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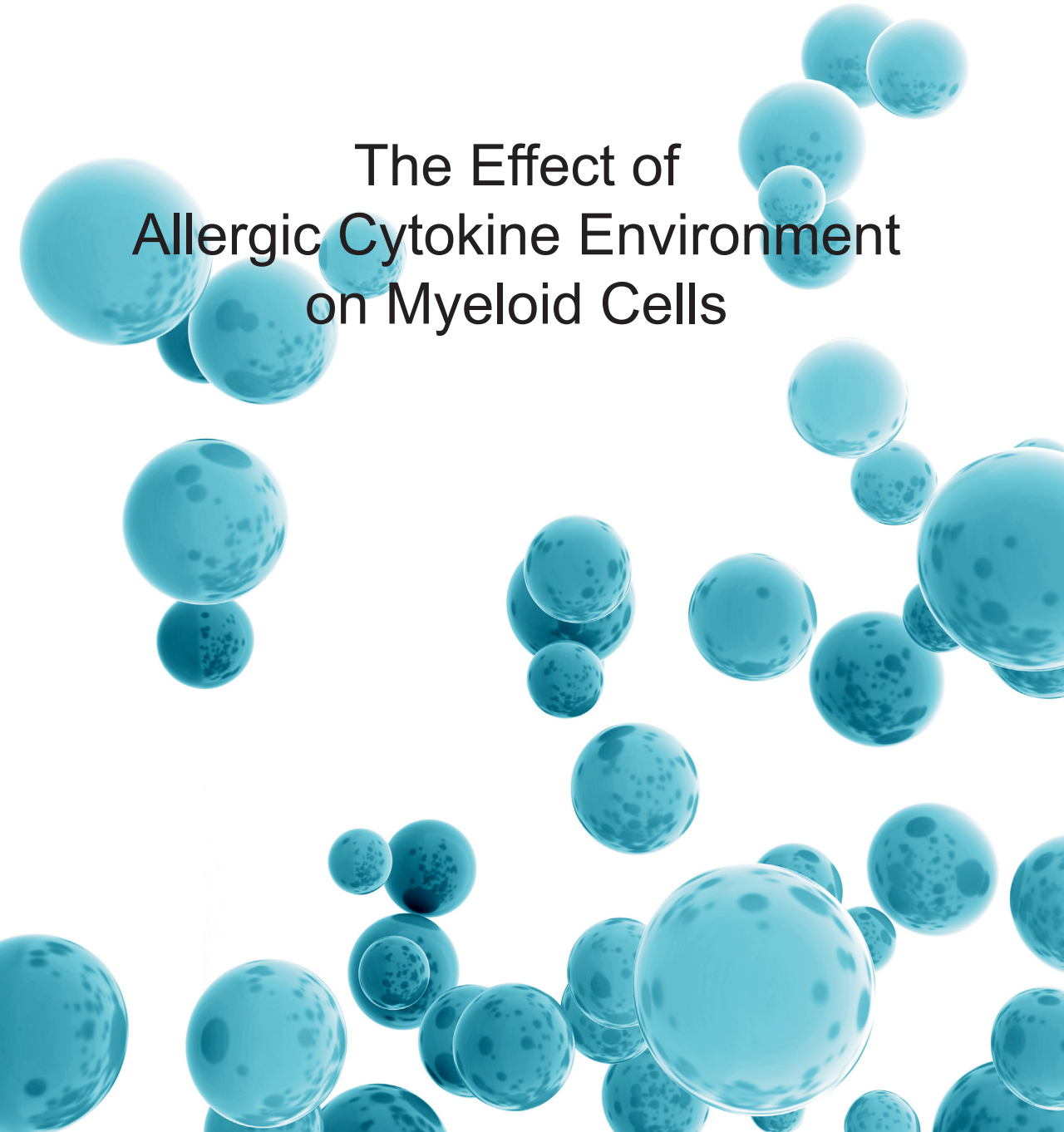
The Effect of Allergic Cytokine Environment on Myeloid Cells



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ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

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I Junttila IS*, Watson C*, **Kummola L**, Chen X, Hu-Li J, Guo L, Yagi R, Paul WE. Efficient cytokine-induced IL-13 production by mast cells requires both IL-33 and IL-3. *J Allergy Clin Immunol.* 2013; 132(3):704-712.e10. doi: 10.1016/j.jaci.2013.03.033.

