to be associated with collagen deposition, lung dysfunction, or both in atopic patients and mice,[57,58] and Oboki et al[42] have generated IL-33 KO mice and suggested IL-33 to be important in the maintenance of airway inflammation in a model of chronic HDM exposure.

Our data, along with those of previous reports using other experimental systems, reveal that TSLP, IL-25, and IL-33 are differentially required to generate T_H2 responses. For example, antihelminth host defense to *Trichuris muris* is dependent on TSLP, IL-25, and IL-33, whereas *Nippostrongylus brasiliensis* requires IL-25 but not TSLP or IL-33.[8,10,50,54,59] In the case of allergic responses, OVA plus alum–driven T_H2 initiation is dependent on all of these cytokines (Fig E1, *G-K*),[26,27,49,53] whereas HDM- and peanut plus CT–induced responses rely on IL-33 but not TSLP or IL-25. Clearly, diverse antigen-host interactions are able to launch distinct molecular programs highlighting a diversity of pathways leading to T_H2 immunity. This might provide a mechanistic basis for the growing clinical recognition that allergic diseases are heterogeneous. Whether these clinicopathologic variants/endotypes are a result of different subtypes of T_H2 cells (eg, TSLP driven, IL-25 driven, and/or IL-33 driven), each with their own molecular signature and effector armamentarium and, ultimately, distinct therapeutic susceptibilities, remains to be explored.

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Key messages

- **TSLP and IL-25 are redundant but IL-33 is central in the initiation of T_H2 responses to the common allergens HDM and peanut.**
- **IL-33–mediated allergic sensitization to these airway and food allergens requires DC OX40L and is associated with expansion of ILCs.**

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METHODS

Sensitization and treatment protocols

HDM administration. Twenty-five micrograms of HDM extract (Greer Laboratories, Lenoir, NC) in 10 μL of saline was administered intranasally to isoflurane-anesthetized mice for 3 or 10 consecutive days.[E1,E2] Mice were killed 24 hours after the last exposure, unless otherwise specified.

Peanut plus CT administration. One milligram of peanut (Kraft, Northfield, Ill) with 10 μg of CT (List Biological Laboratories, Campbell, Calif) in PBS was gavaged intragastrically by using 12-gauge, 2.5-inch intragastric feeding needles (DELVO SA, Biel/Bienne, Switzerland) daily for 3 consecutive days or weekly for 4 weeks.[E3-E5] In some experiments 1 mg of OVA was gavaged instead of peanut, or the route of administration was intraperitoneal instead of intragastric.

OVA or peanut and alum administration. Fifty micrograms of OVA (grade V; Sigma, St Louis, Mo) emulsified in 1.3 mg of aluminum hydroxide (Sigma) was administered intraperitoneally on days 1 and 14.[E6] Anesthetized mice were challenged intranasally with 150 μg of OVA in 40 μL of PBS for 4 days, starting from day 21. Mice were analyzed 24 hours after the last challenge. In some experiments peanut was used instead of OVA, and mice were challenged intraperitoneally with 500 μg of peanut instead of intranasally to induce anaphylaxis and inflammation.

Antibody administration. The following antibodies and corresponding controls (rat, rabbit, or human IgG; Sigma) in PBS were administered intraperitoneally either 24 hours before the first HDM exposure and every 4 to 5 days of the HDM protocol or 24 hours before each peanut sensitization: 1 mg of anti-TSLP or anti–IL-25 (Amgen, with neutralizing activity[E7,E8]), 250 μg of ST2-Fc (R&D Systems, Minneapolis, Minn), 200 or 400 μg of anti-OX40L (R&D Systems), 100 μL anti-asialo GM1 (Wako, Richmond, Va), or 100 μg of intravenous Ba103 (Hycult Biotech, Uden The Netherlands). In some experiments 25 μg of ST2-Fc was administered intranasally.

Cytokine administration. Recombinant mouse TSLP, IL-25, or IL-33 (0.5 μg, R&D systems or eBioscience [San Diego, Calif]) was administered intranasally or intraperitoneally daily for 3 days, and tissues were analyzed 24 hours later.

Induction and grading of peanut-induced anaphylaxis

One to 2 weeks after the last sensitization, 500 μg of peanut (Greer Laboratories) or OVA in PBS was injected intraperitoneally. Mice were monitored for 40 minutes after challenge for changes in rectal (core) body temperature (VWR International, West Chester, Pa). Forty minutes after challenge, heparin-anticoagulated blood was centrifuged at 6000 to 6200 rpm for 1 minute to measure hematocrit. Clinical signs were evaluated as follows: 0, no clinical signs; 1, pruritis (repetitive hind leg ear canal scratching); 2, periorbital/periauricular edema, piloerection; 3, lethargy/decreased activity, lying prone; 4, no response to whisker provocation; and 5, end point (seizure or death).

Collection of specimens

Peripheral blood was collected, and serum was stored at −20°C. Lungs underwent lavage with PBS containing complete protease inhibitor (Roche, Mannheim, Germany). Total cell counts were determined by using a hemocytometer. Cytospin preparations were stained with the Protocol Hema 3 stain set (Fisher Scientific, Hampton, NH), and differential cell counts were determined by using standard hemocytologic criteria. In some cases the left lung was slowly inflated and fixed with 10% formalin for histology.

Seventy-two hours after anaphylaxis, peritoneal lavage was performed with 10 mmol/L ice-cold EDTA in PBS.

Tissue homogenization

Samples were collected in 500 μL of PBS containing complete protease inhibitor (Roche), rotor-stator homogenized (Polytron; Kinematica, Lucerne, Switzerland), incubated with 1% Triton-X100 for 40 minutes at 4°C, and spun, and supernatants were spun again (12,000 rpm for 10 minutes at 4°C). Supernatants were collected, and protein content was quantified by using the Bradford assay (Bio-Rad Laboratories, Hercules, Calif) and stored at −80°C.

Cytokine and immunoglobulin analysis

Cytokines were measured by using ELISA (kits from eBioscience and R&D Systems) or Luminex assay (multiplex kits from Millipore, Temecula, Calif). Total or antigen-specific immunoglobulin levels were measured by using sandwich ELISA.[E1,E4,E5,E9] Antigen-specific immunoglobulin ELISAs are depicted as OD – background, where background = 2*(SD + Average of at least 20 blank wells).

BMDC generation and stimulation

GM-CSF–derived BMDCs were generated[E10] and then incubated with media, HDM, crude peanut extract, 100 ng/mL recombinant mouse (rm) TSLP (R&D Systems), rmIL-25 (R&D Systems), and rmIL-33 (R&D Systems) for 16 to 24 hours and analyzed directly or then stimulated with 10 ng/mL *Escherichia coli* 0111:B4 LPS (cell culture tested; Sigma). BMDC viability was greater than 80%. For intracellular cytokine staining, BMDCs were stimulated with or without LPS for 12 hours and brefeldin A (eBioscience) for the last 6 hours. Some BMDCs were infected with 25 multiplicity of infection VSV-ΔM51, which transduces DCs without significant progeny virus production or effect on viability.[E11]

Cell isolation and culture

Lungs. Lungs were chopped into small pieces and then digested with Collagenase type I (Gibco, Invitrogen, Carlsbad, Calif) in HBSS for 1 hour at 37°C before being pressed through 40-μm cell strainers, and RBCs were lysed with ACK buffer. Cells were washed and filtered again before analysis.

Intestines. After flushing intestinal contents with cold PBS, the intestines were opened longitudinally, fat and mucus were removed manually, and the intestines were cut into approximately 1-cm pieces before epithelium and mucus were removed by means of repeated 15-minute 37°C incubations in PBS with 10 mmol/L EDTA, 10 mmol/L HEPES, 1 mmol/L dithiothreitol, and vortexing. Epithelium-free lamina propria was then incubated with 0.25 Wünsch units/mL Liberase (Roche) for 60 minutes with 20 U/mL DNAse I (Roche), pressed through a 40-μm strainer, and washed before analysis.

Spleens. Spleens were pressed through 40-μm strainers and incubated with ACK before being resuspended in complete RPMI (RPMI supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% β-mercaptoethanol [Gibco, Invitrogen]). Viable cells (8×10^5) were cultured in triplicate in medium alone or with 15.625 μg/mL HDM or 250 μg/mL crude peanut extract in flat-bottom, 96-well plates (BD, San Jose, Calif). After 5 days, triplicates were pooled, and cell-free supernatants were stored at −20°C.

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

Flow cytometry

To minimize nonspecific and Fc receptor–mediated binding, cells were first incubated with anti-CD16/CD32, (2.4G2; eBioscience) in 0.5% to 1% BSA and 10 mmol/L EDTA-supplemented PBS for at least 15 minutes at 4°C. Antibodies were from BD Biosciences, eBioscience, BioLegend (San Diego, Calif), or MD Biosciences (Saint Paul, Minn) and were titrated before use. ST2–fluorescein isothiocyanate (FITC), CD11c–Alexa Fluor (AF) 488, peridinin-chlorophyll-protein complex (PerCP)–Cy5.5, phycoerythrin (PE)–Cy7, or allophycocyanin (APC); OX40L-PE or biotin (eBioscience and Cedarlane [Burlington, NC]); IL-12p40–PE or APC; CD3–PE-Cy5, PerCP-Cy5.5, PE–eFluor 710, PE-Cy7, AF700, or eFluor 450 or V500; CD19–PerCP-Cy5.5, PE-Cy7, or APC-Cy7; c-Kit–PE-Cy5, PE-Cy7, or APC; MHC II (I^A/I^E)–AF700, eFluor 450, or eFluor 650; Sca-1–AF700; CD86-FITC, AF700, or biotin; CD45–APC-Cy7 or eFluor 605; Thy1.2–APC-eFluor 780; CD8–eFluor 450 or PerCP; CD11b–PE-Cy7, AF700, or Brilliant Violet 421; B220–APC-eFluor 780 or V500; CD4-APC, PerCP, eFluor 650, or V500; CD127–PE-Cy7; NK1.1-APC or PE-Cy7; CD49b (DX5)–PE, PE-Cy7 or biotin; CD69-PE or PE-Cy7; IgE-FITC or PE; or inducible

costimulator–FITC were used. Propidium iodide (eBioscience) and forward scatter/side scatter were used to exclude dead cells and doublets. BD Cytofix/Cytoperm was used for intracellular staining. BD CompBeads were used for compensation, except for V500-conjugated antibodies, where cells were stained instead. Data were acquired on a BD LSR II and analyzed with FlowJo software (Tree Star, Ashland, Ore), including software compensation. Fluorescence minus one controls were used for gating.

Histology and morphometric analysis

Lung tissue was embedded in paraffin and cut at a thickness of 4 μm. Sections were stained with hematoxylin and eosin or periodic acid–Schiff. Images of the main airways were analyzed by using a custom system (Northern Eclipse software; Empix Imaging, Mississauga, Ontario, Canada) that calculates the percentage of tissue area that was positively stained within a 30-μm-wide band from the basement membrane extending into the airway lumen.[E9]

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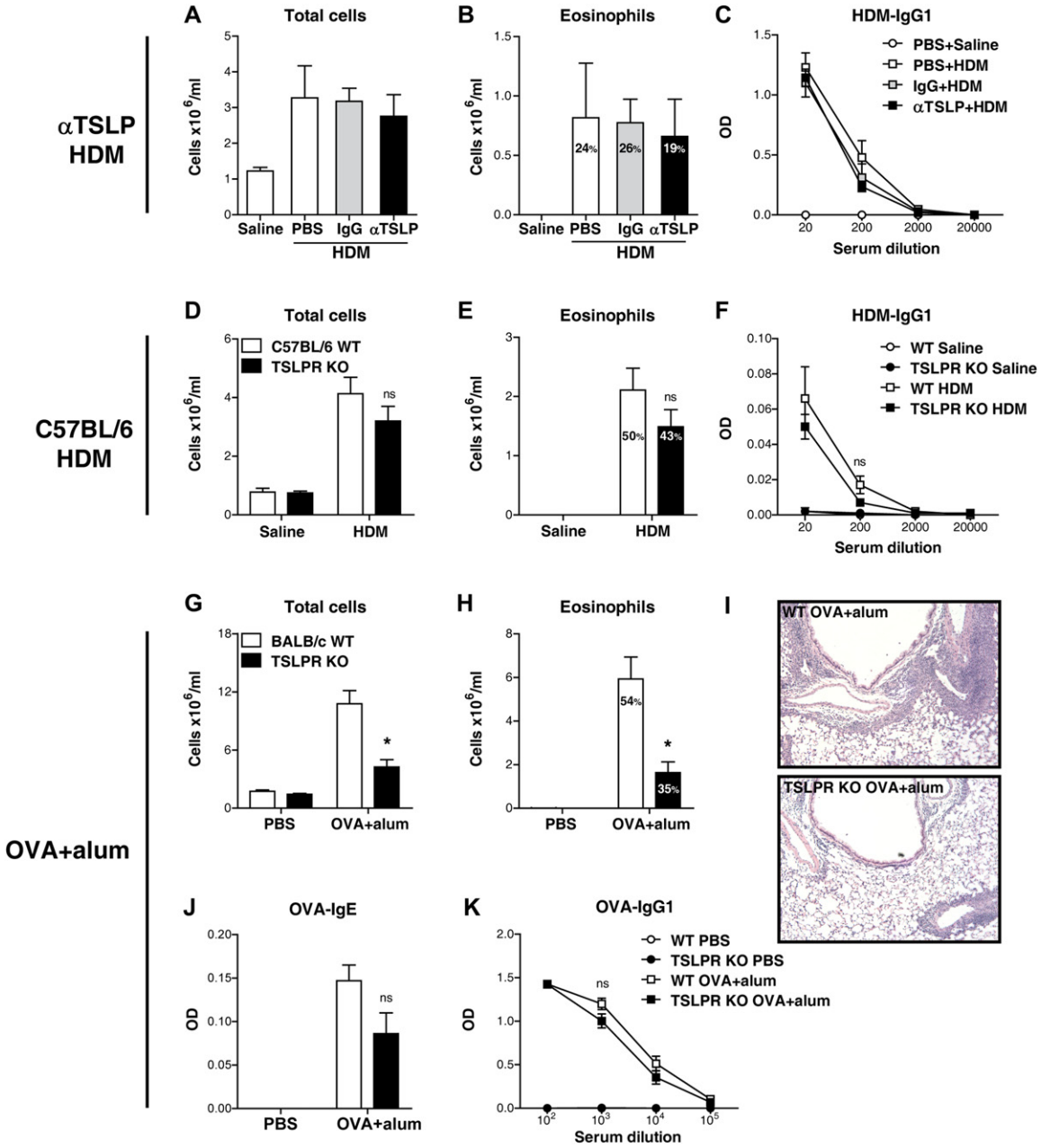

FIG E1. The TSLP-TSLPR axis is differentially required in OVA- and HDM-induced immune-inflammatory responses. **A-C**, BALB/c mice received PBS vehicle, rat IgG, or anti-TSLP blocking antibody 1 day before and throughout the HDM exposure protocol. **D-F**, C57BL/6 WT and TSLPR KO mice were exposed daily to HDM intranasally for 10 days. **G-K**, As previously reported,[E6] BALB/c WT and TSLPR KO mice were sensitized with OVA plus alum intraperitoneally on days 1 and 14 and then challenged with OVA intranasally for 4 days starting on day 21. Total cells (Fig E1, *A*, *D*, and *G*) and eosinophil counts (Fig E1, *B*, *E*, and *H*) in bronchoalveolar lavage fluid. Serum HDM-specific IgG_1 (Fig E1, *C* and *F*) and OVA-specific IgE (Fig E1, *J*) or IgG_1 (Fig E1, *K*) levels. Fig E1, *I*, Representative lung sections stained with hematoxylin and eosin to assess inflammation. One experiment with 2 to 7 mice per group (Fig E1, *A-C*), 2 experiments with 3 to 7 mice per group per experiment (Fig E1, *D-F*), or 2 experiments with 4 to 5 mice per group per experiment (Fig E1, *G-K*). $*P < .05$ versus PBS plus HDM and IgG plus HDM (Fig E1, *A-C*), WT HDM (Fig E1, *D-F*), or WT OVA plus alum (Fig E1, *G-K*). *PN*, Peanut.

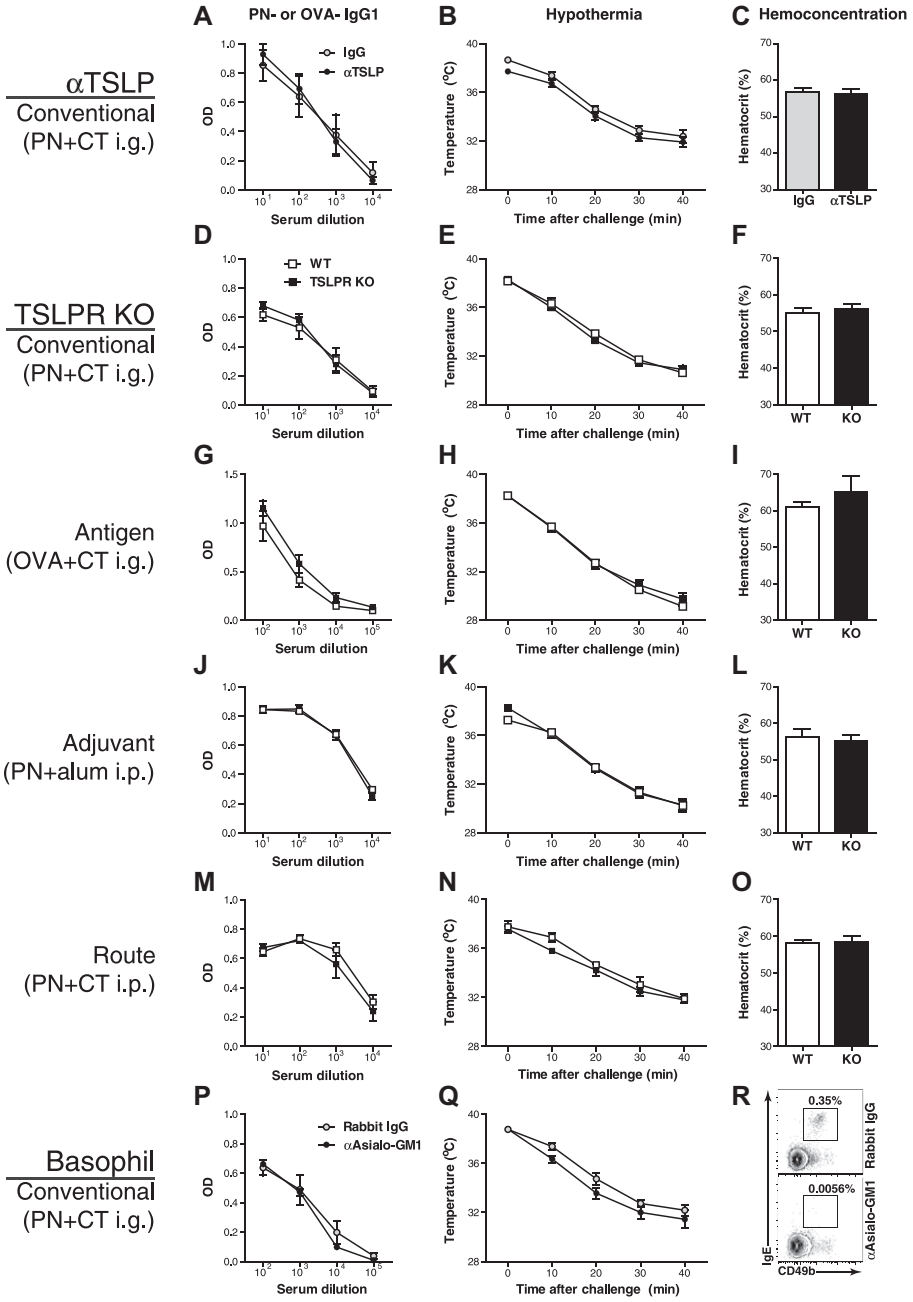

FIG E2. TSLPR independence does not alter with antigen, sensitizing adjuvant, or route of sensitization, and basophils are not required for peanut *(PN)* allergy. WT or TSLPR KO mice were sensitized with peanut plus CT intragastrically (*i.g.*; **A-F** and **P-R**), OVA plus CT intragastrically **(G-I)**, or peanut plus CT intraperitoneally (*i.p.*; **M-O**) and then challenged. Fig E2, *A-C*, Mice received anti-TSLP antibody or rat IgG 1 day before each gavage. **J-L**, Mice were sensitized with peanut plus alum intraperitoneally. Fig E2, *P-R*, Anti-asialo GM1 or rabbit IgG was injected 1 day before each gavage. Fig E2, *A*, *D*, *G*, *J*, *M*, *P*, Serum peanut- or OVA-specific IgG_1 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E2, *B*, *E*, *H*, *K*, *N*, and *Q*) and hematocrit levels (Fig E2, *C*, *F*, *I*, *L*, and *O*). Fig E2, *R*, Proportion of $CD3^-CD19^-NK1.1^-$ $c\text{-}Kit^-CD49b^+IgE^+$ basophils in spleens. Shown are 2 experiments with 3 to 8 mice per group per experiment.

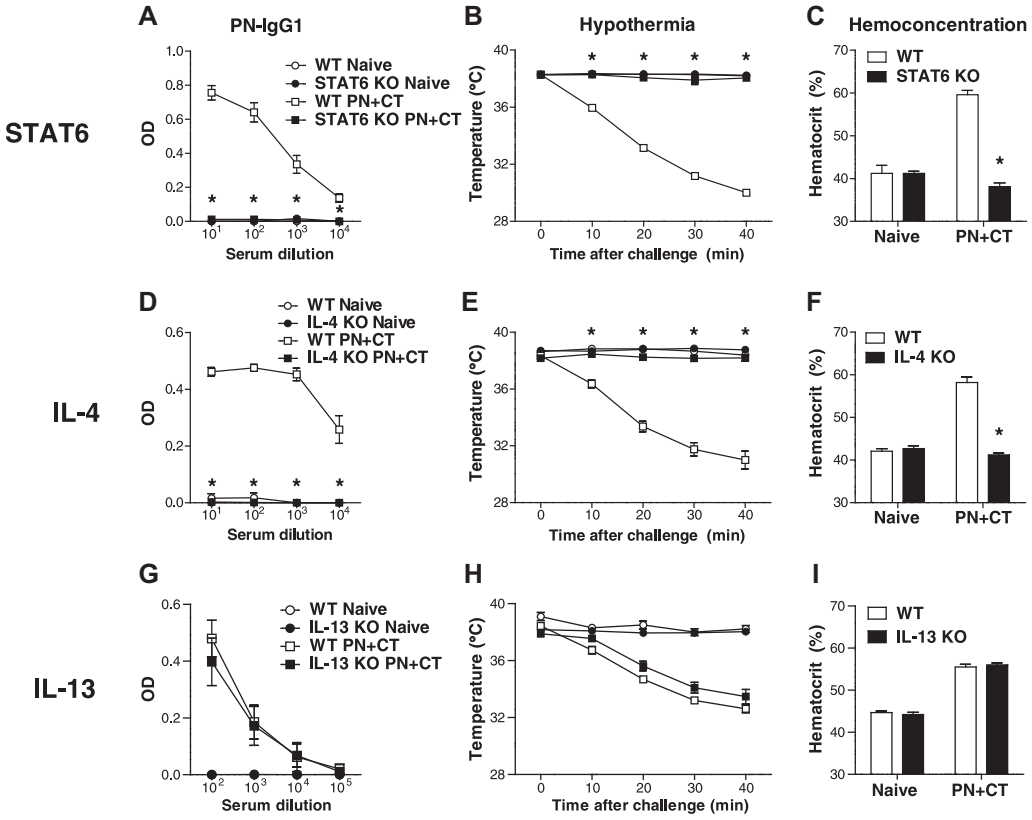

FIG E3. Peanut *(PN)* sensitization follows canonical T_H2 dependency on STAT6 and IL-4. WT **(A-I)**, STAT6 KO (Fig E3, *A-C*), IL-4 KO (Fig E3, *D-F*), or IL-13 KO (Fig E3, *G-O*) mice received peanut plus CT intragastrically weekly for 4 weeks and were challenged with peanut intraperitoneally 1 week later. Fig E3, *A*, *D*, and *G*, Serum peanut-specific IgG_1 levels 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E3, *B*, *E*, and *H*) and hematocrit levels (Fig E3, *C*, *F*, and *I*). Shown are 2 experiments with 3 to 8 mice per group per experiment. $*P < .05$ versus WT peanut plus CT.

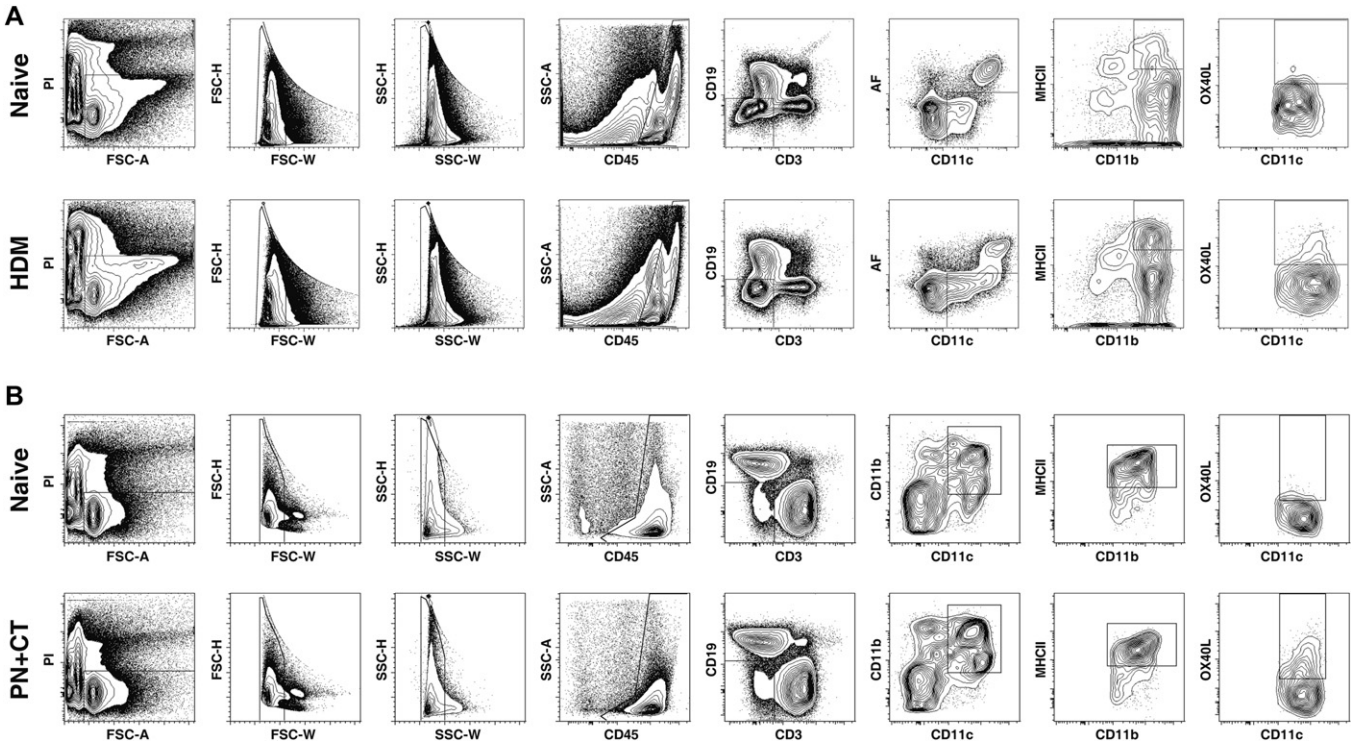

FIG E4. Myeloid DCs expand after 3 days of HDM or peanut *(PN)* plus CT exposure. Flow cytometric gating strategy for myeloid DCs in the lung **(A)** or mesenteric LNs **(B)**. Representative plots are from 2 to 3 experiments. *AF*, Autofluorescence; *FSC*, forward scatter; *PI*, propidium iodide; *SSC*, side scatter.

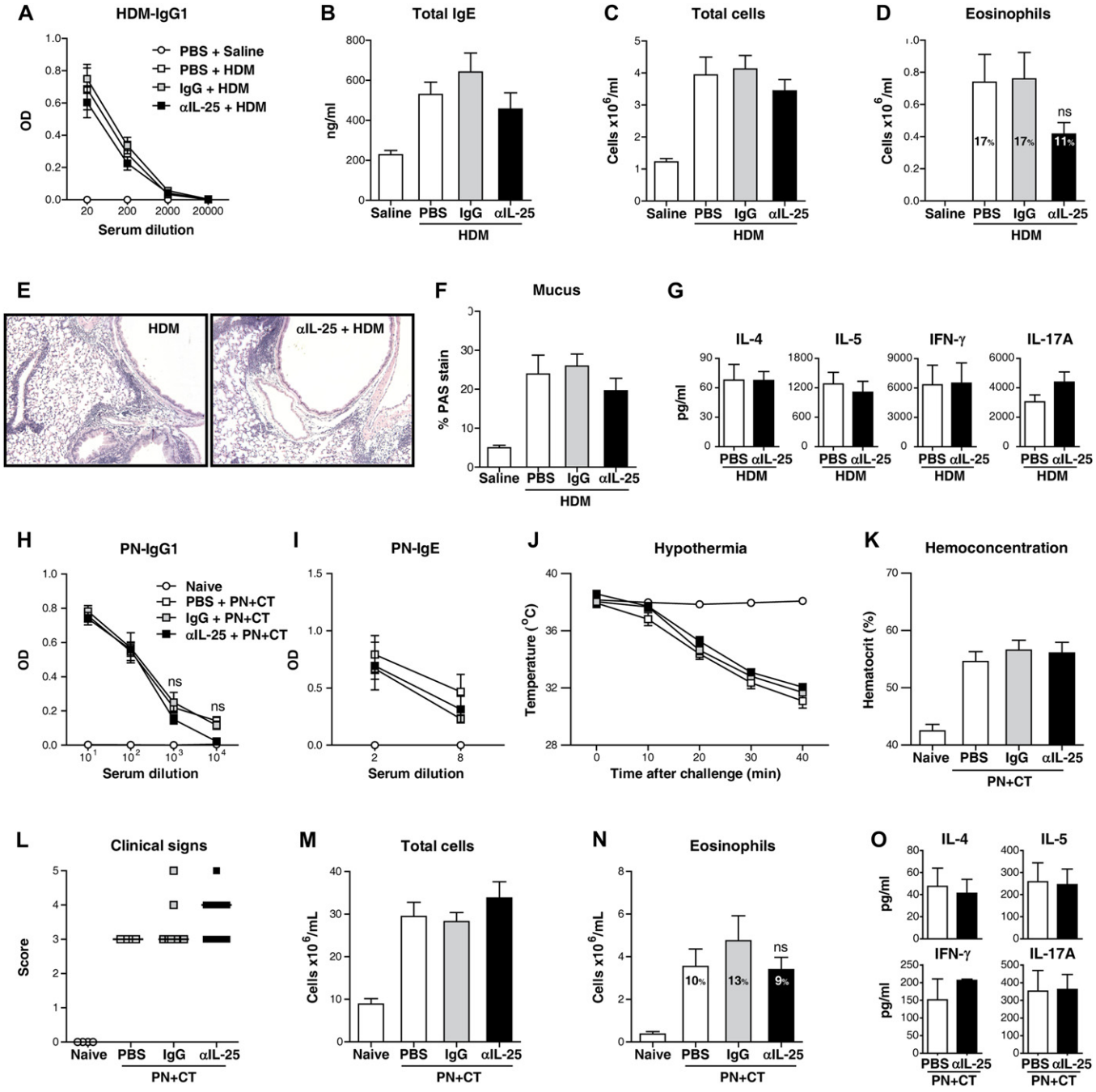

FIG E5. IL-25 neutralization does not significantly affect pulmonary and gastrointestinal allergic sensitization to HDM and peanut *(PN)*. **A-G**, WT mice received PBS vehicle, rat IgG, or 1 mg of anti–IL-25 neutralizing antibody 1 day before and throughout 10 days of HDM exposure. Serum HDM-specific IgG_1 (Fig E5, *A*) and total IgE (Fig E5, *B*) levels. Total cell (Fig E5, *C*) and eosinophil (Fig E5, *D*) counts in bronchoalveolar lavage fluid; mean percentages of eosinophils are shown in *bars*. Fig E5, *E*, Representative lung sections stained with hematoxylin and eosin (×10 magnification) or periodic acid–Schiff *(PAS)* insets (×40 magnification) to assess leukocyte infiltration and mucus production. Fig E5, *F*, Morphometric analysis of periodic acid–Schiff staining. Fig E5, *G*, Cytokine production from HDM-stimulated splenocytes. **H-O**, WT mice received rat IgG or anti–IL-25 antibody 1 day before peanut plus CT intragastrically weekly for 4 weeks and then were challenged with peanut intraperitoneally 1 week later. Serum peanut-specific IgG_1 (Fig E5, *H*) and IgE (Fig E5, *I*) levels 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E5, *J*), hematocrit levels (Fig E5, *K*), and clinical signs (Fig E5, *L*). Total (Fig E5, *M*) and eosinophil (Fig E5, *N*) cell counts in peritoneal lavage; mean percentages of eosinophils are shown in *bars*. Fig E5, *O*, Cytokine production from peanut-stimulated splenocytes. Shown are 2 to 3 independent experiments with 3 to 6 mice per group per experiment. *ns*, Not significantly different.

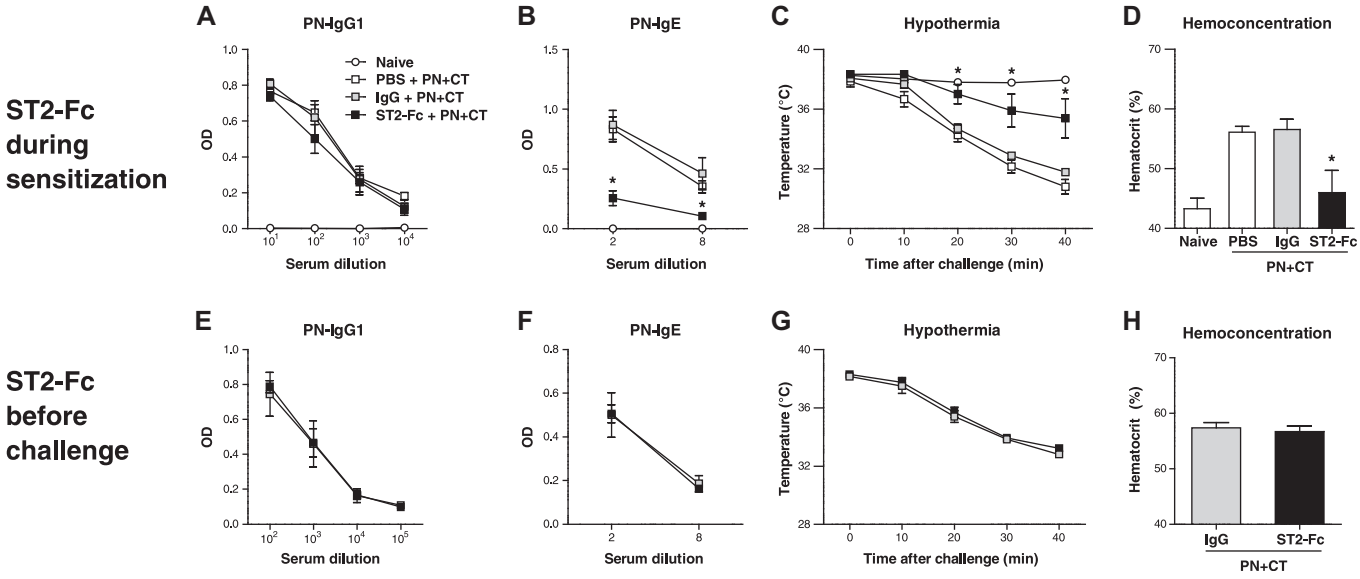

FIG E6. Differential effect of ST2-Fc treatment on peanut *(PN)* sensitization versus challenge. WT mice received PBS vehicle, human IgG, or ST2-Fc 1 day before each sensitization **(A-D)** or challenge **(E-H)**. Serum peanut-specific IgG_1 (Fig E6, *A* and *E*) and IgE (Fig E6, *B* and *F*) levels 1 day before challenge. Anaphylactic assessment was based on core body temperature (Fig E6, *C* and *G*) and hematocrit levels (Fig E6, *D* and *H*). Shown are 2 independent experiments with 3 to 8 mice per group per experiment. $*P < .05$ versus control PBS- or IgG-treated mice exposed to peanut plus CT.

Chapter 3.

T helper cell-intrinsic IL-4 drives intestinal Th2 priming to oral peanut antigen, under the control of OX40L and independent of innate-like lymphocytes

TITLE

T helper cell-intrinsic IL-4 drives intestinal Th2 priming to oral peanut antigen, under the control of OX40L and independent of innate-like lymphocytes

RUNNING TITLE

Th IL-4 for Th2 by DC OX40L not innate lymphocytes

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POTENTIAL CONFLICTS OF INTEREST

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ABSTRACT

Intestinal Th2 immunity in food allergy results in IgG1 and IgE production, and antigen re-exposure elicits responses such as anaphylaxis and eosinophilic inflammation. Although IL-4 is critically required for allergic sensitization, the source and control of IL-4 during the initiation of Th2 immunity *in vivo* remains unclear. Non-intestinal and non-food allergy systems have suggested that NKT or γδ T cell innate lymphocytes can supply the IL-4 required to induce Th2 polarization. Group 2 innate lymphoid cells (ILC) are a novel IL-4-competent population, but their contribution to initiating adaptive Th2 immunity is unknown. There are also reports of IL-4-independent Th2 responses. Here, we show that IL-4-dependent peanut allergic Th2 responses are completely intact in NKT-deficient, γδ T-deficient or ILC-deficient mice, including antigen-specific IgG1/IgE production, anaphylaxis, and cytokine production. Instead, IL-4 solely from $CD4^+$ Th cells themselves induces Th2 immunity. Further, $CD4^+$ Th cell production of IL-4 *in vivo* is dependent on OX40L, a costimulatory molecule on DCs required to induce peanut allergy. However, both Th2 cells and ILCs orchestrated IL-13-dependent eosinophilic inflammation. Thus, intestinal Th2 priming is initiated by an autocrine/paracrine acting $CD4^+$ Th cell-intrinsic IL-4 program that is controlled by DC OX40L, and not NKT, γδ T, or ILC cells.

KEY WORDS

Peanut; allergy; Th2; sensitization; type 2 immunity; anaphylaxis; IL-4; innate-like lymphocyte; γδ T cell; NKT cell; OX40L; IL-15; NK1.1; IgE; 4get; ILC2; group 2 innate lymphoid cell; IL-13.

ABSTRACT WORD COUNT: 200 words

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INTRODUCTION

Allergies are detrimental immune responses to specific environmental antigens that are normally tolerated by most individuals. Food allergies affect 2-10% of the general population, causing symptoms ranging from pruritus and urticaria to systemic, potentially life-threatening reactions, called anaphylaxis [1, 2]. Peanut (PN) allergy is highly associated with anaphylaxis [3]. Central to the pathogenesis of allergies are T helper type 2 (Th2) cells, which are characterized by their production of IL-4, IL-5, and IL-13, provision of B cell help in isotype switching to IgG1 and IgE, and orchestration of eosinophilic inflammation within allergen re-exposed tissues. An important focus of current research is to investigate the mechanisms underlying allergic sensitization, *i.e.*, Th2 generation.

Of the signature Th2 cytokines, IL-4 has been reported to be critically required to mount full Th2 responses, understood as isotype switching to IgE and generation of Th2 cells. IL-4 and TCR activation alone can promote the generation of Th2 cells *in vitro*. However, the source of IL-4 that initiates the development of naïve $CD4^+$ T cells into IL-4 expressing Th2 cells *in vivo* remains to be clarified [4-6] and is unknown in food allergy.

A prominent hypothesis is that naïve T cells receive IL-4 from an innate cell capable of rapid IL-4 production such as mast cells, basophils, or innate-like lymphocytes including γδ T cells or natural killer-T (NKT) cells. Innate lymphoid cell type 2 (ILC2s) are a novel population of IL-4 competent cells that play a critical role in effecting type 2 immune responses through IL-13 production, but their contribution to initiating Th2 immunity is unknown [7]. γδ T cells develop in the thymus from double negative thymocytes undergoing TCRβ, γ and δ gene rearrangement; deficiency of TCRδ or TCRγ blocks γδ T cell development. Following TCR rearrangement, γδ T cells preferentially localize to epithelial sites at barrier surfaces such as the lung and intestine. There, they express a limited TCR-repertoire and when activated, rapidly produce a number of effector molecules, including IL-4 [8]. Indeed, in a model of allergic asthma, where sensitization is achieved through the intraperitoneal (i.p.) route, immunized γδ T cell-deficient mice showed decreased levels of serum ovalbumin (OVA)-specific IgE and IgG1 antibodies, which were restored with IL-4 administration [9]. Hence, it has been suggested that γδ T cells may be a critically required source of IL-4 for the development of Th2 responses.

NKT cells develop in the thymus from double positive thymocytes, are classically defined as $CD3^+NK1.1^+$ or $TCR\beta^+NK1.1^+$ cells and are another innate-like lymphocyte capable of rapidly producing IL-4 upon activation [10]. Development, maturation and survival of NKT cells critically depends on recognition of CD1d-β2 microglobulin (β2m) complexes, as well as IL-15 [11, 12]. Hence, lack of β2m or IL-15 results in a severe lack of NKT cells. The TCR of NKT cells recognize mainly lipid antigens, and lipids within foods have been proposed to be one potential source of NKT cell activation in food allergy [13]. However, the contribution of NKT cells to the

development of food allergy remains unknown. In addition, NKT cells have been proposed to regulate allergic asthma development via their production of type 2 cytokines such as IL-4 [14, 15].

An alternative model of Th2 differentiation states that naïve Th cells do not require IL-4 to be programmed to become Th2 cells. Instead, costimulatory molecules such as OX40L and Jagged2 have been suggested to drive Th2 response development [5, 6, 16]. Here, we used an established model of PN allergy [17-20] to investigate the cellular source and regulation of IL-4 during the initiation of type 2 immunity to oral antigen. We show that neither γδ T or NKT cell nor ILC deficiency impact the development or expression of Th2 immunity to PN. Instead, IL-4 production solely from $CD4^+$ Th cells, under the control of OX40L, allows for full PN allergic Th2 responses to develop. Hence, rather than dichotomous costimulation *versus* IL-4-signalling dependent Th2 generation, we show that *in vivo* development of Th2 cells to a common food allergen follows a pathway requiring both costimulation and IL-4 from $CD4^+$T cells.

RESULTS

IL-4 is required for PN allergy

We previously showed that weekly gavage of PN and the adjuvant, cholera toxin (CT), for 4 weeks induces IL-4 dependent PN-specific IgG1 production by week 5 and establishes, at that time, susceptibility to undergo anaphylaxis upon i.p. PN challenge [17]. Here, we confirm these data, and further show that PN-specific IgE, inflammatory and Th2 cytokine responses are also dramatically impaired in IL-4 deficient (IL-4 KO) mice (**Figure 1**). These data show that Th2 and B cell allergic responses to oral antigen require IL-4.

NKT cells are not required for Th2 responses to PN

We assessed the requirement for NKT cells during allergic sensitization to PN by using IL-15 KO mice, β2m KO mice or by depletion with an anti-NK1.1 antibody in WT mice. Compared to WT mice, NKT cells were reduced by ~75% in IL-15 KO mice, and undetectable in anti-NK1.1 treated mice (**Figure 2i**). Compared to PN-sensitized WT mice, IL-15 KO mice generated comparable PN-specific IgG1 and IgE titres (**Figure 2a**). Clinically, this led to comparable hypothermia and hemoconcentration anaphylactic responses (**Figure 2b**) in both sensitized groups. Peritoneal eosinophilic inflammation (**Figure 2c**) was similarly significantly elevated over naïve levels in both challenged strains. Th2 cytokine production by both sensitized groups was similarly elevated above naïve levels (**Figure 2d**). Anti-NK1.1 treated mice had an even greater level of NKT cell deficiency, with similar results (**Figure 2e-h**). We also found similar results with β2m KO mice (**Supplementary Figure S1**). These data collectively show that Th2 responses to PN develop largely unimpaired in the absence of NKT cells as a result of deficiency in IL-15, β2m or $NK1.1^+$ cells.