II **Kummola L***, Ortutay Z*, Chen X, Caucheteux S, Hämäläinen S, Aittomäki S, Yagi R, Zhu J, Pesu M, Paul WE*, Junttila IS*. Interleukin(IL)-7Rα expression regulates murine dendritic cell sensitivity to Thymic Stromal Lymphopoietin. *J Immunol.* 2017; 198(10):3909-3918. doi: 10.4049/jimmunol.1600753.

III **Kummola L**, Ortutay Z, Vähätupa M, Prince S, Uusitalo-Järvinen H, Järvinen TAH, Junttila IS. R-RAS deficiency does not affect papain-induced IgE production in mice. *Immun Inflamm Dis*. 2017; 5(3):280-288. doi: 10.1002/iid3.168.

*) Equal contribution

ABBREVIATIONS

γc	Common gamma chain
AD	Atopic dermatitis
APC	Antigen presenting cell
AREG	Amphiregulin
ATP	Adenosine triphosphate
BMMC	Bone marrow-derived mast cell
bp	Base pair
CD	Cluster of differentiation
cDC	Conventional/classical dendritic cell
cDNA	Complementary DNA
DAMP	Danger(/damage)-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DsRed	Discosoma sp. red fuorescent protein
EAE	Experimental autoimmune encephalomyelitis
EC	Epithelial cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FcR	Fc receptor
FoxP	Forkhead box P
galK	Galactokinase
GDP	Guanosine diphosphate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GTP	Guanosine triphosphate
HDM	House dust mite
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1 protein
HPC	Hematopoietic progenitor cell
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
ILC	Innate lymphoid cell
IL-R	Interleukin receptor
IRS	Insulin receptor substrate
JAK	Janus kinase
kbp	Kilobase pair
kDa	Kilodalton
KO	Knock-out
LC	Langerhans cell
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene 88
NLRP3	NACHT, LRR and PYD domains-containing protein 3
PAMP	Pathogen-associated molecular pattern
PAR	Protease activated receptor
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
pSTAT	Phospho-signal transducer and activator of transcription
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROR	RAR-related orphan receptor
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription qPCR
SCF	Stem cell factor
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TG	Transgenic
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell

TSLP	Thymic stromal lymphopoietin
TSLPR	Thymic stromal lymphopoietin receptor
WT	Wild type

ABSTRACT

The immune system faces constant attacks from the outside and within its boundaries: bacteria, viruses, parasites, venoms, allergens and cancerous cells of the self all pose a serious threat to the host organism. In order to defend the host, the immune system must successfully control an incredibly complex network of different cells that work in cooperation and equilibrium. In the heart of this immune cell network is signaling by small messenger molecules called cytokines. If the control of immune functions fails, the immune responses may become directed towards innocuous antigens or the cells and tissues of the host itself and the end result is allergy or autoimmunity.

In this study, we aimed to elucidate the mechanisms how the immune system controls allergic responses. We have focused on cytokine signaling and myeloid cells, especially mast cells and dendritic cells. In the first part of the study, we utilized peritoneal mast cells and a transgenic DsRed reporter mouse to demonstrate that mouse mast cells require a two-step signal in order to produce efficiently interleukin (IL)-13. IL-13 is a key cytokine in allergic inflammation, mediating many of the physiological changes associated with allergy. We discovered that these two signals are provided by IL-3 and IL-33. In the second part, we studied how thymic stromal lymphopoietin (TSLP) signals in dendritic cells. TSLP is produced mainly by epithelial cells (ECs) in response to danger signals, such as allergens, and it is thought to drive allergy-associated type 2 responses through dendritic cells. We found that IL-4 induces the upregulation of IL-7 receptor α (IL-7Rα), a subunit of the TSLP receptor complex that is needed for TSLP signaling, both *in vitro* and *in vivo*. Lastly, we investigated in a knockout mouse model how the deficiency of R-Ras might affect allergic immune responses caused by papain. R-Ras is a small GTPase that has recently been associated with the formation of the immunological synapse between dendritic cells and T cells. In our experiments, R-Ras knockout mice were able to mount immunoglobulin (Ig)E responses comparable to those of wild type mice and R-Ras was dispensable in this context. To our knowledge, this was the first study to explore the role of R-Ras in allergy.