γδ T cells regulate IgA, but not allergic responses to PN

To investigate the role of γδ T cells in the development of PN allergic Th2 immunity, WT or TCRδ KO mice were intragastrically (i.g.) sensitized to PN and evaluated for subsequent T and B cell responses. PN-sensitized WT and TCRδ KO mice generated similar PN-specific IgG1 and IgE (**Figure 3a**) titres, resulting in clinically indistinguishable hypothermic and hemoconcentration anaphylactic responses upon challenge (**Figure 3b**). Similarly, both sensitized groups developed comparably robust eosinophilic peritoneal inflammation (**Figure 3c**) and Th2 cytokine responses (**Figure 3d**). In contrast, TCRβ KO mice exhibited none of these features. Similar results to the TCRδ KO were generated when treating mice during sensitization with anti-γδ TCR antibody clone UC7-13D5 (**Supplementary Figure S2**), with one exception: total IgA production in response to PN+CT was markedly decreased in TCRδ KO mice but not WT mice, or WT mice treated with UC7-13D5 (**Figure 3e**). These data show that γδ T cells do not significantly regulate the initiation of Th2 responses to PN, but do contribute to IgA production.

$CD4^+$ T cell-intrinsic IL-4 drives sensitization to PN

To further explore the source of IL-4 responsible for the initiation of PN-induced Th2 immunity we restricted IL-4 expression to $CD4^+$ T cells by sublethally irradiating $CD45.2^+$ WT or IL-4 KO mice and, adoptively transferring purified congenic $CD45.1^+$ IL-4-competent $CD4^+$ Th cells (**Figure 4a and Supplementary Figure S3**). Thus, the sole source of IL-4 in recipient IL-4 KO mice is from CD45.1 marked, $CD4^+$ Th cells that have preserved endogenous T cell (CD45.2) competition for homeostatic/activation signals. Under these conditions, 60-80% of the recipient's $CD4^+$ Th cell compartment were made up of $CD45.1^+$ $il4^{+/+}$ T cells (**Figure 4b and Supplementary Figure S3**). No abnormalities in overall $CD4^+$ T cell frequency or activation status in either $CD45.1^+$ or

$CD45.2^+$ T cell pools were observed (**Supplementary Figure S3**). Compared to control CD45.1 → WT CD45.2 transfers sensitized to PN (WT+T), CD45.1 → IL-4 KO CD45.2 transfers (KO+T) generated comparable levels of antigen-specific IgG1 (**Figure 4c**) and IgE (**Figure 4d**), and upon challenge, anaphylactic responses (**Figure 4e**). Eosinophilic inflammation 72 h after challenge was also similarly elevated (**Figure 4f**), as was cytokine production (**Figure 4g**). These data show that *in vivo*, IL-4 production solely from $CD4^+$ T cells allows for full development of Th2 responses to PN, and that non-$CD4^+$ sources of IL-4 are dispensable during the development of type 2 immunity in this system.

OX40L controls T-cell intrinsic IL-4 production during PN sensitization

We have previously shown that OX40L, expressed on dendritic cells (DCs), is required to initiate PN allergic Th2 responses [17]. To test if OX40L controls T cell-intrinsic IL-4 production *in vivo*, we used 4get *il4* reporter mice, which express eGFP from a bicistronic locus shared with *il4* [21]. These mice were exposed to an acute protocol of PN+CT, with or without neutralizing anti-OX40L antibody. Draining mesenteric lymph nodes (MLNs) were analyzed for *il4* expression, marked by eGFP. PN+CT exposure resulted in approximate doubling of the percentage of IL-4eGFP$^+$CD3$^+$CD4$^+$ T cells, which was prevented with OX40L neutralization (**Figure 5a**). Gating and quantification of this population showed that γδ T cells and $CD49b^+$ NKT cells made up a minority (<2%) of this population (**Figure 5a**), and that neither changed in percentage or number (**Figure 5b, c**) during Th2 priming to PN, or OX40L blockade. In contrast, $CD49b^-TCR\gamma\delta^-$ Th2 cells markedly expanded approximately threefold after PN sensitization, which was inhibited by OX40L blockade (**Figure 5d**). Hence, IL-4 production during PN sensitization is primarily from conventional Th cells and is under the control of OX40L.

Innate lymphoid cell depletion impacts eosinophilic inflammation, but not Th2 initiation

ILC2s are a novel population of non-B, non-T, $CD11c^-$, $CD11b^-$ cells expressing c-Kit, Sca-1 and CD90 that plays a critical role in effector type 2 immune-inflammatory responses to allergens and helminths [7]. They are potent sources of IL-5 and IL-13, but have also been found to be IL-4 competent and to be present within LNs [17, 22, 23], but their contribution to regulating adaptive Th2 immunity remains unknown. We previously observed expansion of ILCs during PN sensitization[17]. Consistent with this, after PN+CT priming we observed a minor expansion of $IL\text{-}4^+$ non-B/T cells (**Figure 5a**), and hence evaluated whether ILCs contributed to Th2 priming. To accomplish this, we generated CD90-disparate chimeras (**Supplementary Figure S4**), which allow for antibody-mediated depletion of $CD90.2^+$ ILCs, but not $CD90.1^+$ Th cells[24]. ILC depletion (**Figure 6a**) was not accompanied by any impact on IgE/IgG1 production, anaphylactic responses, or Th2 cytokine production (**Figure 6b, c, e**). However, ILC depletion significantly impaired allergic eosinophilic inflammatory responses (**Figure 6d**). Consistent with the potent IL-13 producing ability of ILCs[23, 25], anti-CD90.2 treated mice exhibited reduced levels of IL-13 within their peritoneal cavities (**Figure 6f**). Although IL-13 KO mice become comparably sensitized to PN as to WT mice[17], we found that IL-13 KO mice mounted markedly impaired eosinophilic inflammatory responses (**Figure 6g**). Thus, although ILC2s play an important role in effecting type 2 immunity within antigen-exposed tissues, likely through IL-13, they do not regulate the initiation of adaptive Th2 immunity in this system.

DISCUSSION

Th2 induction to oral antigen causes food allergy, a growing health problem with potentially fatal consequences. Although IL-4 is often thought to be critical for the development of Th2 responses, the original source of IL-4 in the initiation of type 2 responses to foods remains unknown. Herein, we report that the development of type 2 immunity during PN allergic sensitization is independent of γδ T cells, NKT cells or ILC2s and is, instead, driven by T-helper cell-intrinsic IL-4 under the control of OX40L.

A large body of literature supports the notion that non-T helper cell derived IL-4 is required to initiate Th2 responses [4, 5]. Indeed, classical *in vitro* polarization of naïve T cells to Th2 cells requires supplementation with exogenous IL-4 in addition to TCR stimulation. Thus, a popular paradigm is that innate cell types capable of rapidly producing IL-4 after stimulation play critical roles in driving Th2 polarization *in vivo*. Such a function has been ascribed to mast cells, basophils, NKT cells, and γδ T cells. Notably, we have previously shown that mast cells, B cells and basophils are not required to induce intestinal Th2 immunity to PN [17, 20].

The role of NKT cells in the initiation of food allergy has not been examined previously, but these cells have been shown to be activated by food lipids [13]. Further, NKT cells can produce type 2 cytokines and, thus, regulate the development of Th2 immunity, in particular, in IgE production and allergic asthma [14, 15, 26]. Here, we found that IL-15 KO mice, β2m KO mice and anti-NK1.1 treated mice had comparable levels of PN-specific IgE, Th2 cytokine production and eosinophilic inflammatory responses to WT or non-depleted NKT-sufficient mice (**Figure 2 and Supplementary Figure S1**). Collectively, our IL-15 KO, β2m KO and anti-NK1.1 data support the idea that NKT cells do not augment or inhibit Th2 responses in this system.

γδ T cells are abundant within mucosal surfaces in both mice and humans. Bol-Schoenmakers *et al.* have recently proposed that γδ T cells negatively regulate the induction of PN allergy based on observations that PN+CT exposure decreases intestinal γδ T cell proportions, and administration of anti-TCRγδ clone UC7-13D5 during PN+CT sensitization resulted in increased serum PN-IgE, but not PN-IgG1 or PN-IgG2a levels [27]. Unfortunately, other biological or clinical readouts (*i.e.*, anaphylaxis) were not examined in these studies. In contrast, here, we found that TCRδ KO mice sensitized to PN and their WT controls mount not only comparable PN-specific IgG1 and IgE but also similar clinical anaphylactic (hypothermia, hemoconcentration, clinical signs), inflammatory and cytokine responses (**Figure 3**). We obtained similar results to our KO data using UC7-13D5 antibody throughout our PN sensitization protocol (**Supplementary Figure S2**) or the one described by Bol-Schoenmakers *et al*. TCRδ KO mice, as expected [28], had impaired IgA responses at baseline and in response to CT-mediated immunization (**Figure 3e**). In contrast, UC7-13D5 treated mice had markedly increased IgA responses in response to PN+CT. Hence, UC7-13D5 treatment and genetic γδ T cell deficiency do not appear to phenocopy each other, or comparably impair γδ T cells. Altogether, our observations lead us to propose that γδ T cells do not significantly regulate the initiation of PN allergy in this system. In line with this, $Kit^{Wv/v}$ mice are also deficient in intestinal γδ T cells [29], and we have previously shown that these mice mount robust Th2 immunity including IgE production, inflammation, and cytokines [18, 20]. Other studies using adoptive transfer, genetic and antibody-based approaches have reported that γδ T cells may be required to promote Th2 responses and/or IgE production [8, 9]. For example, γδ T cell-deficient mice showed decreased levels of serum OVA-specific IgE and IgG1 antibodies after i.p. immunization with OVA, which were restored with administration of IL-4 [9].

However, we could not find any impact of TCRδ deficiency or anti-TCRγδ treatment on multiple immunological, clinical and physiological parameters of Th2 immunity and PN-induced anaphylaxis, notwithstanding its possible role in IgA production.

ILCs are a novel cell type that lack lineage markers (non-B, non-T, including CD4, CD11c-, CD11b-), express c-Kit, Sca-1, and CD90[7, 25] and have been reported to be increased in the intestine and draining LN during PN sensitization [17]. CD90 is a T cell maker with genetic polymorphisms across mouse strains which allows for adoptive transfer and tracking studies. Although CD90 is also expressed by non-T cells such as stromal cells, generation of CD90 disparate chimeras is a recently established method to selectively deplete ILCs[24]. Indeed, it has been in such a system that ILCs have been shown to regulate T cell responses[24]. Although ILC2s are known to be important to effecting anti-helminth immunity[25], the role of ILCs in regulating the initiation of Th2 responses remains unknown. The main cytokines produced by these cells are IL-13 and IL-5, and to a much lesser extent, IL-4 [7, 25]. Herein, we also generated CD90-disparate chimeras and found that ILCs are not required to initiate Th2 immunity, but are important for effecting type 2 inflammatory responses, including the accumulation of eosinophils, likely through IL-13 production (**Figure 6**). Further, although multiple non-T cell types are potentially IL-4 competent (NKT cells, γδ T cells, ILCs, basophils, mast cells and eosinophils[30]) we conclusively show that all non-$CD4^+$ sources of IL-4 are not critical for the initiation of Th2 responses *in vivo* by employing IL-4-sufficient $CD4^+$ T cell transfer into IL-4 KO mice (**Figure 4**). Hence, IL-4 derived solely from $CD4^+$ T helper cells is fully sufficient to induce clinically and biologically meaningful Th2-polarized allergic responses *in vivo*.

Competing and dichotomous models of Th2 differentiation argue for IL-4 dependent *versus* IL-4 independent pathways [21, 31]. The latter has been proposed to be driven by costimulation, and the former, by IL-4R signalling [16, 32]. The outcome of either pathway is upregulation of lineage determining factors such as GATA3, leading to initiation of Th2 phenotype differentiation and secretion of signature cytokines such as IL-4, IL-5, and IL-13. In response to PN allergic sensitization we not only found that $CD4^+$ T helper cell-intrinsic IL-4, alone, drove this process, but also that *in vivo* IL-4 upregulation required OX40L costimulation (**Figure 5**). Hence, we provide evidence for a pathway that requires OX40L costimulation for IL-4 production, yet also IL-4 for complete Th2 development. OX40L has been shown to regulate IL-4 secretion early after *in vitro* T cell stimulation or primary helminth infection [33, 34], potentially through NFAT2, which is also known as NFATc1 [32]. Independent from this, multiple reports have shown that early, low levels of IL-4 secretion from $CD4^+$ T cells can be independent of IL-4, IL-4Rα or STAT6, but that intact IL-4 signalling is required for a maximal, terminal Th2 effector profile [21, 31, 35, 36]. Thus, impairments in Th2 development due to IL-4 signaling deficiency typically do not become apparent until later time points, such as in effector tissue responses or secondary infections. Altogether, these and our data support an at least two-step model of IL-4 production, whereby naïve $CD4^+$ T cells initially produce IL-4 in response to costimulation (such as through OX40-OX40L), and that subsequent autocrine/paracrine signaling of $CD4^+$ T helper cell-intrinsic IL-4 amplifies and stabilizes the Th2 state (**Figure 7**), in a manner somewhat analogous to IL-23 mediated stabilization of Th17 differentiation, or IFN-γ during Th1 differentiation [6]. Thus, DC OX40L mediated triggering of IL-4 production from naïve T cells initiates a self-driven

Th2 differentiation/amplification pathway that leads to the development of PN allergy and anaphylaxis.

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METHODS

Mice

Age, sex and strain-matched controls were used in all experiments. CD45.1^{+} mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ), IL-4 KO (B6.129P2-*Il4*^{tm1Cgn}/J), TCRδ KO (B6.129P2-*Tcrd*^{tm1Mom}/J), TCRβ KO (B6.129P2-*Tcrb*^{tm1Mom}/J), CD90.1^{+} mice (B6.PL-Thy1^{a}/CyJ) and 4get (C.129-*Il4*^{tm1Lky}/J) mice were from JAX laboratories (Bar Harbor, Maine). β2m KO (B6.129-*B2m*^{tm1Jae}) mice were from Taconic. IL-15 KO (C57BL/6NTac-*IL15*^{tm1Imx}) and IL-13 KO (C.129P2-Il13^{tm2Anjm}) mice were bred in-house. All procedures were approved by the McMaster University Research Ethics Board.

PN allergy model

Intragastric gavage (Delvo) of PN (Kraft) with CT (List Biologicals) was performed weekly for 4 weeks, with challenge by crude PN extract (Greer) one week later. Serum was collected 24h before challenge and analyzed for total or PN-specific immunoglobulins [17]. In some experiments, anti-TCRγδ antibody UC7-13D5 (BioXCell), or anti-OX40L (R&D systems), was given 24h before each gavage. 1 mg anti-Thy1.2 (30H12, made in-house) was administered every 3-4d beginning 24h before the first gavage. 450 μg anti-NK1.1 (clone PK136 [37], hybridoma HB191 from HTCC, made in-house), was given 3d and 1d before the first gavage, and every 3d thereafter until the last gavage. In some anti-TCRγδ antibody UC7-13D5 experiments, the protocol described by Bol-Schoenmakers *et al.* [27] was employed. Temperature was assessed by rectal probe, and hematocrit by centrifuging anticoagulated blood at 40' after challenge. Peritoneal lavage was performed with PBS-EDTA. Total cells were counted using Turks and hemocytometer. Eosinophils were quantified using flow cytometry as $F4/80^{lo}$, Siglec-F^{+} cells or manual differential cell counting of at least 500 cells from cytospins (Thermo Shandon) stained using Hema 3 (Fisher Scientific), with good agreement between both methods.

Cytokine production

Spleens were collected 72h after challenge and 800,000 live splenocytes were cultured for 120h in media alone or supplemented with 250 μg/mL CPE and cytokines in supernatants were quantified using Luminex (Millipore) or ELISA (R&D). Peritoneal lavage cell-free supernatants were analyzed using ELISA (R&D).

Adoptive transfer

$CD4^+$ T cells were isolated from pooled spleens and lymph nodes by MACS (Stemcell) to >95% purity and 10^7 cells were i.v. transferred to $Thy1.2^+$ TCRβ KO or 5.5 Gy (Cs^{137} source) sublethally irradiated WT or IL-4 KO mice. Sensitization was initiated 2 weeks post-irradiation.

Flow cytometry

In all assays, cells were incubated with anti-FcγRII/IIIb before incubation with fluorochrome-conjugated antibodies, dead cells excluded by propidium iodide (PI) uptake, and gated on singlets. Whole blood was anticoagulated with EDTA and lysed with ACK lysis buffer before staining. Antibodies used were from eBioscience, BD Biosciences, or Biolegend: CD3-FITC or Pacific Blue; CD4-APC, eFluor605 or eFluor650; CD19 PE-Cy5; CD45.1-eFluor 605; CD45.2-Pacific Blue; MHC II-eFluor 650; F4/80-eFluor 450 or Pacific Blue; Siglec-F-PE; NK1.1-PE-Cy7; CD49b (DX5)-PE or PE-Cy7; TCRβ-FITC or Pacific Blue; TCRγδ-PE; Sca-1-Alexa Fluor 700; CD44-V500 or Alexa 700; CD62L-APC-eFluor 780; B220-eFluor650 or V500; CD90.1-Alexa Fluor 700 or eFluor605; CD90.2-APC-eFluor780 or FITC.

Data were acquired on an LSR II (BD Biosciences) and analyzed using FlowJo (Treestar Inc.).

Statistics

Comparisons were made using unpaired t-tests or one or two-way ANOVA. Repeated measures was used for temperature data. $P<0.05$ was considered statistically significant. The number of experiments performed are indicated at the end of each figure legend.

DISCLOSURE

AAH and RK are employees of MedImmune LLC. DKC is a CIHR Vanier Scholar. ALG was supported by a Fundación Caja Madrid doctoral scholarship (Spain). MJ holds a Senior Canada Research Chair in Immunobiology of Respiratory Diseases and Allergy. This work was funded by grants from Anaphylaxis Canada, MedImmune LLC, and AllerGen NCE. All other others do not have conflicts of interest to disclose.

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FIGURE LEGENDS

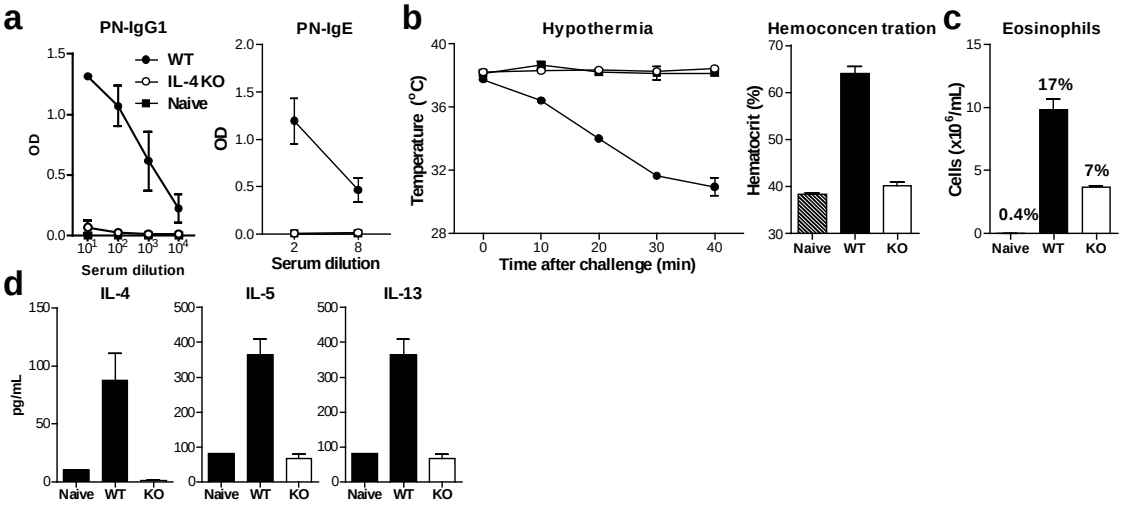


FIGURE 1

Figure 1. IL-4 is required for PN allergy. WT or IL-4 KO mice were exposed to PN+CT i.g., weekly for 4 weeks, or kept naive. (**a**) Serum PN specific-IgG1 (PN-IgG1, left) and -IgE (right) at wk 5. (**b**) Anaphylactic hypothermia (left) and hemoconcentration at 40' (right) after i.p. PN challenge at wk 5. (**c**) Peritoneal eosinophilia recovered 72h after challenge. (**d**) Th2 cytokine production from PN-stimulated splenocytes. Mean +/- SEM, n=3-5/group/experiment, 2 pooled experiments.

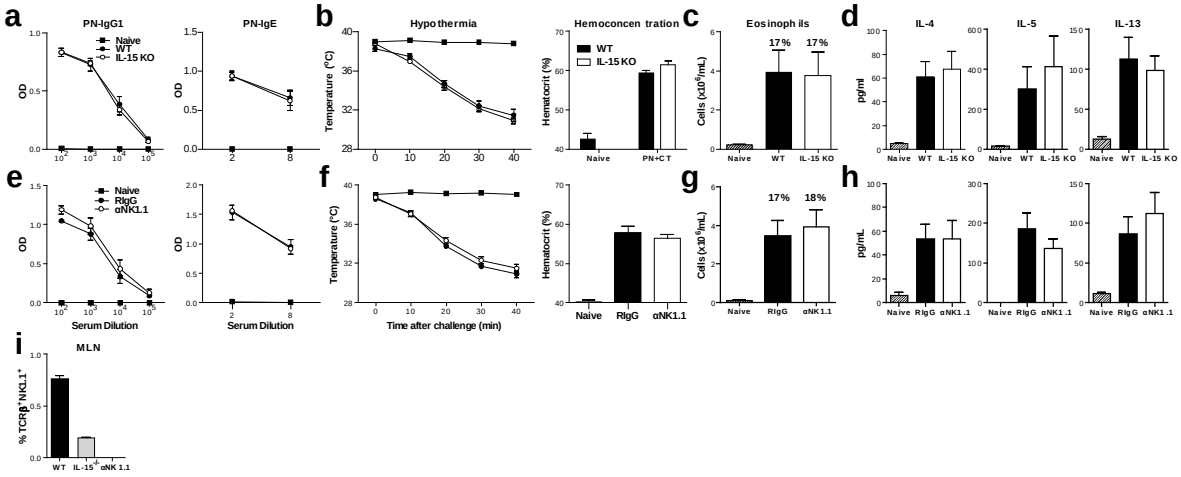


FIGURE 2

Figure 2. IL-15 or NK1.1$^+$ cells are not required to induce food allergy and anaphylaxis to PN. WT mice receiving rat IgG (RIgG) or anti-NK1.1 (αNK1.1), or IL-15 KO mice were exposed to PN+CT i.g. weekly for 4 weeks, or kept naïve. (**a, e**) Serum PN-IgG1 (left) and PN-IgE (right). (**b, f**) Anaphylactic hypothermia (left) and hemoconcentration (right). (**c, g**) Peritoneal eosinophilia. (**d, h**) Th2 cytokine production. (**i**) NKT cell quantification in mesenteric lymph nodes (MLN) by flow cytometry. Mean +/- SEM, n=3-8/group/experiment, 2-3 pooled experiments.

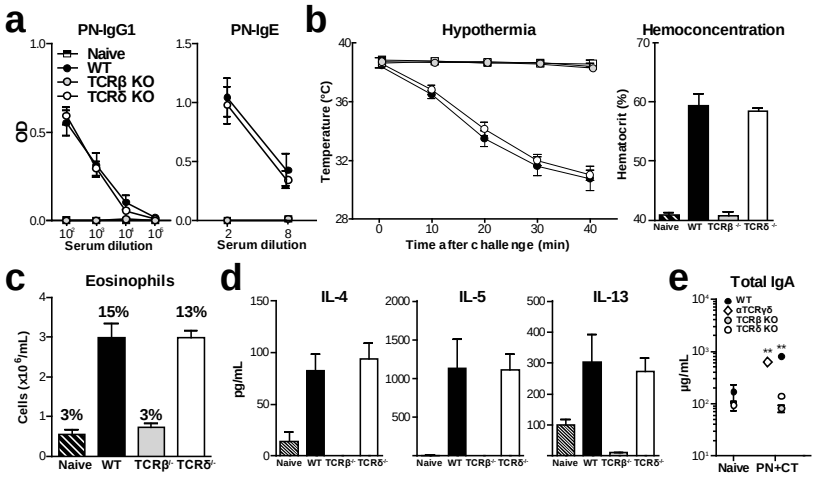


Figure 3

Figure 3. γδ T cells are not required to induce food allergy and anaphylaxis to PN. WT, TCRδ or TCRβ KO mice were exposed to PN+CT i.g. weekly for 4 weeks, or kept naïve. (**a**) Serum PN-IgG1 (left) and PN-IgE (right). (**b**) Anaphylactic hypothermia (left) and hemoconcentration (right). (**c**) Peritoneal eosinophilia. (**d**) Th2 cytokine production. (**e**) Serum total IgA titres at wk 5 after weekly oral exposure to PN+CT for 4 wks with or without anti-TCRγδ antibody injection before each gavage. Mean +/- SEM, **p < 0.01 vs TCRδ KO. n=3-8/group/experiment, 2-3 pooled experiments.

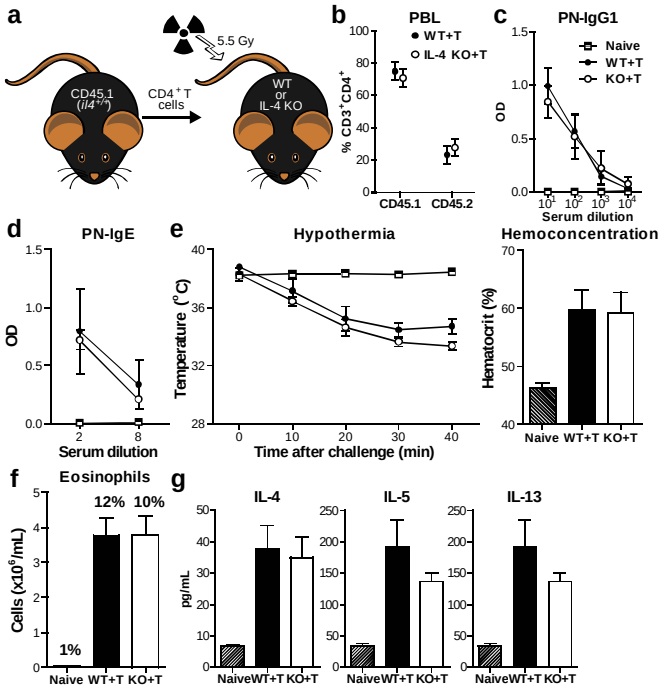


Figure 4

Figure 4. CD4$^+$ T cell-intrinsic IL-4 induces PN allergy and anaphylaxis. (**a**) Transfer of *il4*$^{+/+}$ CD45.1$^+$ CD4$^+$ T cells to sublethally irradiated CD45.2$^+$ WT (WT+T) or IL-4 KO recipients (IL-4 KO+T). (**b**) Peripheral blood (PBL) T helper cell composition at wk 0 of PN sensitization. Mice were exposed to PN+CT i.g. weekly for 4 weeks, or kept naïve. (**c**) Serum PN-IgG1 at wk 5. (**d**) Serum PN-IgE. (**e**) Anaphylactic hypothermia (left) and hemoconcentration (right). (**f**) Peritoneal eosinophilia. (**g**) Th2 cytokine production. Mean +/- SEM, n=2-5/group/experiment, 2 pooled experiments.

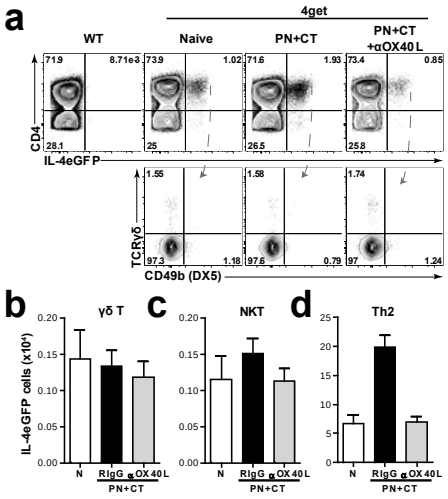


Figure 5

Figure 5. IL-4 originates from conventional Th cells during PN sensitization, and IL-4 production is controlled by OX40L *in vivo*. Mice were exposed to PN+CT i.g. with or without anti-OX40L administration and then evaluated 96 h later. (**a**) Representative flow cytometric plots after PN sensitization with or without OX40L neutralization. Quantification of IL-4 expressing (**b**) γδ T cells. (**c**) NKT cells. (**d**) Th2 cells. Mean +/- SEM, n=2-5/group/experiment, 2 pooled experiments. N, Naïve.

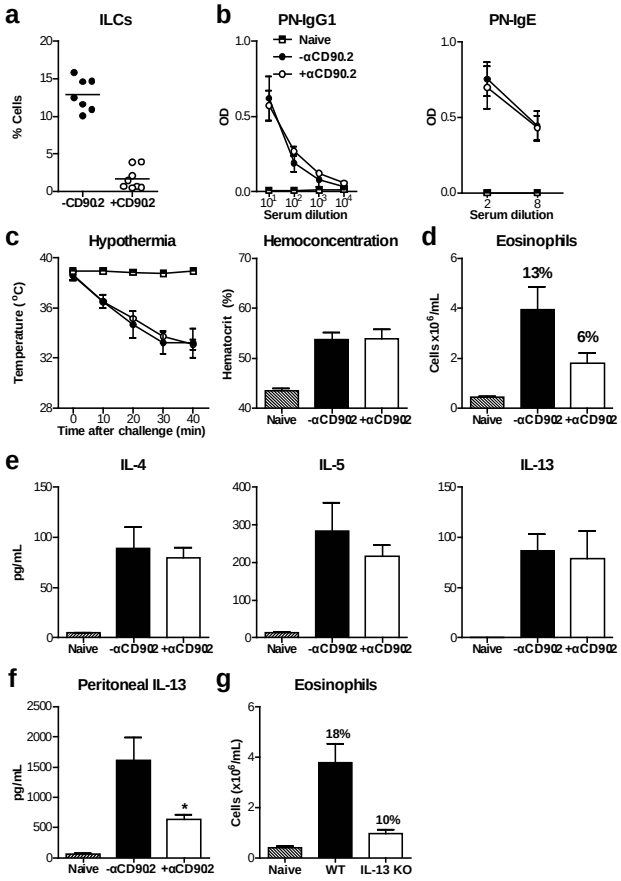


Figure 6

Figure 6. Innate lymphoid cell depletion prevents allergic inflammation but not sensitization. CD90-disparate chimeras were treated with anti-CD90.2 antibody throughout sensitization (PN+CT i.g. weekly for 4 weeks) and challenge to PN, or kept naïve. (**a**) ILC depletion. (**b**) Serum PN-IgG1 (left) and PN-IgE (right) at wk5. (**c**) Anaphylactic hypothermia (left) and hemoconcentration (right). (**d**) Peritoneal eosinophilia. (**e**) Th2 cytokine production. (**f**) Peritoneal lavage IL-13 after 72h after challenge. (**g**) Peritoneal eosinophilia in sensitized and challenged WT and IL-13 KO mice. Mean +/- SEM, n=2-8/group/experiment, 2 pooled experiments.

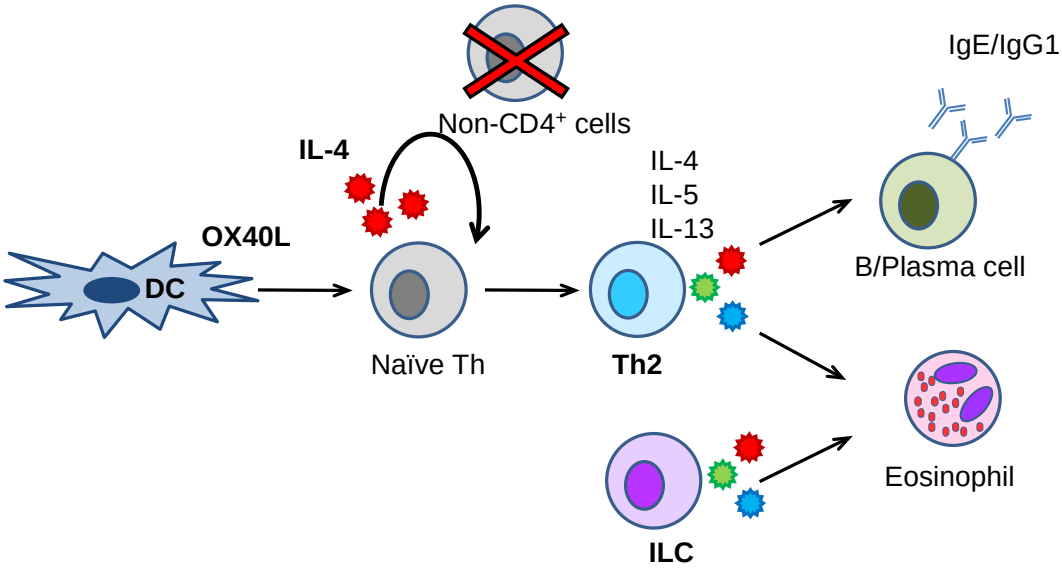
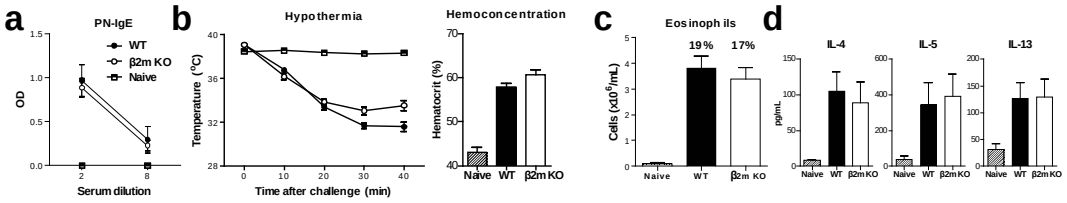


Figure 7

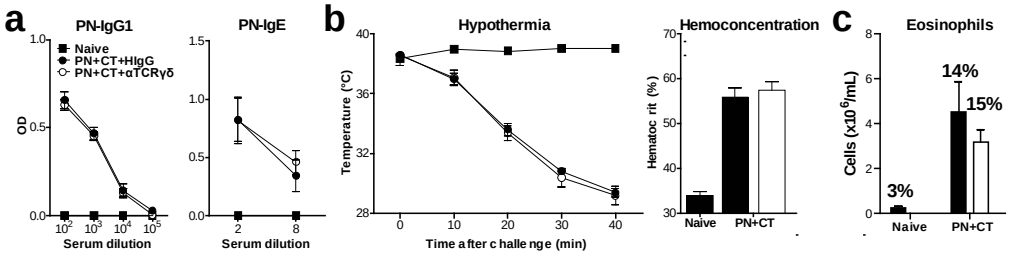
Figure 7. Model of Th2 induction during PN allergic sensitization. Allergen exposure at epithelial sites activates DCs to express OX40L in an IL-33 dependent manner [17]. Within the MLN, DC OX40L triggers naïve T cells to secrete IL-4, which then acts in an autocrine/paracrine manner to amplify and stabilize Th2 differentiation. Differentiated Th2 cells then act within LNs to help B cells class switch to IgE and IgG1. Effector Th2 cells exit the lymph node to induce IL-13-dependent eosinophilia at sites of allergen exposure in cooperation with ILCs. Non-CD4$^+$ sources of IL-4, such as mast cells, basophils, γδ T cells, NKT cells, and ILCs are dispensable to induce adaptive Th2 immunity in PN allergy.

Supplemental Figure Legends



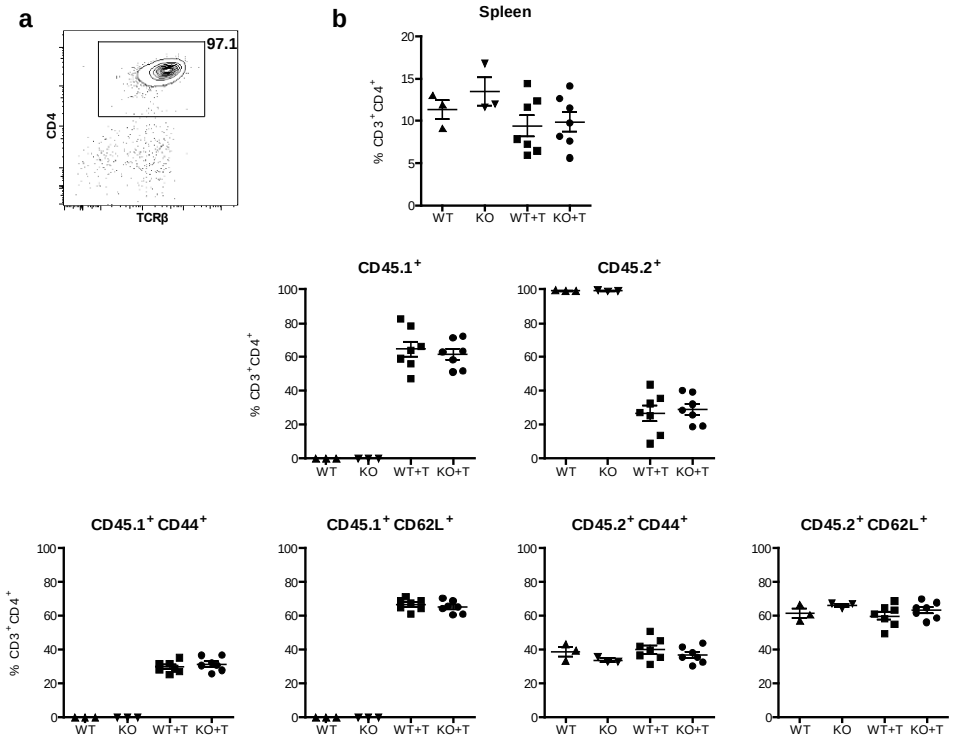
Supplementary Figure S1

Supplementary Figure S1. β2 microglobulin deficiency does not impact the initiation of Th2 immunity to PN. WT or β2m KO mice were exposed to PN+CT i.g., weekly for 4 weeks, or kept naïve. (**a**) PN-IgE (right) at wk 5. (**b**) Anaphylactic hypothermia (left) and hemoconcentration (right). (**c**) Peritoneal eosinophilia. (**d**) Th2 cytokine production. n=3-8/group/experiment, 2-3 pooled experiments



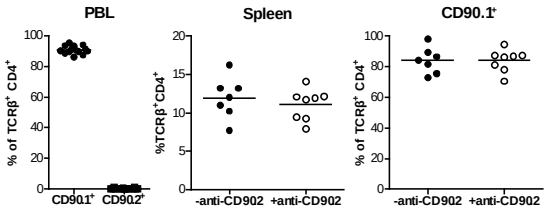
Supplementary Figure S2

Supplementary Figure S2. Impact of anti-TCRγδ clone UC7-13D5 or control hamster IgG (HIgG) on PN allergic responses. WT mice received anti-TCRγδ or control IgG during exposure to PN+CT i.g. weekly for 4 weeks, or were kept naïve. (**a**) Serum PN-IgG1 (left) and PN-IgE (right) at wk 5. (**b**) Anaphylactic hypothermia (left) and hemoconcentration (right). (**c**) Peritoneal eosinophilia. Mean +/- SEM, n=3-8/group/experiment, 2-3 pooled experiments.



Supplementary Figure S3

Supplementary Figure S3. Spleen $CD3^+CD4^+$ T cell composition. Irradiated WT or IL-4 KO mice received $il4^{+/+}$ $CD4^+$ T cells (WT+T or KO+T, respectively). (**a**) Purity of adoptively transferred $CD4^+$ Th cells. **(b)** Overall $CD3^+CD4^+$ T cell percentages (top). Quantification of CD45.1 and CD45.2 pools that make up T cells (middle). Activation status of CD45.1 and CD45.2 T cells (bottom). Mean +/- SEM, n=3-7/group/experiment, 2 pooled experiments.



Supplementary Figure S4

Supplementary Figure S4. CD90-disparate chimeras. (**Left**) Peripheral blood (PBL) composition of Th cell compartment in CD90-chimeras before initiation of study. (**Middle and Right**) Th cell compartment composition at sacrifice in spleen. Mean, n=2-8/group/experiment, 2 pooled experiments.

Chapter 4.

Indigenous Enteric Eosinophils Are Essential to Initiate a Primary Th2 Immune Response in vivo

Title

Indigenous Enteric Eosinophils Are Essential to Initiate a Primary Th2 Immune Response *in vivo*

Running Title

Enteric Eosinophils Initiate Th2 Immunity *in vivo*

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Abstract:

Eosinophils natively inhabit the small intestine, but a functional role for them has remained elusive for over 100 years. Here, we show that eosinophil-DC communication is essential to initiate adaptive Th2 immunity to intestinal immunization. Eosinophil-deficient mice were protected from induction of Th2-mediated food allergy and anaphylaxis, which was restored by reconstitution with either *il4*$^{+/+}$ or *il4*$^{-/-}$ eosinophils. Eosinophils controlled CD103^{+} dendritic cell (DC) activation and CCR7-dependent migration from the intestine to draining lymph nodes, events necessary for Th2 priming. Eosinophil activation *in vivo* and *in vitro* led to degranulation of eosinophil peroxidase, a granule protein which promoted DC upregulation of CD86, OX40L and CCR7, and mobilization to lymph nodes *in vivo*. Thus, eosinophil-granule-DC shaping of adaptive immune responses can be therapeutically targeted in food allergic, and potentially other immune-mediated diseases.

Character count: 27789

Introduction

Eosinophils evolved hundreds of millions of years ago. Indeed, eosinophils have been identified in tissues of lower vertebrates and eosinophil-like cells in some invertebrates, *i.e.*, before adaptive T and B cell immunity emerged, thus suggesting that they are part of an ancient innate immune apparatus (McGarry, 2013). Eosinophils may have evolved from phagocytic cells in primitive invertebrates when digestion and immunity became specialized functions (Lenzi et al., 1997). In this regard, the intestine is a unique immune site that natively houses an abundance of eosinophils. Although Ehrlich described the presence of rich magenta eosin staining granules that characterize eosinophils over 130 years ago (Erlich, 1879), the precise roles of eosinophils in health and disease remain controversial (Rosenberg et al., 2013).

As eosinophil granulocytes are thought of as specialized effector cells recruited from the blood and bone marrow to tissues during adaptive Th2 responses, most healthy tissues are devoid of eosinophils (Barnes, 2004; Rosenberg et al., 2013). Once in the tissue, recruited eosinophils are activated, produce a number of cytokines, and release characteristic cationic granules such as eosinophil peroxidase (EPO) in a process termed degranulation. Eosinophils have been ascribed both beneficial and harmful functions. For example, there is evidence of eosinophil attachment to helminths and subsequent degranulation, and cytotoxicity towards helminths. Despite these associations, an indispensible role for eosinophils in host defense against helminths remains controversial (Cadman

and Lawrence, 2010; Rosenberg et al., 2013). With respect to allergic disease, eosinophils are thought to play a pathogenic inflammatory role in diseases such as allergic asthma, atopic dermatitis, or food allergy. In response to helminths or allergens, Th2 responses in the gastrointestinal (GI) tract, and elsewhere, are characterized by the late influx of eosinophils into the tissue to mediate inflammation. However, a role for the abundant native GI eosinophil population has remained enigmatic (Drury, 1915; Duran-Jorda, 1947; Mowat, 2010).

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

activation conditions, and T cell polarization factors such as the source of the 'initial' IL-4 for Th2 cell priming remain unclear.

In stark contrast with the conventional paradigm that eosinophils participate in immune responses as terminal effector leukocytes whose production, migration and activation is under the control of adaptive Th2 immunity, we report just the opposite process. Here, we found a crucial role for indigenous SI eosinophils in the initiation of Th2 immunity, with critical importance to the development of food allergy. Exposure to peanut (PN) along with the classical mucosal Th2 adjuvant, cholera toxin (CT), resulted in marked activation of eosinophils and degranulation of EPO. Oral Th2 priming also resulted in CCR7-dependent migration of activated $CD103^+$ DCs to the intestine-draining MLN, but these responses were absent in either eosinophil- or EPO-deficient mice. This resulted in impaired DC-mediated Th2 priming and cytokine production, antigen-specific IgE and IgG1 secretion and, ultimately, clinical anaphylaxis; all parameters were restored upon reconstitution of the eosinophil compartment. Thus, eosinophil activation during intestinal Th2 priming results in the release of EPO that controls $CD103^+$ DC activation and CCR7-dependent migration to the MLN, where DCs then promote Th2 responses. Collectively, these data identify a novel and critical *in vivo* role for indigenous eosinophils in shaping DC responses and initiating adaptive immunity.