Collectively, our results offer a few more pieces to the puzzle of allergic inflammation. While we could not find a place for R-Ras in the induction of allergic

inflammation, we revealed important details of the induction of IL-13 by mast cells. Our findings also introduce a new mechanism for the regulation of TSLP signaling. As the prevalence of allergic diseases has risen dramatically in western and westernized countries during the last few decades - reaching a point where these disorders have become a substantial burden on the individual and societal level – all effort is needed in investigating the underlying molecular mechanisms.

TIIVISTELMÄ

Immuunipuolustus kohtaa jatkuvasti hyökkäyksiä ulkopuoleltaan ja rajojensa sisäpuolelta: bakteerit, virukset, loiset, eläinperäiset myrkyt, allergeenit ja kudoksissa syntyvät syöpäsolut ovat vakava uhka eliöille. Puolustaakseen elimistöä, immuunipuolustuksen on kontrolloitava onnistuneesti monimutkaista verkostoa, joka koostuu erilaisista soluista jotka toimivat yhteistyössä ja tasapainossa. Tässä immuunipuolustuksen verkoston keskiössä on solujen välinen signalointi pienillä, sytokiineiksi kutsutuilla molekyyleillä. Jos immuunipuolustuksen mekanismien ja solujen kontrollointi epäonnistuu, immuunivasteet saattavat suuntautua kohti vaarattomia antigeenejä tai kehon omia kudoksia ja lopputuloksena voi syntyä allergia tai autoimmuunitauti.

Tässä väitöskirjassa selvitettiin mekanismeja, joilla immuunijärjestelmä kontrolloi allergisia vasteita. Olemme keskittyneet sytokiinisignalointiin ja myeloisiin soluin, erityisesti syöttösoluihin ja dendriittisoluihin. Väitöskirjan ensimmäisessä osatyössä hyödynsimme hiiren vatsaontelon syöttösoluja sekä transgeenistä DsRed-reportterihiirtä näyttääksemme, että hiiren syöttösolut tarvitsevat kaksivaiheisen sytokiinisignaalin tuottaakseen tehokkaasti interleukiini (IL)-13:a. IL-13 on avainsytokiini allergisessa tulehduksessa, ja sillä on tärkeä merkitys allergiaan liittyvien fysiologisten muutosten aikaansaamisessa. Nämä kaksi signaalia tulevat syöttösolulle IL-3:lta ja IL-33:lta. Toisessa osatyössä selvitimme, miten TSLP (thymic stromal lymphopoietin) signaloi dendriittisoluissa. TSLP:tä tuotetaan erityisesti epiteelisoluissa vasteena vaarasignaaleihin, kuten allergeeneihin, ja sen on ajateltu ajavan allergiaan liittyviä, tyypin 2 auttaja-T-soluvasteita dendriittisolujen kautta. Osoitimme, että IL-4 indusoi IL-7-reseptori α:n (IL-7Rα) ylössäätelyä dendriittisoluissa sekä *in vitro* että *in vivo*. Tämä reseptori on osa TSLP-reseptorikompleksia, joka on välttämätön TSLP-signaloinnille. Viimeisessä osatyössä käytimme poistogeenistä hiirimallia tutkiaksemme, miten R-Rasin poistaminen vaikuttaisi allergisiin immuunivasteisiin papaiini-immunisaation aikana. R-Ras on pieni GTPaasi, joka on hiljattain yhdistetty immunologisen synapsin muodostumiseen dendriittisolujen ja T-solujen välille. Kokeissamme R-Rasin suhteen poistogeeniset hiiret kykenivät tuottamaan immunoglobuliini (Ig)-E-vasteita normaaleihin hiiriin verrattavalla tavalla, joten tässä kontekstissa R-Ras ei ollut

vasteelle välttämätön. Tietääksemme tämä on ensimmäinen tutkimus, jossa selvitettiin R-Rasin roolia allergiassa.