Results

Definition of indigenous eosinophils along the intestinal tract

Eosinophils along the intestinal tract were histologically quantified in 1999 (Mishra et al., 1999). We took advantage of flow cytometry's ability for precise quantitation and multiparameter analysis to better define eosinophils along the GI tract. Eosinophils were most prevalent within the SI, ranging from ~10-15% of all lamina propria (LP) cells in the duodenum and ileum, and ~15-25% in the jejunum (Fig. 1A). In contrast, the large intestine (LI) contained ~3-5% eosinophils in the caecum, ~7% in the colon, and <1% in the rectum. These patterns were maintained when quantifying eosinophils as a proportion of $CD45^+$ leukocytes. Phenotypically, although both SI and LI eosinophils were Siglec-F^+, $CD11b^+$, $CD44^+$, $CD11c^{int\ to\ neg}$, and ~30% $Ly6G^+$, SI and LI eosinophils also had distinct cell surface phenotypes (Fig. 1B). SI eosinophils expressed ST2 (IL-33 receptor), CD69, and Ly6C, whereas LI eosinophils did not. Therefore, different compartments of the intestine harbor different numbers and phenotypes of eosinophils at baseline. As the intestinal microbial load increases in burden from the SI to the LI, these data also show that eosinophil prevalence was not directly correlated with microbial load. Given the prominence of eosinophils in the SI, we next sought to evaluate the impact of eosinophil ablation on SI function and immunity.

Indigenous eosinophils promote induction of Th2 immunity *in vivo*

ΔdblGATA1 mice (Fattouh et al., 2011; Humbles et al., 2004; Yu et al., 2002), lack a high-affinity GATA binding site in the GATA-1 promoter, resulting in strong, but submaximal GATA-1 promoter activity and, ultimately, complete ablation of the eosinophil lineage (Yu et al., 2002). Other GATA-1 dependent lineages, namely erythrocytes, megakaryocytes and mast cells, are not affected as their lineages do not require maximal GATA-1 promoter activity.

Intragastric (i.g.) immunization to the common food allergen, PN, with the classical oral Th2-inducing adjuvant, CT (Lycke and Holmgren, 1986; Snider et al., 1994), induces GI Th2 responses, such that subsequent allergen challenge elicits systemic anaphylaxis (Chu et al., 2013). Whereas wild-type (WT) mice mounted robust antigen-specific IgE, IgG1, and Th2 cytokine responses and, clinically, anaphylactic hypothermia and hemoconcentration, ΔdblGATA1 mice did not (Fig. 2A). This defect was corrected by adoptive transfer of eosinophils into ΔdblGATA1 mice (Fig. 2A). The requirement for eosinophils was site-dependent as immunization via the peritoneum, skin, or rectum, tissues with little to no eosinophils at baseline, resulted in robust intestinal Th2 priming (Fig. 2B-D). These data also showed that eosinophils were not required to induce anaphylaxis *per se*. Eosinophil deficiency (Fig. 3A) did not impair conventional parameters of intestinal and adaptive immunity, including Peyer's patch organogenesis (Fig. 3B), intestinal IgA levels (Fig. 3C), macronutrient uptake (Fig 3D), antigen-specific IgE production in response to intraperitoneal

immunization, or oral tolerance induction (Fig. 3E). Lastly, although eosinophils in the SI-draining MLN are *il4* competent (Svensson et al., 2011), mixed bone marrow chimeras showed that reconstitution of the eosinophil compartment with IL-4-deficient eosinophils fully restored Th2 priming to i.g PN+CT (Fig. 4). In summary, eosinophils are required for the induction of Th2 responses that are initiated in the SI, irrespective of their ability to produce IL-4.

CD11c$^+$ cells are required for intestinal Th2 priming

Aside from being a potential source of IL-4, there is also evidence that granulocytes such as eosinophils may supplant DCs in their requirement as APCs to launch Th2 immune responses (Akuthota et al., 2010; Perrigoue et al., 2009; Sokol et al., 2009). We tested the requirement for DCs in this system by generating CD11c-diptheria toxin receptor (DTR) transgenic → WT chimeras, which allow for the depletion of CD11c$^+$ cells with repeated injection of diphtheria toxin (DT). Such chimeras treated with DT lacked SI DCs but not CD11c$^+$ eosinophils (Fig. 5A,B), likely due to the higher expression of CD11c (and therefore the DTR transgene) by DCs. Depletion of DCs throughout sensitization protected CD11c-DTR → WT chimeras from i.g. PN+CT sensitization and anaphylaxis (Fig. 5C-F) as indicated by antigen-specific IgG1 and IgE, IL-4 production and, clinical hypothermia or hemoconcentration. Altogether, these data show that CD11c$^+$ DCs are required to initiate intestinal Th2 priming.

CD103$^+$ DCs prime for Th2 immunity to oral antigen

Adaptive immunity is primed in the draining lymph nodes, where naïve T cells interact with APCs that have migrated from peripheral sites carrying antigen and are equipped with a particular package of immunological instructions (Ritz et al., 2002). Thus, we further evaluated whether eosinophils or DCs acted as APCs by migrating from the SI to the MLN to activate CD4$^+$ T cells. In response to PN+CT, DCs, but not eosinophils, increased in the MLN (Fig. 6A). Similarly, DCs, but not eosinophils, expressed MHC II (Fig. 6B). These data suggest that DCs are the primary APC in this system.

SI CD103$^+$ DCs preferentially capture ingested food antigens and then home to the draining MLN in a CCR7-dependent fashion to induce oral tolerance and T regulatory cells (Schulz et al., 2009; Worbs et al., 2006). Whether similar mechanisms take place during pathogenic Th2 priming to oral antigen is unclear. We found that CCR7-mediated homing of CD103$^+$ DCs, but not eosinophils, to the MLN also drove pathogenic Th2 responses to i.g. PN+CT (Fig. 6C,D). Likewise, IRF8(R249C) mutant mice, which are deficient in the SI CD103$^+$ DC subset due to a block in differentiation (Edelson et al., 2010), were sufficient in eosinophils (Fig. 6C) and protected from allergic sensitization (Fig. 6D). Thus, CD103$^+$ DCs are elicited from the SI to the MLN in a CCR7-dependent manner and these cells act as a critically required APC to initiate adaptive Th2 immunity in response to i.g. priming.

Eosinophils control DC activation *in vivo*

That ablation of either eosinophils or $CD103^+$ DCs prevented the induction of Th2 responses suggested that these two cell types acted in series rather than in parallel. The capability to induce adaptive immunity by non-oral immunization routes (Fig. 2 and Fig. 3E) and to inhaled house-dust mite (HDM) (Fattouh et al., 2011), suggested that the defect in immunizing ΔdblGATA1 mice i.g. was upstream of T or B cell activation and differentiation. Consistent with this reasoning, we found that eosinophils were required for $CD103^+$ DC mobilization to the MLN in response to i.g. PN+CT (Fig. 7A). The ability to mobilize SI DCs was not inherently disabled in eosinophil-deficient mice because $CD103^+$ DCs migrated to the MLN in response to i.p. LPS (Fig. 7A), an established parenteral method to mobilize SI DCs (Schulz et al., 2009).

To better understand how eosinophils control DC activation and migration, we undertook histological analysis of the intestine within 24h after i.g. PN+CT priming. Electron microscopy revealed that i.g. PN+CT caused marked morphological changes in LP eosinophils *in vivo*, including the development of cytoplasmic protrusions and cell membrane ruffling (Fig. 7B,C,), marked decreases in granule density (Fig. 7B,C) and even the occasional degranulation chamber undergoing exocytosis (Fig. 7D). We also observed membrane-bound granules and semi-dissolved extracellular granules amidst extracellular cell debris (Fig. 7E). Together, these data illustrate that Th2 priming with PN+CT induces eosinophil degranulation by means of both active granule release (Fig. 7C,D), as

well as eosinophil cytolysis (Fig. 7E). Notably, a number of granules lost their electron-lucent matrix (Fig. 7D-F), which contains the eosinophil-specific granule protein, EPO. Further, eosinophils activated *in vitro* with PN+CT released EPO (Fig. 7G). Thus, eosinophils control $CD103^+$ DC migration to the MLN in response to i.g. PN+CT, and this is associated with degranulation of EPO.

Eosinophil granule-mediated activation of DCs and Th2 immunity

In response to i.g. PN+CT, EPO-deficient mice phenocopied ΔdblGATA mice. Indeed, the lack of EPO resulted in the inability of DCs to be mobilized to the MLN (Fig. 8A) and become activated, including upregulation of CCR7, CD86 and OX40L (Fig. 8B-D), a costimulatory molecule required for intestinal Th2 priming (Chu et al., 2013). As it would then be expected, EPO deficiency resulted in a lack of induction of antigen-specific IgE (Fig. 8E), and full protection from anaphylaxis (Fig. 8F,G), peritoneal delayed-type hypersensitivity eosinophilic inflammation (Fig. 8H) and Th2 cytokine production (Fig. 8I). Similar to eosinophil-deficient mice, parenteral immunization did not rely on EPO for DC mobilization, IgE or cytokine production, or induction of anaphylaxis (Fig. 9). Thus, DCs rely on eosinophil cues, at least in the form of EPO, to become activated and migrate to the MLN to initiate adaptive immunity.

Discussion

Immigrant eosinophil granulocytes are a hallmark of Th2-mediated immune responses. In contrast, we have found a novel, critical role for indigenous

SI eosinophils in the initiation of Th2 immunity with direct relevance to the development of food allergy. Eosinophil activation during intestinal immunization results in the release of EPO that triggers $CD103^+$ DC activation and CCR7-dependent migration to the MLN, where DC CD86 and OX40L then control induction of Th2 responses. In support of these data, human intestinal eosinophils also degranulate during infection with CT-producing *Vibrio cholera* (Qadri et al., 2004) and *in vitro* co-cultures of human DCs and eosinophils result in enhanced DC activation to CpG-ODN stimulation (Lotfi and Lotze, 2008).

Aside from their classical cytotoxic/destructive inflammatory functions, eosinophils have also been ascribed various immunodulatory and tissue remodeling activities (Rosenberg et al., 2013). Notably, these reports mainly stem from *in vitro* work or data on inflammatory eosinophils infiltrating helminth infected or allergen exposed tissues such as the lung, skin, or peritoneum. First, direct APC activity for T cell priming has been shown for eosinophils after they were Ag-pulsed *in vitro* and then placed in coculture, or transferred to otherwise naive mice (Akuthota et al., 2010; Shi et al., 2000). Second, eosinophils have been shown to express IL-4 (Lacy and Moqbel, 2000; Shinkai et al., 2002; Voehringer et al., 2004), and as the initial source of IL-4 required for naïve $CD4^+$ T to Th2 differentiation remains contentious, eosinophils have been speculated to be a potential candidate for this. In support of these data, eosinophils have long been recognized to localize to the T cell zone of LNs (Litt, 1964). Here, we found that intestinal LN eosinophils did not express MHC II or increase in number under

naïve or immunized conditions *in vivo* and, further, that selective deletion of IL-4 in eosinophils did not impair the induction of Th2-cytokine mediated immunity. The defect in intestinal immunization of eosinophil-deficient mice was instead more proximal to the level of T cell activation as these mice could fully mount Th2 responses to priming through the lung (Fattouh et al., 2011), skin or peritoneum. These data highlight unexpected and essential contributions of indigenous eosinophils to shaping adaptive immunity.

Tolerance and immunity are viewed as opposing outcomes after intestinal antigen encounter with the immune system (Pabst and Mowat, 2012). DCs are also thought to be central to this process, with Ag-bearing $CD103^+$ DCs migrating in a CCR7-dependent manner to induce T regulatory cells. How Th2 responses are induced is less clear. Indeed, Th2 responses to the intestinal helminth *Trichuris muris* require basophils to act as APCs instead of DCs (Perrigoue et al., 2009). We have previously shown that basophils are not required for Th2 priming to oral antigen (Chu et al., 2013). We found here that ablation of oral tolerance-associated $CD103^+$ DCs or CCR7 prevented Th2 immunization. Thus, these data identify DCs as being critical for Th2 immunity in this system and illustrate the potential Janus nature of intestinal $CD103^+$ DCs as inducers of either tolerance or immunity to oral antigen.

Granulocyte-DC cooperation has been reported in papain-induced Th2 responses, with antigen-presenting DCs cooperating with IL-4^+ basophils during T cell priming (Tang et al., 2010). Herein, we identify a different level of

granulocyte-DC cooperation: eosinophils and EPO mediate activation of DCs, and these activated DCs then migrate to the LN to prime T cells. These data fortify the notion that DCs may heavily rely upon signals from the microenvironment, now including eosinophil cues, to initiate specific classes of adaptive immunity. Thus, not only DC sensing of foreign antigens but also the demographics and activity of innate cells neighboring DCs articulate the induction of adaptive immunity. Consistent with this, eosinophils did not regulate oral tolerance induction.

We have analyzed the contribution of eosinophils to multiple parameters of intestinal innate and adaptive immunity and have found that normally-resident eosinophils initiate primary adaptive immune responses through pathways conventionally associated with oral tolerance. These data identify a novel role for indigenous eosinophil populations as well as a Th2 induction pathway whereby the hallmark effector cell type also acts as an incipient early inducer. That we identify a molecular link between food, eosinophils, DCs and T cell priming, suggests that eosinophil activation in the intestine may represent a primitive digestive-immune mechanism that has been co-opted during evolution to aid in the initiation of adaptive immunity. Indeed, DC activation required degranulation of the microbicidal peroxidase EPO, consistent with an innate defense role for intestinal eosinophils (Yousefi et al., 2008). More broadly, the identification of a mechanism that shapes adaptive immunity through cooperative eosinophil-DC interaction should encourage investigation into the role of eosinophils, EPO, or

other eosinophil products in the (patho)genesis or treatment of immune mediated processes such as allergy, autoimmunity, vaccines and infectious disease.

Materials and Methods

Mice

Age, sex and strain-matched controls were used in all experiments. ΔdblGATA1 (C.Cg-Gata1^{tm6Sho}) and CD11c-DTR mice were bred in-house. IL-4 KO (BALB/c-Il4^{tm2Nnt}/J), CCR7 KO (B6.129P2(C)-*Ccr7*^{tm1Rfor}/J), IRF8(R249C) (BXH2/TyJ), and EPO-deficient (Duguet et al., 2001) (NZW/LacJ) mice were from JAX laboratories (Bar Harbor, Maine). IL-5 transgenic (IL-5Tg) mice were from Charles River. All procedures were approved by the McMaster University Research Ethics Board.

Intestinal cell isolation

Intestines were opened longitudinally, cut into 2-3 mm pieces and incubated in 1mM DTT (Sigma) in PBS for 15 min, then three times of 15 min 10% FBS in PBS and 2mM EDTA before digestion in 0.239 mg/mL Collagenase A (Roche) with DNAse (Roche) for 50-60 min and 40/70% Percoll (GE Healthcare) separation.

Flow cytometry

In all assays, cells were incubated with anti-FcγRII/IIIb before incubation with fluorochrome-conjugated antibodies, dead cells excluded by propidium iodide (PI) uptake, and gated on singlets. Whole blood was anticoagulated with EDTA and lysed with ACK lysis buffer before staining. Antibodies used were from eBioscience, BD Biosciences, or Biolegend: CD3-FITC or Pacific Blue; CD4-APC, eFluor605 or eFluor650; CD19 PE-Cy5; MHC II-eFluor 650; F4/80-eFluor

450 or Pacific Blue; Siglec-F-PE; CD44-V500 or Alexa 700; B220-eFluor650 or V500; Ly6G-Alexa 700 or APC-Cy7; Ly6C-Alexa Fluor 700 or APC-Cy7; ST2-FITC or biotin; CD11b-PerCP-Cy5.5, Alexa Fluor 488, PE-Cy7 or Alexa Fluor 700; CD11c-APC, PerCP-Cy5.5 or PE-Cy7; CD69-PE-Cy7; CD45-eFluor605 or APC-eFluor780; CD103-APC, Brilliant Violet 421, or biotin; CD86-PE-Cy7 or eFluor605; OX40L-biotin; CCR7-biotin; Steptavidin-APC, Qdot-800 (Invitrogen), or PE. Fluoresence minus one (FMO) controls were used for gating. Data were acquired on an LSR II (BD Biosciences) and analyzed using FlowJo (Treestar Inc.).

Ig measurements

Intestines were flushed with 5 mL ice cold soybean trypsin inhibitor (Sigma) in PBS-EDTA-PMSF, centrifuged at 2000 rpm for 10 min, and supernatants analyzed for total IgA.

Glucose uptake

Following an overnight fast, 2 g/kg D-glucose was administered by oral gavage. Blood glucose was measured at various time points using a hand-held glucomeneter (Accu-CheckActive, Roche Diagnostics, Laval, Canada).

Oral Tolerance

Mice were fed with PBS or 1 mg OVA (Grade V, Sigma) i.g. daily for d0-5, then injected with 200 µg OVA adsorbed to 1 mg aluminium hydroxide i.p. on d7 and 14. Serum was collected on d28 and analyzed for OVA-IgE (Cayman).

PN allergy model

PN (Kraft) with CT (List Biologicals) was administered i.g. (Delvo), i.p., p.r., or s.c. weekly for 4 weeks, with challenge by crude PN extract (Greer) one week later. Serum was collected 24h before challenge and analyzed for total or PN-specific Igs (Chu et al., 2013). Temperature was assessed by rectal probe, and hematocrit by centrifuging anticoagulated blood at 40 min after challenge. Peritoneal lavage was performed with PBS-EDTA. Total cells were counted using Turks and hemocytometer. Eosinophils were quantified using flow cytometry as $F4/80^{lo}$, Siglec-F^{+} cells.

Adoptive Transfer

Spleen and peritoneal lavage from IL-5Tg mice were labeled with biotin anti-CD3, CD19, Thy1.2, B220, CD11c, F4/80, c-Kit, TER-119 and >90% pure eosinophils isolated using anti-biotin microbeads and LS MACS columns (Miltenyi) before $5x10^7$ eosinophils were transfered i.v. to GATA mice 16-24h before each gavage.

Cytokine production

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

Bone Marrow Chimeras

Mice irradiated twice with 5.5 Gy (Cs^{137} source) received $5x10^6$ T-cell depleted BM cells i.v., rested for 8 wks and then used in experiments. 100 ng DT was injected i.p. 24h before each gavage for CD11c-DTR experiments. For mixed BM

chimeras, i.v. injections were composed of 80% WT or ΔdblGATA1 BM mixed with 20% IL-4 KO BM.

Lymph node processing

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

Transmission Electron Microscopy

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

EPO assay

With minor modifications from previously described EPO assays (Dyer et al., 2010; Humbles et al., 2002), eosinophils were isolated as described above, and incubated for 24h with media, lyso-platelet activation factor (L-PAF, Sigma), and 1 or 10 ug/mL PN+CT and assayed for EPO activity by oxidation of o-phenylenediamine (Sigma), stopped with sulphuric acid and absorbance measured at 492 nm. Results were normalized to maximal activity from Triton-X100 lysed eosinophils.

Statistics

Comparisons were made using unpaired t-tests or one or two-way ANOVA. Repeated measures were used for temperature data. $P<0.05$ was considered statistically significant.

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DKC designed and performed experiments, analyzed data and wrote the manuscript. TW, SG, ALG, NGB, JDB, and RJS performed experiments. JSE performed TEM experiments and analysis. RF, RK, AAH, and KDM provided reagents and input. SW and MJ obtained funding. MJ oversaw the project and edited the manuscript.

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Figures and Legends

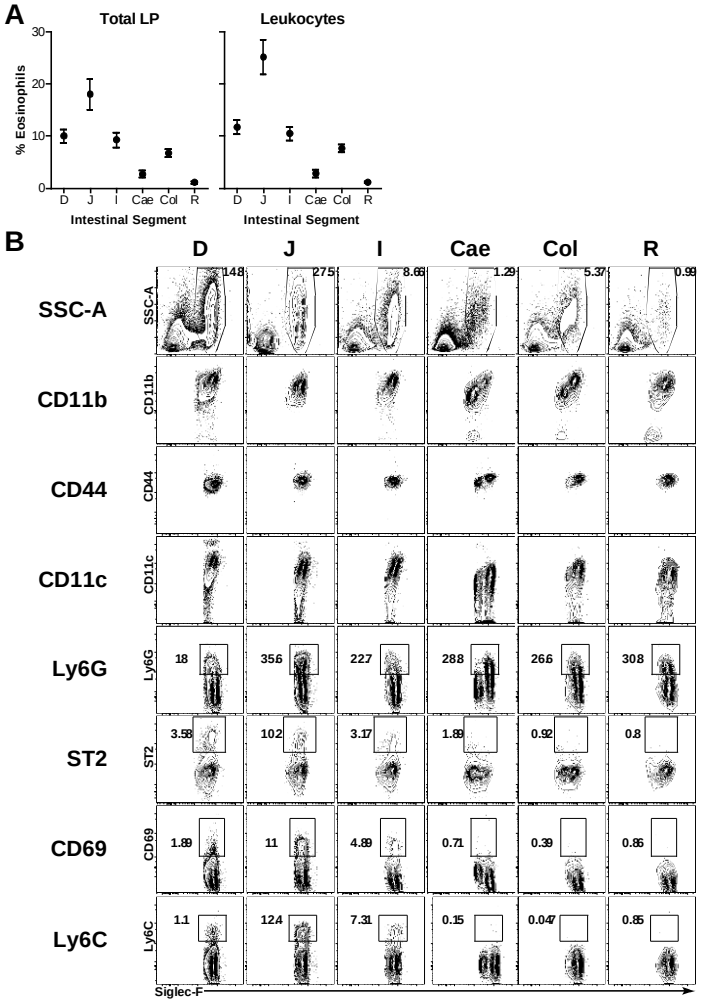

Figure 1

Figure 1. Characterization of eosinophils along the intestinal tract. (A) Proportion of eosinophils of total LP cells or $CD45^+$ leukocytes along the intestinal tract. (B) Phenotypic characterization of eosinophils along the intestinal tract. Mean ± SEM, n = 6-8, with each sample being pooled from 3-4 mice, from 2 experiments. D, duodenum; J, jejunum; I, ileum; Cae, caecum; Col, colon; R, rectum.

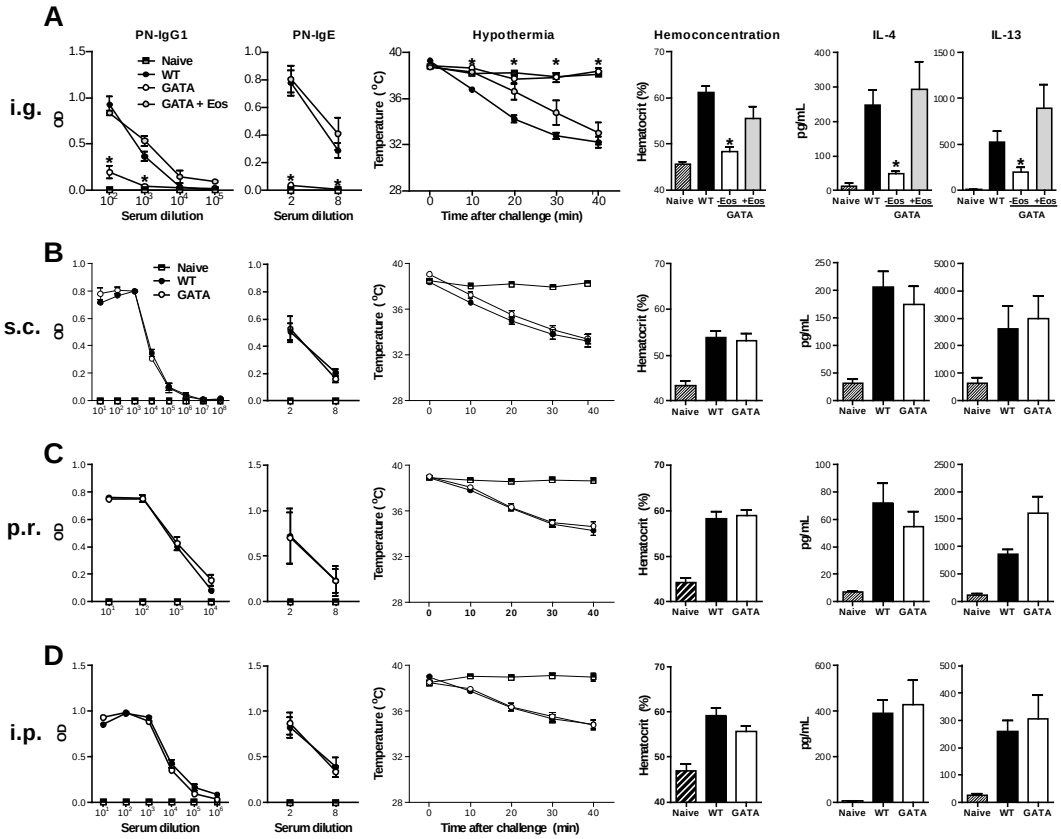

Figure 2

Figure 2. Eosinophils are required for induction of Th2 immunity to oral antigen. WT, ΔdblGATA1 (GATA), or ΔdblGATA1 mice that received eosinophils weekly (GATA + Eos), were administered PN+CT (A) i.g., (B) s.c., (C) p.r. or (D) i.p. weekly for four weeks. (Left) Serum PN-specific (PN-) IgG1 and PN-IgE at week 5. (Middle) Clinical anaphylaxis assessment of hypothermia and vascular leakage after i.p. challenge at week 5. (Right) Th2 cytokine production from PN-stimulated splenocytes. Mean ± SEM, n = 3-10 from 3-5 experiments. *p<0.05 vs WT.

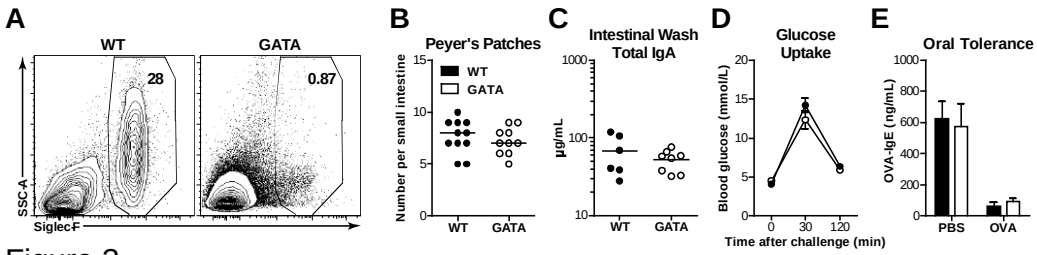

Figure 3

Figure 3. Conventional features of intestinal and adaptive immunity are intact in the absence of eosinophils. (A) Siglec-F^{+} cells in WT and ΔdblGATA1 (GATA) mice. (B) Median number of Peyer's Patches with individual data shown. (C) Mean intestinal wash IgA with individual data shown. (D) i.g. glucose challenge and blood glucose over time. (E) Serum ovalbumin (OVA)-specific IgE at d28 from mice i.g. fed OVA or PBS daily from d0 to d5 before i.p. immunization on d7 and d14 with OVA adsorbed to aluminium hydroxide. Mean ± SEM, n = 6-12 from 2 experiments.

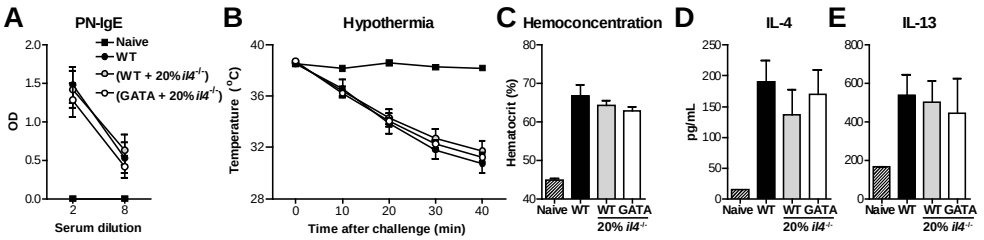

Figure 4

Figure 4. IL-4-deficient eosinophils restore Th2 responsiveness. Bone marrow chimeras were generated by mixing 80% WT or ΔdblGATA1 bone marrow (BM) with 20% *il4*$^{-/-}$ BM, resulting in mice with *il4*$^{+/+}$ or *il4*$^{-/-}$ eosinophil compartments, respectively. Naïve, WT, 80% WT + 20% *il4*$^{-/-}$ (WT + 20% *il4*$^{-/-}$) chimeras, and 80% ΔdblGATA1 + 20% *il4*$^{-/-}$ (GATA + 20% *il4*$^{-/-}$) chimeras received i.g. PN+CT weekly for 4 weeks. (A) Serum PN-specific PN-IgE at week 5. (B) Clinical anaphylaxis assessment of hypothermia and (C) vascular leakage after i.p. challenge at week 5. (D, E) Th2 cytokine production from PN-stimulated splenocytes. Mean ± SEM, n = 5-12 from 3 experiments.

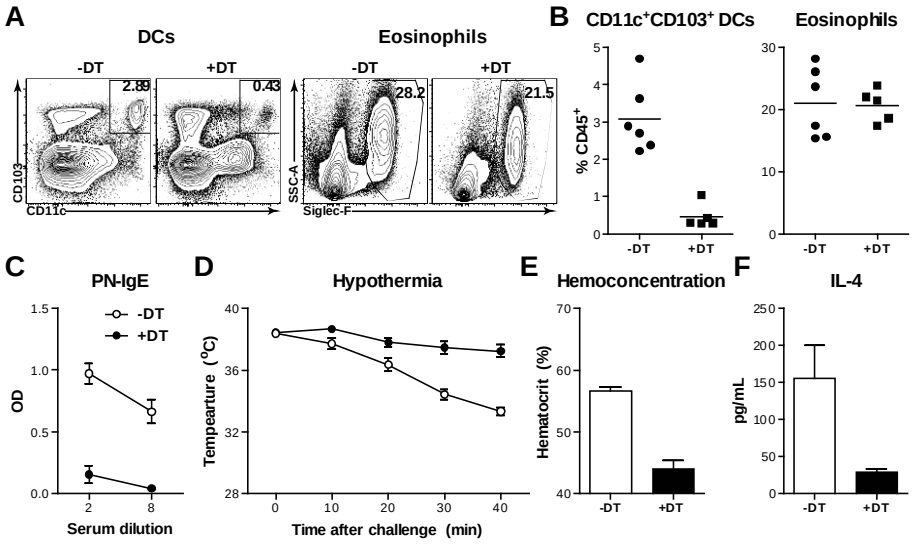

Figure 5

Figure 5. CD11c-DTR → WT chimeras show that DCs are required for induction of Th2 immunity to oral antigen. CD11c-DTR → WT chimeras received i.p. PBS or DT 24h before each i.g. PN+CT, weekly, for four weeks. (A, B) Depletion of SI DCs but not eosinophils. (C) Serum PN-specific PN-IgE at week 5. (D) Clinical anaphylaxis assessment of hypothermia and (E) hemoconcentration after i.p. challenge at week 5. (F) Th2 cytokine production from PN-stimulated splenocytes. Mean ± SEM, n = 5-10 from 2 experiments.

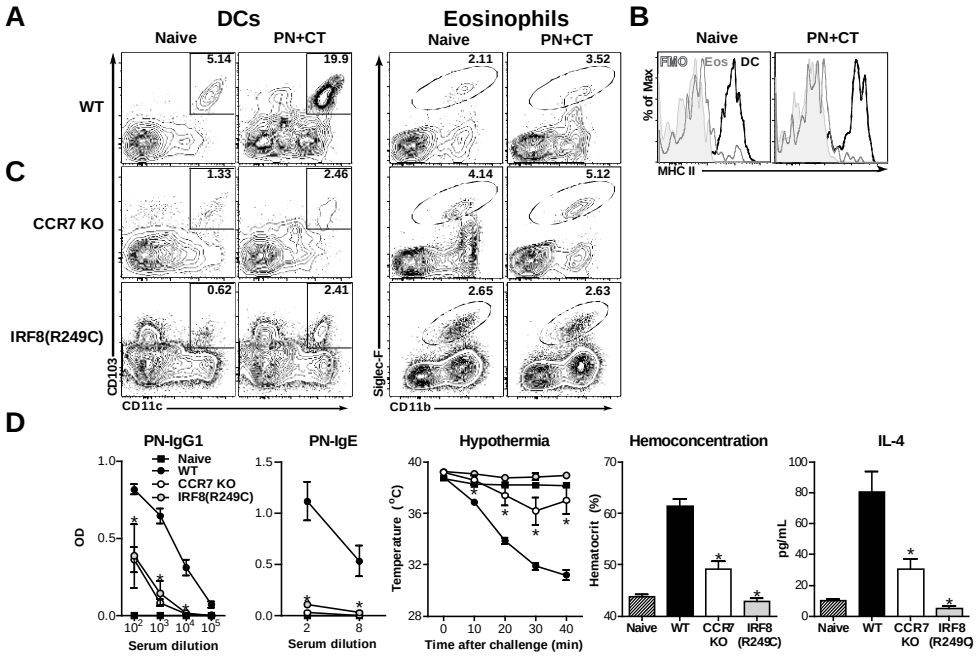

Figure 6

Figure 6. CD103$^+$ DCs are required for induction of Th2 immunity to oral antigen. WT, CCR7 KO or IRF8(R249C) mice were administered PN+CT i.g. for three consecutive days, or weekly for four weeks. (A) DC, but not eosinophil, migration to MLN in response to PN+CT (B) Expression of MHC II on DCs but not eosinophils. (C) Migratory DCs and eosinophils in CCR7 KO and IRF8(R249C) mice. (D) Immunological and clinical responses in the absence of CD103$^+$ DCs. Mean ± SEM, n = 3-10 from 2 experiments. *$p<0.05$ vs. WT.

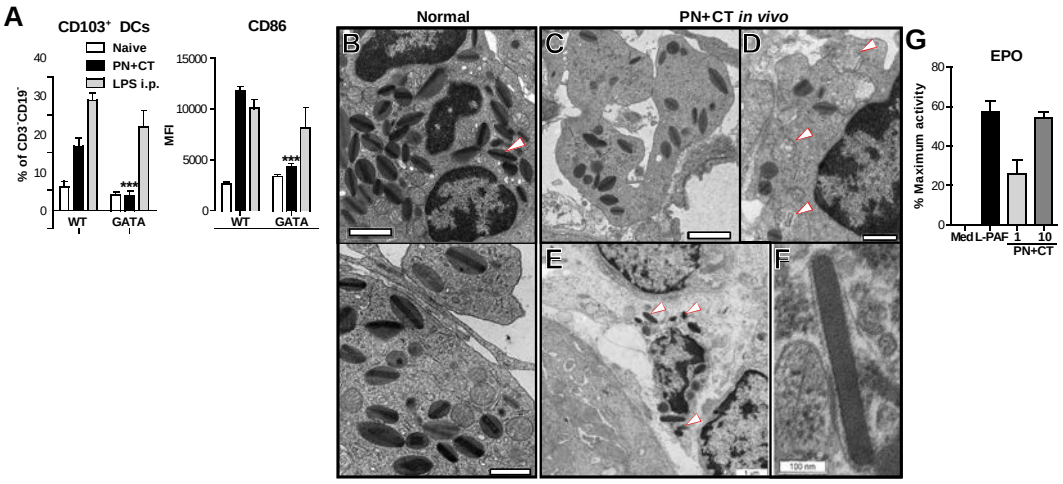

Figure 7

Figure 7. Eosinophil control of CD103$^+$ DC migration and activation is associated with elicitation of degranulation *in vitro* and *in vivo*. WT or ΔdblGATA1 mice received PN+CT i.g. for three consecutive days, or i.p. LPS 16 h before sacrifice. (A) Proportion and activation status of CD103$^+$ DCs in MLN. (B, top and bottom) Normal SI eosinophil transmission electron microscopy ultrastructure, showing bi-lobed nuclei and a high density of granules composed of an electron-dense core surrounded by an electron-lucent matrix rich in EPO (arrowhead). (C) Intact eosinophil with marked membrane protrusions and ruffling with loss of granule density. (D) Formation of degranulation chambers (arrowheads). (E) Extracellular free granules amidst apoptotic/necrotic eosinophil debris (arrowheads). (F) Dissolution of eosinophil granule matrix. (G) Purified eosinophils were incubated for 24h with Lyso-platelet activating factor (L-PAF), or increasing amounts of PN+CT, and assayed for EPO activity. Mean ± SEM, n = 3-8 from 2-4 experiments.

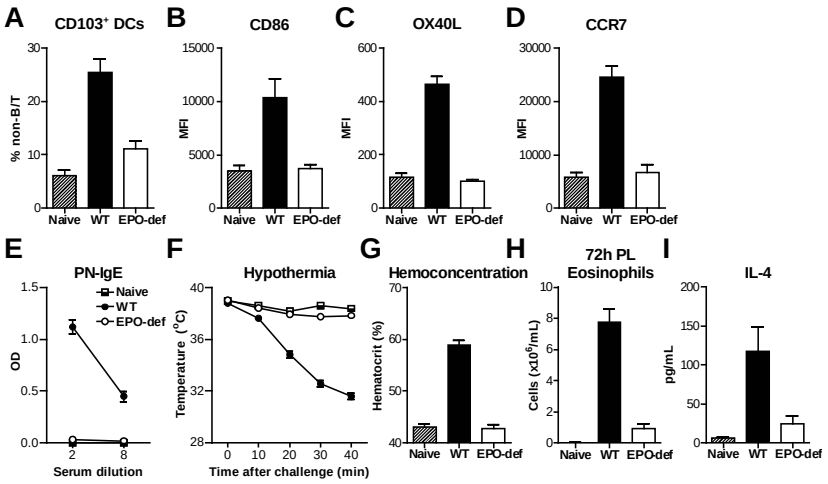

Figure 8

Figure 8. The peroxidase, EPO, controls CD103$^+$ DC migration, activation, and induction of Th2 immunity *in vivo*. WT or EPO-deficient mice were administered PN+CT i.g. daily for three days, or weekly for 4 weeks. (A) Proportion of CD103$^+$ DCs in the MLN. (B) CD86, (C) OX40L and (D) CCR7 expression. (E) Serum PN-IgE at week 5. (F) Clinical anaphylactic hypothermia and (G) hemoconcentration. (H) Peritoneal eosinophilic inflammation 72h after i.p. challenge. (I) Th2 cytokine production. Mean ± SEM, n = 3-8 from 2-3 experiments. PL, peritoneal lavage.

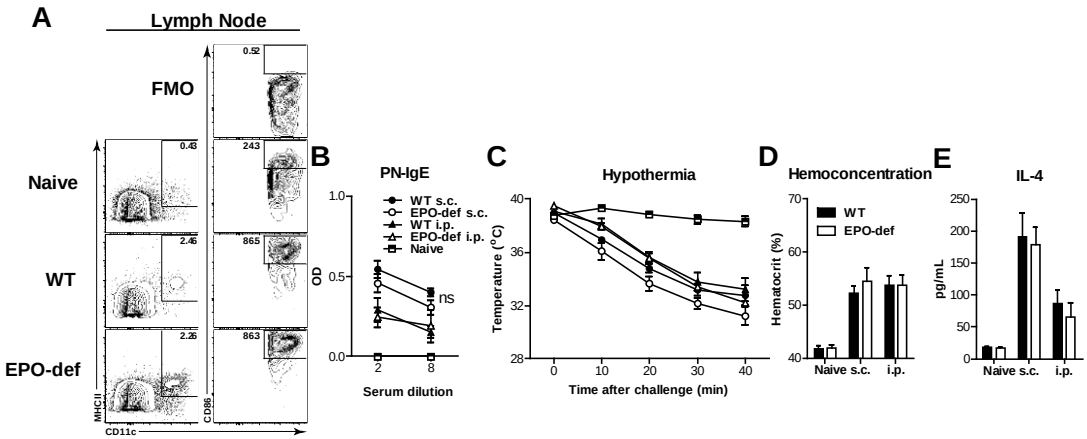

Figure 9

Figure 9. Parenteral immunization bypasses the requirement for EPO in DC activation and Th2 immunity. (A) WT or EPO-deficient mice received s.c. PN+CT or were kept naïve, and draining inguinal lymph nodes analyzed 16-18h later. Mice were immunized s.c. or i.p. weekly for 4 weeks, or kept naïve. (B) PN-IgE at wk 5, and (C,D) clinical responses after antigen challenge. (E) IL-4 production. Mean ± SEM, n = 3-8 from 2 experiments. ns, not significant.

Chapter 5.
Discussion

Summary

Food allergy is a growing health problem, can cause life-threatening anaphylactic reactions and has a poorly understood mechanistic basis. Using a murine model of PN allergy, this Thesis identifies mechanisms of Th2 immunity driving allergic sensitization: SI epithelial/IL-33 and eosinophil/EPO activation mobilizes $CD103^+$ DCs to migrate to the MLN in a CCR7-dependent fashion (**Chapters 2 and 4**); subsequently, DC OX40L induces Th-cell intrinsic IL-4 autocrine/paracrine signalling and, thus, Th2 differentiation (**Chapter 3**). This then leads to B cell activation and class switch to IgG1/IgE, which binds to systemically dispersed MCs and results in the sensitized state of allergy. Innate-like lymphocyte γδ T cells regulate IgA induction, whereas ILC2s contribute to allergic inflammation (**Chapter 3**). Lastly, these processes act independently of TSLP, IL-25, basophils (**Chapter 2**), IL-15, NK and NKT cells (**Chapter 3**). My unpublished work has also ruled out the requirement of a number of other cells/molecules, including IL-31R (IL-31 and oncostatin M), β2-microglobulin (MHC I and related molecules, $CD8^+$ T cells, mucosal associated invariant T cells), monocytes, neutrophils, CX3CR1 (intestinal møs), TLR4, TLR2, tryptophan hydroxylase 1 (serotonin/5-HT), CD101, IRF3/7/9 (IFN signalling), IL-6 (including Th17), indolamine-2,3-dioxygenase (IDO), non-hematopoietic MHC II, IL1R1, and ASC (inflammasome signalling). In sum, this Thesis provides a better understanding of how food allergens, such as PN, interact with the immune system to cause allergy by delineating a pathway stemming from epithelial activation and terminating in Th2 differentiation.

Basic Science Implications – How are Th2 Responses Initiated?

Current paradigms of Th2 immunity *in vivo* (*37, 81, 82, 84*) stem from mainly two branches of investigation: i.p. alum-driven allergic airway inflammation, and host-defense

against parasites such as the eukaryotic helminths (*28, 85, 86*) *Nippostrongylus brasiliensis* (*Nb*) (*87*), *Schistosoma mansoni (88)*, *Heligmosomoides polygyrus* (*aka H bakeri or H polygyrus bakeri or Hp*) (*89*), and *Trichuris muris (90)*, or unicellular protozoa such as the *Phlebotominae* subspecies sandfly-associated *Leishmania major (91-95)*. Common to these systems are the ideas that: 1) Ag-bearing, activated APCs are needed to activate naïve T cells to become Th2 cells; 2) eosinophilic inflammation and IgE are terminal readouts of ongoing Th2 responses; and 3) IL-4 and IL-13 are key cytokines orchestrating Th2 responses. Beyond this, significant variability between systems is observed. Hence, here I will attempt to place this Thesis' findings on food allergy in context with basic Th2 biology, while aiming to limit repetition from the discussions sections in **Chapters 2-4**.

TSLP, IL-25, and IL-33

TSLP, IL-25, and IL-33 are epithelial-associated cytokines that are each capable of promoting Th2 responses. These three cytokines have been reported to be important for i.p. alum-induced airway allergic inflammatory responses. With respect to Th2 responses in the GI tract, TSLP is required for Th2-mediated protection from *T. muris*, but not *Nb* or *Hp*; only IL-25 is required for *Nb*; and there are no reports on IL-33 or IL-25 and *Hp*. In **Chapter 2**, we show that both HDM-induced airway allergic responses and GI-induced PN allergy require IL-33, but not TSLP or IL-25, for full development of type 2 immunity, including Ab and cytokine generation and eosinophilic inflammation. A question that then arises is, "If all these systems share the same end phenotype (a Th2 response), what then dictates when TSLP is required, or when IL-25 is, or when IL-33 is?" Possible answers include: the inciting stimuli are different, the cytokines have different activities, and/or the responses/outcomes are different.

One hypothesis that has been put forth to explain the differential requirement of TSLP in different Th2-driven systems has been the relative contribution of DC IL12p40 for Th1/Th17 polarization. Indeed, compromised Th2-induction in TSLPR KO mice infected with *T. muris* can be restored with concomitant anti-IL-12p40 treatment, and TSLP-independent systems such as *Nb*, *Hp*, HDM or PN all contain factors that inhibit DC IL-12p40 production (*96*), thereby making TSLP redundant. Interestingly, although there are multiple reports on TSLP's ability to limit DC IL-12p40, there do not appear to be any addressing whether or not IL-25 or IL-33 are also able to do this. Thus, these data might explain situations when TSLP is or is not required to initiate Th2 responses, but do not explain when IL-25 or IL-33 are required.

We could not identify reports comparing the activity of TSLP, IL-25 and IL-33, and so we examined this *in vitro* and *in vivo*. Although TSLP, IL-25 and IL-33 were all capable of inducing OX40L on DCs *in vitro*, only IL-33 was able to do this *in vivo*. Thus, at least for HDM and PN sensitization, reliance on an IL-33-OX40L pathway is critical to generate Th2 responses. It is notable that *Hp*, but not *Nb*, requires OX40-OX40L signalling for Th2 induction (*97, 98*). One might then hypothesize that Th2 immunity would be compromised in IL-33 or ST2 KO mice infected with *Hp*; this remains untested. Another possibility is that the partial reductions in Th2 responses to HDM and PN in anti-OX40L treated mice, or *Hp* infected OX40L KO mice (*97*), might suggest the existence of additional costimulatory molecules important to Th2 induction. Indeed, although TSLP, IL-25 and IL-33 are capable of inducing DC OX40L *in vitro*, it is not known what other molecular changes take place after stimulation with each respective cytokine. Comparative gene array or proteomic studies *in vitro* or *in vivo* might be able to provide some initial insight in this regard.

Lastly, we propose the possibility that different epithelial-Th2-initiating cytokines might be required for <u>different</u> Th2 responses. Indeed, there are no known comparative

studies on the molecular phenotype of developing Th2 cells, or type 2 inflammatory responses, during TSLP versus IL-25 versus IL-33-driven processes. In support of this hypothesis is the observation that exogenous TSLP, IL-25 and IL-33 can induce distinct populations of cells (*77, 99*), including basophils (TSLP-associated) or group 2 ILCs (ILC2; IL-25 and IL-33 associated) (*15, 100, 101*) that are composed of natural helper cells, nuocytes, innate helper type 2, and a related, but distinct, population called multipotent progenitor type 2 cells (MPP^{type2}; IL-25 associated). Thus, it remains plausible that a particular type of inciting stimulus requires a specific pro-Th2 epithelial cytokine to induce a unique type 2 immunity program tailored to respond to that inciting stimulus. Nevertheless, formal proof of different types of Th2 responses (*e.g.* TSLP-driven, IL-25-driven, IL-33-driven) requires further investigation.

Contribution of Innate versus Adaptive Cells to the Generation of Type 2 Immunity

It is generally thought that IL-4 is important to the development of Th2 responses *in vivo*, but what is not known is precisely how Th cells become Th2 cells. Indeed, multiple reviews by prominent immunologists have repeatedly admitted that how Th cells become Th2 cells remains to be elucidated (*81, 102*). In particular, it has remained contentious for over 20 years (*103, 104*), how Th2 differentiation occurs. A major question has stemmed from the fact that *in vitro* polarization of naïve T cells to Th2 cells requires exogenous IL-4, which has led many researchers to conclude that another non-Th cell type must supply IL-4 to naïve Th cells during priming *in vivo*. A number of cell types critical for Th2 development have been identified in a variety of systems, such as NKT cells, γδ T cells and, even just a few years ago, basophils (References #4-11, 13-15, 22, 26 in **Chapter 3**). Melissa Brown summarizes this well, "In their discussion, they also asked the inevitable question: if IL-4 is needed for its own production by naive [sic] T cells, what is the source of the initial priming

IL-4 in vivo [sic]? Although several IL- 4-producing cells are candidates, including naive T cells themselves, this question still has not been satisfactorily answered." (*105*). Further, William E Paul, the discoverer of IL-4, himself admits the lack of knowledge in this area, "Identifying the initial source of IL-4 is crucial to understanding the initiation of IL-4-dependent TH2 cell responses. […] *In vitro*, autocrine and/or paracrine IL-4 induces and consolidates TH2 cell differentiation, but whether it does so *in vivo* is not known." (*81*). Robert Coffman has made similar statements (*106*). Hence, the identification of a precise mechanism that explains what happens in a model of food allergy provides novel insights into the pathogenesis of this disease, as well as contributes fundamentally to Th2 immunology.

Further, there is recent evidence that Th2 induction in particular systems does not actually require IL-4 but, instead, may require costimulatory molecule signalling such as OX40L (References 4-6, 16 and 35 of **Chapter 3**). Herein, we show clearly that *in vivo* Th2 generation to oral Ag results from an autocrine/paracrine acting IL-4 signalling loop generated by $CD4^+$ T cells, and that this pathway is controlled by OX40L signalling. Hence, these data not only address the aforementioned paragraph's longstanding question of the cellular source of "the initial IL-4" during naïve T cell polarization, but they also show that a 3rd possible pathway of Th2 generation exists: 1) exclusively IL-4 dependent, 2) exclusively co-stimulatory molecule dependent and, as shown in our study, 3) co-stimulatory molecule-dependent and IL-4 dependent.

If the generation of adaptive Th2 immunity to food allergens is orchestrated exclusively through the interaction between DCs and $CD4^+$ T cells, followed by $CD4^+$ T cells communicating to themselves through IL-4, then what is the role of IL-4 competent innate cells such as basophils, γδ T cells, NKT cells and ILC2s? Considerable controversy exists, and ILC2s have emerged as a novel cell type with uncharacterized contributions to the

generation of adaptive immunity. However, using CD90-disparate chimeras, the related group 3 ILCs have been shown to be capable of regulating $CD4^+$ Th adaptive immune responses through MHC-II-dependent interactions (*107*).

Basophils are rare $Fc\varepsilon RI^+$ IL-4 competent granulocytes that were initially reported to contribute to IgG1-mediated anaphylaxis, be more important APCs than DCs in initiating Th2 responses to *T. muris* and papain (a cysteine protease from papaya), but have more recently been shown to be dispensable for initiating Th2 responses to *Nb, S. mansoni*, papain, alum/OVA and HDM (*108*). Instead of acting at the priming stage, basophils may contribute to the effector stage of disease, including secondary helminths or tick parasite infections and minor, if any, contributions to anaphylaxis (*108, 109*). In **Chapter 2**, we showed that basophil depletion throughout sensitization to PN does not impact Th2 priming. Together, these data suggest that basophils play little role in initiating primary Th2 responses but may play a more prominent role in effecting secondary immune responses, including a minor role in anaphylaxis.

γδ T cells are innate-like lymphocytes predominantly located within the epithelial layer of barrier surfaces including the skin, lung and intestine (*110*). These cells are capable of rapid cytokine secretion, including IL-4, to initiate Th2 responses in the lung (see **Chapter 3** references). Response to epithelial stress has also linked γδ T cell responses to skin Th2 priming (*111*). γδ T cells have also been shown to be able to orchestrate B cell activation, germinal centre formation, isotype switching and Ig synthesis in the absence of conventional αβ T cells, particularly in the lung (*112-117*). In terms of food allergy and Th2 responses, Bol-Schoenmakers reported that anti-TCRγδ treatment during PN allergic sensitization resulted in augmented Th2 responses, concluding that γδ T cells negatively regulate Th2 priming in the intestine (*118*). In contrast, we observed that mice treated with anti-TCRγδ or control IgG treatment during sensitization mounted indistinguishable Th2

responses, and further, that this was also seen in WT and TCRδ KO mice. In terms of total IgA, anti-γδ TCR treatment did not produce the same effects as those seen in TCRδ KO mice by us or others (*119*). The technical issue in the study by Bol-Schoenmakers and colleagues may be the use of anti-γδ TCR Abs throughout priming to PN. Although the group claimed that this Ab was inhibitory/depleting, there is evidence that this Ab is actually activating (*120*). In sum, γδ T cells do not appear to regulate intestinal Th2 priming, but may regulate IgA levels. γδ T cells may affect IgA through their close communication with epithelial cells, which regulate IgA through mechanisms such as a proliferation inducing ligand (APRIL)/B cell activating factor (BAFF) (*121, 122*).

NKT cells are TCRαβ or TCRγδ-bearing innate-like lymphocytes capable of rapid cytokine secretion after activation. The most commonly studied subset of NKT cells is the CD1d-dependent subset, which recognizes glycolipid Ags presented by a CD1d-β2-microglobulin (β2m) complex. Within this classification, NKTs with invariant TCRs are the most commonly studied as reagents for detection of such cells and genetic tools for ablation are readily available. In the context of Th2 responses, invariant NKT (iNKT) cells have been proposed to be critical to asthmatic responses, potentially through production of IL-4 (*123-125*). In **Chapter 3**, we show that loss of NKT cells (CD1d-dependent or not, as well as invariant or variant) due to deficiency in IL-15-mediated maturation or anti-NK1.1 Ab-mediated depletion did not impact intestinal Th2 priming to PN. Our unpublished data with β2m KO mice paralleled these data. Similarly, NKT cells do not appear to play a role in Th2 priming to alum/OVA (*126, 127*), *S. mansoni*, *Nb*, *L. major (128)* or *T. muris (129)*. Thus, NKT cells do not play a major role in priming for Th2 responses to PN+CT, but whether there is a subset of food allergic patients where NKT cells play an important role in disease, similar to that observed in a subset of asthmatic patients, remains to be determined.

A new subset of innate-like lymphocyte related to NKT cells (*130*) was recently discovered to recognize vitamin B metabolites (*131, 132*) in the context of β2m and the MHC-I-like molecule, MR1. These cells were termed mucosal-associated invariant T (MAIT) cells due to their prominence in mucosal tissues and invariant TCR. There are no reports on MR1 KO mice to directly test the requirement of these cells in Th2 responses, but a number of aforementioned studies, including our own, have found comparable Th2 responses in WT and β2m KO mice, which are also deficient in MR1, and therefore MAIT cells (*130*).

ILCs are a diverse group of lymphoid-lineage cells that do not express a TCR/BCR, but parallel Th1, Th2, Th17 adaptive immune responses in terms of cytokine secretion upon activation. Consensus nomenclature was proposed in February 2013 (*101*): Group 1 ILCs produce IFN-γ and include NK cells and a new non-cytotoxic cell population referred to as ILC1. Group 2 ILCs produce IL-13 and IL-5, and to a lesser extent, IL-4, and include natural helper cells, nuocytes and innate helper type 2 cells; the related population, MPP^{type2} is associated with these. Group 3 ILCs produce IL-17A and/or IL-22 and include (secondary) lymphoid-tissue inducer (LTi) cells, and non-LTi ILC3 cells. To the best of my knowledge, an innate lymphoid regulatory cell has not been identified. All ILC groups highly express CD90 (Thy1), which is also highly expressed on all T cells. CD90 has at least two alleles, with CD90.2 being expressed in most mice, and CD90.1 expressed in less common strains, or mutant mice. Notably, using CD90-disparate chimeras, group 3 ILCs have recently been shown to be capable of regulating $CD4^+$ Th adaptive immune responses through MHC-II-dependent interactions (*107*). In **Chapter 2**, we showed that the expansion of ILCs were associated with HDM and PN sensitization. Using CD90-disparatate chimeras, we show in **Chapter 3** that depletion of ILCs does not impact Th2 priming, but that ILCs contribute to effecting allergic eosinophilic inflammation. This is consistent with reports that ILC2s can provide an important source of IL-13 and IL-5 in Ag-exposed tissues to

orchestrate host-defense against parasitic worms or allergic airway inflammation (*100*). Indeed, in **Chapter 2**, we show that IL-13 is dispensable for Th2 priming, and our unpublished data show that IL-13-deficiency severely impairs post-anaphylaxis peritoneal allergic inflammatory responses. Thus, ILCs do not affect Th2 priming to PN but do contribute to type 2 immunity by effecting inflammatory responses. Further study of ILCs will require application of CD90-disparate chimera systems (*e.g.* to different disease models), or new tools for Ab-mediated or genetic ablation of ILCs.

To summarize, basophils, γδ T cells, NKT cells (likely including MAIT cells), and ILCs do not contribute to intestinal Th2 priming to PN, a program that is instead driven by $CD4^+$ Th cell-intrinsic IL-4 autocrine/paracrine signalling under the control of DC OX40L. During the effector stage of inflammation (*i.e.* a secondary immune response), ILCs contribute to recruiting eosinophils, likely through their production of IL-13 and IL-5. Finally, γδ T cells regulate IgA production. These data are consistent with the idea, recently well-substantiated and articulated by Locksley and colleagues (*133*), that IL-4 and IL-13 have divergent cell and tissue expression, and therefore functional properties. IL-4 mainly functions within lymphoid organs to achieve Th2 differentiation and B cell activation (isotype switch, *etc*), while IL-13 mainly functions within non-lymphoid tissues to mediate inflammation (eosinophil infiltration, goblet cell hyperplasia, mø activation, *etc*).

In **Chapter 2**, we showed that OX40L was required for PN allergic sensitization, and in **Chapter 3**, we showed that OX40L controlled T cell production of IL-4 early after Th2 priming. We also evaluated IL-6 as a potential Th2 polarization factor (*134-136*), but our unpublished data show that IL-6 drives inflammation, but not Th2 priming to PN. As stated previously, partial reductions in Th2 responses to PN may suggest the existence of additional costimulatory molecules important to Th2 induction. In this context, our unpublished data have confirmed that CD28-CD86/CD80 interactions are important for the generation of

type 2 immunity to PN, with CD86 KO mice having a slightly more severe defect than CD80 KO mice. Beyond OX40 and CD28, there are a multitude of DC-T cell interactions, as well as T cell-B cell interactions that may govern Th2 priming and subsequent B cell activation. Although many costimulatory molecules and their basic signalling pathways have been discovered, it is quite clear that there remains much to be understood about the importance of these molecules to specific diseases, the timing of their importance, and their potential for therapeutic immunomodulation (*137-142*). In reference to food allergy, CD30 (TNFRSF8) has been shown to regulate alum/OVA-induced Th2 responses (*143, 144*) and thus, may be important to food allergic responses. Indeed, elevated levels of CD30 have been observed in the sera of patients allergic to cow's milk compared to controls (*145*). Another candidate is the Jagged-Notch signalling system, given that some Th2 responses have been shown to be regulated by Jagged2, that CT upregulates Jagged2 mRNA (*69*), and that unpublished preliminary Basic Local Alignment Search Tool (BLAST) search data showed that PN proteins may have homology to Notch signalling proteins. Jagged2 KOs exhibit perinatal mortality due to cleft palate and hence, a conditional KO approach (*146*), or fetal liver chimeras (*147*) will be required to examine if Jagged2 is important for Th2 priming to PN. Lastly, inducible costimulator (ICOS) regulates Th2 responses to airway allergens (*148-150*), and is a marker of Tfh cells (*151*), which control at least IgG Ab class switch and secretion. Thus, blockade of ICOS or ablation of $ICOS^+$ cells might abrogate the development of PN allergy. Whether other costimulatory molecules are involved will require additional investigation. Given the large number of possible molecules and low sensitivity of protein detection by flow cytometry (*e.g.* low-affinity Abs), costimulatory molecule discovery in PN allergy might best be approached by first evaluating mRNA expression *in vitro* and *in vivo* using high-throughput RT-PCR techniques such as those offered by Fluidigm, followed by protein detection techniques and functional *in vivo* studies/phenotype evaluation.

Eosinophils

Eosinophil granulocytes are classically thought of as cells recruited during ongoing Th2 responses to mediate tissue inflammation through elaboration of microbicidal and cytotoxic granule proteins, cytokines and lipid mediators (such as leukotrienes and prostaglandins) (*27, 152*). The intestine, best known for its importance to food digestion and absorption, houses the highest density of eosinophils. However, any biologically significant function for them there has remained enigmatic (*12*). In **Chapter 4**, we examined multiple parameters of intestinal immunity in eosinophil-sufficient and eosinophil, EPO, or DC–deficient mice and show that eosinophils promote intestinal Th2 priming through EPO-mediated cooperation with $CD103^+$ DCs without a need for eosinophil-IL-4 or APC activity.

Eosinophils were required for small intestinal Th2 priming, but not Th2 priming through the skin, peritoneum, or large intestine. Further, Th2 priming in the lung also does not require eosinophils, at least in response to HDM (*153*). These data are important to note because previous studies have concluded that eosinophils have no role in intestinal Th2 immunity when helminths infections of the small or large intestine are examined (*e.g. Nb* and *T. muris* respectively). However, it is important to consider that the route of infection that many helminths follow, including *Nb,* is not strictly enteric. *Nb* is a hookworm whose 620-750 μm long infective third-stage larvae (L3) infect through the skin, enter the vasculature, migrate to pulmonary alveoli within 12 hrs, cause a marked lung Th2 inflammatory response during moulting and maturation to L4 larvae over 1-3 days post-infection (pi), before migration cephalad and being swallowed into the proximal SI (often, jejunum), where at days 3-5 pi, mature (L5) worms take up residence between villi, hook onto and penetrate epithelia to feed on blood; they produce eggs at 6-9 pi and are expelled at days 10-16 pi (*86, 154*). *S. mansoni* also infects through the skin, enters the blood, travels to the lung, and then the heart to be pumped to the liver to mature and mate. After this, they secrete eggs from the large

intestinal mesenteric vasculature into the intestinal lumen and liver (*88*). In terms of helminths that reside within the intestine, *T. muris* infects the caecum and large intestine (*90*). *Hp* 300-600 μm long L3 larvae invade through the duodenal/jejunal mucosa, migrate deep to the intestinal musculature within 6h pi, where they undergo their third and fourth moult until full maturation and migration into the intestinal lumen at approximately day 8 pi. There, they establish a chronic infection, feed on the epithelium, mate, and produce eggs for several months unless mice are treated with anthelminthic medications (*86, 89*). Thus, helminths that migrate during infection (*e.g.* parenteral followed by enteral migration) do not require eosinophils for Th2 priming because they infect tissues with little to no eosinophils at baseline. Instead, DCs are likely activated and conditioned by alternative cells within the skin and lung. A similar rationale can be made for *T. muris* and infection of the large intestine. Consistent with these proposals, eosinophil-deficient ΔdblGATA1 mice exhibit little to no defect in Th2 responses to *Nb (155)*, *S. mansoni (156)*, *or T. muris (157).*

Hp infection affects the same intestinal segments as CT (duodenum, jejunum mainly), and would then be hypothesized to require eosinophils to initiate Th2 responses and host protection. To the best of my knowledge, there are no studies that have used ΔdblGATA1 mice to examine *Hp* infection, likely due to studies using anti-IL-5 or IL-5 KO mice that concluded that eosinophils do not affect worm clearance during *Hp* infection *(158, 159).* Notably, IL-5 deficient mice have slightly reduced, not absent, levels of eosinophils in the intestine (*160*), and there does not appear to be any formal flow cytometric comparison of enteral eosinophils in IL-5 KO mice versus ΔdblGATA1 mice (which in **Chapter 4** we show are intestinal eosinophil deficient). Therefore, a re-evaluation of the role of eosinophils in *Hp* infection might be warranted with the use of ΔdblGATA1 mice. Alternatively, it is notable that very early on during *Hp* infection, larvae penetrate beneath the muscular layer of the intestine, beneath both the mucosal and submucosal compartments and just adjacent to the

visceral peritoneal lining. When adult helminths emerge from the intestinal tissue, they penetrate and feed on epithelia. In comparison, CT directly activates epithelial cells, with likely partial direct activation of mucosal cells just adjacent to the epithelia. Interestingly, eosinophils express high levels of the CT receptor, GM1, on their cell surface (*161*). Given the high surface area of the mucosal epithelium as well as their tight junctions, it is likely that few, if any, cells of the submucosa or muscularis propria are directly activated by CT. This is consistent with immunolocalization data (*162*), albeit in ligated loops at a single time point. Further, *Hp* is a living, eukaryotic organism while CT is a microbial-derived protein toxin. Thus, the distinct localization and biology of CT *versus Hp* might impact the requirement for eosinophils in promoting intestinal Th2 priming. In terms of non-helminth responses and eosinophils, *G. lamblia* infection (*163-165*) stimulates enteric eosinophils (*166*) and *G. lamblia* excretory-secretory products may act similarly to CT (*63*). Therefore, it would be interesting to evaluate how ΔdblGATA1 mice respond to *G. lamblia* infection and excretory-secretory products. There are no reports of this. Likewise, whether eosinophils would also impact the enteral induction of non-Th2 classes of immunity remains to be determined, but at least oral tolerance (*46*) is unimpaired in ΔdblGATA1 mice.

We also show that EPO is critical to induce Th2 immunity through activation of DCs. Whether other eosinophil granule proteins are also required, and whether EPO directly or indirectly activates DCs requires further investigation. EPO may activate DCs through its peroxidase activity, which uses hydrogen peroxide to generate free radicals and acids to preferentially oxidize proteins with bromine, nitrite and thiocyanate (*167-169*). This could activate nearby epithelia or DCs, but also could cause cell lysis, damage-associated molecular pattern release (*170-176*), and thereby activation of DCs. Alternatively, enzymatic activity may not be required (*177*). EPO is a homolog of neutrophil myeloperoxidase (MPO) (*178*), and MPO has been shown to activate DCs (and neutrophils) through direct binding to cell

surface proteins such as CD11b (*179-182*). As well, EPO has been shown to activate bronchial epithelial cells (*183, 184*), including epidermal growth factor receptor-2 and mitogen-activated protein kinase (MAPK) signalling (*185*). Further, other features of eosinophil activation that often take place concomitantly with degranulation include respiratory burst through NADPH oxidase activity and the generation of reactive oxygen species (ROS), as well as ejection of DNA webs laden with antimicrobial intracellular, intranuclear and granule proteins and peptides (*186, 187*). Further, DNA is negatively charged and EPO, like all eosinophil granule proteins, is highly positively charged. Our unpublished data suggest that both ROS and extracellular DNA are important in mediating Th2 responses to PN, suggesting that multiple facets of eosinophil activation contribute to intestinal Th2 priming. Nevertheless, formal dissection of the molecular mechanism by which EPO activates DCs will require dedicated and extensive biochemical studies, but it is notable that EPO has been shown to activate human-monocyte derived DCs and møs in a hydrogen peroxide-independent manner (*188*) and eosinophil granule inclusions within phagocytes are commonly seen in eosinophilic inflammatory states (*181, 182, 189, 190*), including asthma (Dr. Parameswaran Nair, personal communication).

To my knowledge, such eosinophil-EPO-DC cooperation *in vivo* has not previously been identified. To date, the only other granulocyte-DC cooperation model proposed referred to basophils providing IL-4 directly to Th cells while DCs acted simultaneously as APCs (*191*). There are numerous reports on direct immunomodulation of Th cells by eosinophils, either through eosinophil-derived IL-4 or eosinophil APC activity (*192-194*). Notably, support for these ideas stems from reports based on *in vitro* co-culture systems, or *in vitro* Ag-loading of eosinophils that are then transferred to mice and T cell activity read out. It is also important to note that there does not appear to be any report utilizing MHC II deficient eosinophils or IL-4 deficient eosinophils without *in vitro* priming, to evaluate if

eosinophil APC or IL-4 activity truly is required *in vivo*. We generated mixed bone marrow chimeras such that the entire eosinophil compartment was deficient in IL-4 and concluded that eosinophil-derived IL-4 is not required for intestinal Th2 priming (**Chapter 4**). Lastly, there is *in vitro* evidence that the eosinophil granule RNAse, eosinophil-derived neurotoxin (EDN), can activate DCs to enhance Th2 priming through a TLR2-dependent pathway (*195*). There is also evidence that co-culture of eosinophils and DCs during CpG stimulation results in enhanced activation of DCs compared to DCs cultured alone (*196*). Although our unpublished data show that TLR2 is not required for Th2 priming to PN and suggest that EPO activation of DCs is a distinct mechanism from that performed by EDN, these data support the idea that eosinophils have the capability of regulating DC activity. In sum, we demonstrate *in vivo* that a cell conventionally thought to be a terminal event in the allergic/Th2 cascade plays a critical role in initiating that same cascade in PN allergy.

Miscellaneous Mechanisms Associated with the Induction of Th2 Immunity

A number of other mechanisms that have been proposed to be critical for inducing Th2 responses *in vivo* include IFN regulatory factor 3 (IRF3) (*197, 198*), TLR4 (*199*), IL-1 signalling (*200*), and uric acid (*201*). In short, our unpublished data show that deficiency of IRF3, TLR4, or IL1R1 leads to comparable Th2 priming in WT and respective KO mice. However, mice depleted of uric acid appear to be impaired in inducing Th2 responses to PN. Further evaluation of the role of uric acid in PN allergy is being pursued by Msc candidate Joshua Kong in our laboratory.

How Does CT Coupling to ER Result in Th2 responses?

CT is the mucosal adjuvant used to drive Th2 priming to PN in this Thesis. Beyond understanding which cells are activated by CT, it is equally important to understand how CT activates cells to initiate Th2 responses as this may identify immune or signalling derangements that promote allergy in humans. Additional considerations for this modelling approach are discussed in, "How is PN allergy induced in humans?", while basic science implications are considered here. As previously stated in **Chapter 1**, CT binds to cells via GM1, is internalized and after entering the ER, CTA is released into the cytosol to activate adenylate cyclase for excessive production of cAMP. Whether CTA enzymatic activity is required for Th2 priming remains to be tested, and already established CTA mutants may be helpful in this regard (*202*). However, classical studies by Snider and colleagues showed that CTB alone was not sufficient to cause Th2 priming to oral Ag (*49*). Secondly, the intracellular signalling events activated by CT to cause allergy remains poorly understood, but recent data suggest that CT may be sensed by the ER sensor inositol-requiring-1a which then activates cytosolic nucleotide sensors such as RIG-I (*57*). It has also recently been shown that innate immune activation takes place in response to intracellular detection of bacterial or viral DNA by c-GMP-AMP (cGAMP) synthase (cGAS), which produces cyclic dinucleotide second messengers such as cAMP to activate stimulator of IFN genes (STING, or TMEM173) (*203-207*). Importantly, while both RIG-I and STING are well known for their ability to stimulate type I IFNs, they are able to stimulate other pathways, including NFκB activation (*208*), which regulates multiple cell activation and cytokine production programs. Indeed, our unpublished data show that IFN signalling is not required for Th2 priming to PN. Preliminary examination of STING KO mice (n=3) suggest an impairment in Th2 priming. Clearly, further investigation into the intracellular signalling pathways engaged by CT may prove useful in identifying novel mediators of type 2 immunity. It

certainly would be interesting if such investigations yielded mechanistic proof that (food) allergies are, at least in part, due to the evolution of intracellular DNA sensors against bacteria and viruses. Notably, CT is a bacterial-derived molecule.

Clinical Implications

Tenets of medicine include the prevention, treatment, management and cure of disease. To a large extent, efforts to achieve these goals rely upon an understanding of the process(es) that cause disease. That is, disease initiation, perpetuation, exacerbation, or progression/transformation. Therapeutics need targets. For example, health care guidelines need to warn against risk factors. For a disease such as food allergy, the prevailing understanding has been rather generic: a Th2 response, likely activated by DCs and in an environment rich in IL-4, is responsible for generating allergy. In this Thesis, we delineate a pathway extending from allergen ingestion and encounter with intestinal epithelial cells, to naïve T cell to Th2 differentiation and subsequent B cell help. SI epithelial/IL-33 and eosinophil/EPO activation mobilizes $CD103^+$ DCs to migrate to the MLN in a CCR7-dependent fashion; subsequently, DC OX40L induces Th-cell intrinsic IL-4 autocrine/paracrine signalling and thus, Th2 differentiation. Such a mechanism provides a number of additional targets for prevention or treatment for food allergy. For example, it is conceivable that individuals with overactive IL-33 (either intrinsically or due to some exogenous stimulus), eosinophils, EPO, $CD103^+$ DCs, or OX40L would be more susceptible to acquire food allergy by having a propensity to promote Th2 differentiation. Identification of such high-risk individuals and/or disease-promoting factors would allow for more focused targeting and monitoring by medical personnel and may help prevent the development of food allergy. As well, therapeutics that inhibit or modulate, even

temporarily, such pro-food allergic molecules/cells may also be able to prevent the induction of food allergy.

In terms of disease management and potential cure, desensitization strategies offer effective, albeit temporary, relief from food hypersensitivity. Precisely why discontinuation of the desensitization protocol leads to reactivation of food hypersensitivity is unclear, but one possibility is that re-differentiation of Th2 cells takes place, and therefore food-specific IgE is regenerated. A question then raised by this Thesis is whether or not inhibition of IL-33/eosinophils/OX40L might aid in extending the duration of effect of current desensitization strategies, and even possibly converting temporary desensitization into permanent tolerance.

How is PN Allergy Induced in Humans?

Food allergens are commonly characterized as being resistant to proteolysis by digestive enzymes, and this is thought to be central to their allergenicity. However, it remains uncertain precisely how food allergy in humans first begins. Indeed, other factors that might contribute to the genesis of food allergy include the cutaneous route of immunization and the role of the GI microbiota in shaping immune responses. There is considerably less discussion by the scientific community regarding the role of non-allergen immunogens, such as PN agglutinin, or the common fungal contaminant in PN, aflatoxin. Without clear direction from genetic studies or clinical trials, it remains unknown precisely what factors result in food allergy rather than tolerance.

In this Thesis, we utilize the classical mucosal adjuvant, CT, to induce allergy to co-ingested PN. While overt *V. cholera* infection is unlikely to be the basis for food allergic sensitization, this does not preclude the usefulness of CT to model allergic disease. Indeed, CT-like molecules span bacterial and protozoan taxonomic kingdoms and also include

viruses. This suggests that molecules similar to CT in terms of mode of binding, mechanism of action, or both, may also play a relevant role in human food allergy. In this regard, it would be interesting to evaluate the possible contribution of other GM1-binding or AB_5 toxins, such as those from *Giardia* or *E. coli,* to the development food allergy in humans or mice.

The immune pathways engaged by CT may also be operative during sensitization mediated by non-CT adjuvants. Perhaps most obvious is the essential contribution of IL-4 for the induction of allergic disease, whether this is driven by i.g. PN+CT, i.p. alum/OVA, or i.n. HDM. Likewise, we show clearly that an IL-33-OX40L axis is operative during both CT-mediated priming to PN, as well as to i.n. HDM. Thus, CT serves as a useful and relevant tool to probe mechanisms of Th2 induction and food allergic sensitization.

EoE versus Food Allergy and Heterogeneity in Immunology

TSLP has been considered to be a critical pro-Th2 epithelial derived cytokine and is commonly thought to be central to the development of allergic disease. In contrast, we showed that TSLP is not required to mount Th2 immunity in allergic airway inflammation to HDM or food allergy and anaphylaxis to PN. Superficially, this might be viewed as implying that TSLP actually is not important to human Th2 responses. I do not submit to this line of thinking. Instead, what this reveals is that different Th2 responses require different initiating cytokines; a particular outcome can have more than one cause. One intriguing possibility that then stems from this is whether different initiating cytokines can result in phenotypically different Th2 responses. In the context of asthma, we discussed this possibility within the manuscript.

In the context of food allergy, one GWAS study has linked TSLP polymorphisms to the atypical food allergy, eosinophilic esophagitis (EoE). Although both classical and non-

classical food allergies are associated with Th2 responses, classical allergies are associated with anaphylaxis or immediate/Type I hypersensitivity, while EoE is mainly viewed as inflammatory and more akin to delayed/Type IV hypersensitivity. Thus, that TSLP does not appear to regulate Th2 induction and anaphylactic reactions to food does not negate the possibility that TSLP may be important to inflammatory-type atypical food allergies such as EoE. These data support the notion that different epithelial cytokines may be capable of inducing different types of Th2 responses that can lead to clinically different outcomes such as anaphylaxis *versuss* esophageal inflammation.

Clinical Implications, Outside of Food Allergy

Apart from promoting disease, the pathways identified in this Thesis may also have beneficial therapeutic potential. Th2 responses are classically thought to be critical for host-defense against helminths parasites. Most human vaccines use aluminium hydroxide to induce protective immune responses, and this is a classical Th2 inducing adjuvant. Lastly, if autoimmune disease therapy is viewed from an immune deviation perspective, strategies for reprogramming autoimmune Th1 or Th17 cells to a benign or less pathogenic Th2 phenotype may be beneficial. This Thesis then identifies mechanisms to promote Th2 responses. For example, administration of IL-33, eosinophils, EPO, or OX40L activating agents along with, or in place of aluminum hydroxide, would be expected to result in a potently protective vaccine. In support of this, TSLP has recently been reported to be capable of acting as a vaccine adjuvant against HIV-1 (*209*). In sum, the mechanisms identified in this Thesis may help prevent or cure food allergy, as well as impact other fields such as host-defense and autoimmunity.

Considerations: Lessons from Primary Immunodeficiency/Genetic Defects

The ultimate test of whether or not basic science research is truly reflective of human biology has been argued to be human experiments of nature. For example, the *in natura* test of whether or not IL-12 and Th1 cells are truly important to defense against intracellular pathogens is to examine naturally IL-12 or Th1-deficient humans and evaluate if they are susceptible to infection. Indeed, such humans are described as having Mendelian susceptibility to mycobacterial disease. This line of thinking has been championed by Jean-Laurent Casanova and colleagues and has resulted in a number of confirmatory, as well as contradictory results to common immunology theory (*32-34*). For example, since MyD88 and IRAK4 are required for signalling by IL-1 family members (IL-1, IL-18, IL-33, and likely more) as well as all TLRs except TLR3 and partially TLR4, it was assumed that MyD88 or IRAK4 deficiency would be absolutely incompatible with life, but Casanova and others have shown that human MyD88 or IRAK4 deficiency mortality declines with age, such that survivors into their teenage years tend to live normal lives (*i.e.* without continued antibiotic prophylaxis), presumably due to compensatory adaptive immune mechanisms (*32*) such as those identified in the intestine (*210*). Notably, these advances have been almost exclusively in the realms of infectious and autoimmune disease. This then raises the question, "Are there humans with single gene defects that should (based off of a wealth of human/animal *in vitro* and animal *in vivo* research) result in susceptibility or protection from the development of allergy?"

Descriptions of human deficiency of DCs (*211, 212*), B cells or T cell number or function have not reported clear protection from allergy. Human deficiency of GATA3, a master transcription factor required for Th2 differentiation, has a defined clinical phenotype (hypoparathyroidism, sensorineural deafness, and renal dysplasia syndrome) without evidence of immune abnormality, including Th2 immunity (*213*). Likewise, human deficiency

of EPO appears to be without any immune consequence (*214-225*). However, early studies on OX40 deficiency show poor $CD4^+$ T cell number, memory and activation and a predisposition to Kaposi's sarcoma (*226*). Whether there are other, undiscovered, deficiencies in Th2 immunity genes, such as IL-4 or STAT6, are also not clear. Certainly, identification of genes that lead to immune aberrancy/deficiency is not impossible (*32*), but there are no clear genetic defects that lead to allergy or protection from it. The reason(s) for a lack of association between these deficiencies and protection from allergy are not clear. One potential explanation is that defects in Th2 immunity are clinically silent in developed societies. Indeed, a patient without allergy might be labelled as being, from an allergy perspective, 'healthy', even if their clinical phenotype is due to a lack of an entire arm of adaptive immunity. Thus, a true *in natura* test of the translatability of basic science findings of Th2 immunity to human biology may be extremely difficult to perform.

The evidence for human genetic defects that lead to a propensity to cause food allergy is equally not robust. Since oral tolerance and food allergy are thought to be opposing outcomes that take place after intestinal Ag encounter, defects in tolerogenic immune responses are thought to result in food allergy. However, although oral tolerance responses critically rely on IL-10 and FoxP3, human deficiency of either of these does not lead to a clinical phenotype with prominent food allergic features (*227, 228*). For example, FoxP3 deficiency leads to the syndrome termed immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX). The enteropathy is inflammatory and more similar to inflammatory bowel disease rather than allergic diarrhea to foods. IPEX is not associated with anaphylactic reactions to foods or drugs. Likewise, IPEX is variably associated with skin rash, which is sometimes atopic dermatitis. However, dermatitis itself can have a number of causes including allergy, autoimmunity, mechanical, dehydration, infection, or others and the dermatitis of IPEX is not necessarily rich with eosinophils, MCs, and lymphocytes. This

illustrates that defects in oral tolerance do not necessarily result in food allergy and anaphylaxis. However, this also illustrates the need to better understand immunology. Looking forward, it would be interesting to perform detailed genetic analyses (DNA, epigenetic and RNA) on patients with multiple comorbid allergic disease, or food allergy clustering within families, in an attempt to identify single gene deficiencies that promote Th2 immunity in allergic sensitization.

Outlook for Food Allergy Research

A myriad of questions and problems remain to be solved in order to aid in the prevention, management and cure of food allergy. From a basic science perspective, a better understanding of the immunological mechanisms underlying food allergy may lead to new concepts, paradigms, research tools, and strategies to, in general, understand biology and promote health over disease.

In terms of the main model for food allergy, the CT-driven model, two main questions exist: 1) how does CT activate cells intracellularly? and 2) how does CT-mediated food hypersensitivity function? I discuss the former in "How Does CT coupling to the ER Result in Th2 responses?" In regards to the latter, this can be subdivided into 3 sub-questions (that are still quite broad in scope): 1) How is Th2 immunity initiated? 2) How is Th2 immunity effected, *i.e.* How does anaphylaxis take place? and 3) How is Th2 immunity and IgE sustained?

For the first question, a detailed understanding of epithelial cell-DC-eosinophil interactions, as well as DC-T cell and T cell-B cell interactions is required. This Thesis provides a basis for further investigation for increased molecular detail as well as discovery of additional mechanisms (*e.g.* other costimulatory molecules). Much remains to be understood regarding how PN-specific Abs are generated due to T cell-B cell interactions,

especially the role, if any, of Tfh cells. Detailed *in vivo* characterization of T cell and B cell activation events *in vivo* along with mice deficient in key Tfh machinery (*e.g.* ICOS, SLAM) may be helpful. Likewise, precise localization of where exactly Th2 priming and B cell activation takes place, and where it is required to take place (*e.g.* Peyer's Patches, MLN, both or elsewhere), in PN allergy are relatively unexplored areas. Notably, unpublished data examining NOD1/2 KO mice, which are deficient in the number and maturation of isolated lymphoid follicles (*229*), appear to show that Th2 priming is unimpaired. Further, unpublished data show that hematopoietic MHC II is required for Th2 priming and that non-hematopoietic MHC II (*e.g.* epithelial derived) is insufficient to allow for Th2 priming. As mentioned above, gene/protein profiling of cells activated with PN+CT *in vitro* or tissues/cells from mice undergoing PN allergic sensitization may inform on new or unexpected pathways of Th2 induction. Comparative profiling of this with different model Th2 systems (*e.g.* alum/OVA, HDM, *Hp, Nb, S. mansoni*) will inform on the diversity of pathways to induce Th2 immunity and potentially shed light on the possibility of different types of Th2 immunity.

The main mediators of anaphylaxis have been identified to be MCs and phagocytes, IgE, IgG1 (in mice), platelet-activating factor, and to a lesser extent, histamine (*30, 51, 109*). However, much remains to be understood about the pathophysiology and cellular communication events that take place to cause PAF and histamine to drive anaphylactic distributive shock. PAF receptor KO chimeras, histamine receptor or histidine decarboxylase KOs, cytosolic phospholipase KOs and tools to quantify PAF might be helpful in this regard. Further, identification of mediators of anaphylaxis through lipidomics, metabolomics and potentially, proteomics might prove fruitful. Serotonin (5-hydroxytryptophan or 5-HT) is a commonly cited potential mediator of anaphylaxis but our unpublished data show that 5-HT is not required to drive allergic reactions to PN. Additionally, if it can be understood

precisely why epinephrine rescues patients undergoing anaphylaxis, then perhaps new rescue medications can be created for patients. It is notable that there is significant emphasis on IL-33 as a mediator of anaphylaxis due to publication in PNAS by Pushparaj and colleagues (*230*). However, this article was retracted, along with at least 6 others by the same first author and/or principal investigator (Alirio J. Melendez) on grounds of re-use of the same data/figures in multiple publications and potential data manipulation (*231-236*). Dishearteningly, this retracted IL-33 paper continues to be cited to support a role for IL-33 in anaphylaxis. We directly address this in **Chapter 2** and show that IL-33 is not required to mediate PN-induced anaphylaxis *per se*.

In regards to persistence of food allergy, a conservative estimate is that 80% of individuals will remain allergic to PN for their lifetime. Very little is understood about the persistence of humoral immunity, and even less about long-lived IgE responses. Although we have preliminary data that show that murine PN allergy begins to wane by approximately one year post-sensitization, and that a single re-challenge at 6 months post-sensitization can result in lethal responses at one year post-initial sensitization, a great deal requires to be understood regarding plasma cell biology as well as T and B cell memory in PN allergy. Particularly useful tools might include IgE reporter mice (*237, 238*), transgenic B cells (*e.g.* specific to henn egg lysozyme, JAX #002595, or a limited number of protein Ags), Blimp-1 reporter mice, and APRIL/BAFF reagents. Human data on transfer of allergy by body fluid or solid organ transplantation may be informative in this regard. Beyond this, even less is known about the immunological pathways that lead individuals to naturally outgrow their food allergies. Examination of individuals that are losing hypersensitivity to PN is likely not a straightforward or simple objective, but is certainly one in dire need of investigation. Indeed, understanding in this area may lead to the creation of a tolerogenic adjuvant that could promote tolerance to allergenic foods in infants or patients already allergic to foods.

Overarching these themes are means to generate different models of food allergy, including sensitization through the skin, or with the use of alternative adjuvants or transgenic mice. As well, the relationship of the immune system to the commensal microbes (microbiota) and its impact on susceptibility to food allergy is a highly speculated, but poorly investigated area to date. However, our unpublished data show that PN allergy and anaphylaxis can be induced in germ-free mice without the use of an adjuvant, and that eosinophils are much more abundant in the LP of germ-free mice compared to colonized mice. Ongoing research in the lab is investigating the potential connection between these two findings.

From a clinical point of view, the dominant themes are protocols on desensitizing patients to PN using oral immunotherapy (OIT; grading feeding of PN to PN allergic patients), and when to feed PN to infants in order to prevent allergy from developing (*e.g.* early *versus* late feeding). The main barriers to widespread use of OIT are that it is highly associated with adverse events, including risk of anaphylaxis, and that it is temporary and highly sensitive to perturbation such as menstruation, sleep/awakening, and exercise. We are currently conducting a randomized control trial to evaluate the efficacy, safety and immunological mechanisms of OIT with concomitant antihistamine use *versus* OIT alone *versus* placebo OIT. Whether inhibition of some of the mechanisms that drive allergic sensitization would benefit OIT by increasing the duration of desensitization or inducing durable clinical tolerance to PN remains to be tested. A randomized control trial by Lack and colleagues aims to evaluate whether early introduction of PN to an infant's diet will protect from the development of PN allergy.

Concluding Statements

This Thesis advances PN allergic sensitization from a concept of APC activation of Th cells in an environment rich in IL-4, to epithelial secretion of IL-33 and eosinophil degranulation of EPO result in $CD103^+$ DC activation and CCR7-dependent migration to the MLN where OX40L activates naïve Th cells to produce IL-4 in an autocrine/paracrine fashion such that Th2 differentiation, B cell IgE/IgG1 class switch, Ab production and MC sensitization take place, collectively resulting in allergen hypersensitivity and upon re-exposure, anaphylaxis and partially ILC-mediated eosinophilic inflammation take place. Certainly, a great deal of more information is needed to better define and expand upon these findings in the basic science and clinical realms; I discuss some of these objectives and implications here. Translation of basic science concepts and mechanisms of Th2 immunity in PN allergic sensitization to clinical practice may benefit patients with immune-mediated diseases, such as PN allergy, for which the only medical treatment is avoidance.

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