Yhteenvetona, tuloksemme asettavat oikeille paikoille muutaman palan lisää allergisen tulehduksen palapelissä. Vaikka emme löytäneet osaa R-Rasille allergisen tulehduksen aikaansaamisessa, paljastimme tärkeitä yksityiskohtia syöttösolujen IL-13-tuoton säätelystä. Tuloksemme esittelevät myös uuden mekanismin TSLP-signaloinnin säätelyyn. Koska allergiset sairaudet ovat yleistyneet dramaattisesti viime vuosikymmeninä länsimaissa ja länsimaistuneissa maissa – siinä määrin, että niistä on tullut merkittävät taakka sekä yksilö- että yhteiskuntatasolla – on ehdottoman tärkeää pyrkiä huolellisesti selvittämään allergian taustalla olevat molekulaariset mekanismit.

CONTENTS

1 INTRODUCTION

The human immune system has evolved to continuously encounter and process antigens from the environment and the cells and tissues of body itself, eliminating viruses, bacteria, parasites and cancer cells efficiently without evoking responses against innocuous antigens or self-antigens. All this is accomplished with an extremely fine-tuned and organized co-operation between the innate and adaptive immunity. Evolutionally, the innate immunity developed earlier and is shared by all living creatures, characterized by a limited capacity to recognize certain molecular patterns of pathogens, allergens and self-antigens. The adaptive immunity evolved in vertebrates to further enhance the efficiency of immune defense, now introducing a specific response to any possible antigen, accompanied by immunological memory(1,2).

Unfortunately, the same defense mechanisms, crucial to the survival of the individual, many times give rise to different disorders. One of them is allergy, defined as a hypersensitivity reaction of the immune system to something that would otherwise be a harmless environmental substance. The prevalence of allergy has increased for the last 50 years with the Western lifestyle and now roughly 25% of the people in the developed countries suffer from disorders such as asthma, atopic dermatitis, allergic rhinitis and anaphylaxis(3,4).

The culprit behind allergic inflammation is the part of the immune system that evolved to fight extracellular parasitic infections. Especially in countries where poor health standards exist, helminth infections still pose a major threat along with snake, tick and insect bites that provoke the same immune responses(5). It has been postulated, that the lack of microbial and parasitic stimuli is the driving force behind the rise of allergic diseases(6).

Allergic responses have been named type 2 responses, according to the type 2 helper T cells (Th2 cells) that are involved in them. The type 2 pathway is initiated when the immune system encounters an antigen which is taken up by a cell of the innate immune system: an antigen presenting cell (APC), such as a dendritic cell. The APC then presents the antigen on its MHCII molecule to the adaptive immune system: the encounter directs a naïve T cell towards a Th2 phenotype, a key feature of which is the secretion of cytokines such as interleukin-4 (IL-4), IL-5, IL-9 and IL-

13. The cytokines promote mucus secretion, smooth muscle contraction and vascular permeability, help B cells produce IgE antibodies specific to the antigen and recruit mast cells and mediate the maturation of eosinophils and basophils. The binding of antigen and IgE-complex to mast cells and basophils leads to the release of inflammatory mediators such as serotonin, heparin and histamine that are responsible for the initial allergic symptoms, such as itching, urticaria and oedema. Cytokines and chemokines encourage the further migration of Th2 cells and other leukocytes to the site of allergen exposure. The end result is called allergic inflammation(4,7).

The exact mechanisms of how type 2 responses are initiated, maintained and suppressed are not yet fully elucidated and further research is still needed. It is clear that the cytokine milieu is a key player in shaping immune responses and that cytokines from non-lymphoid sources are important for the differentiation and control of immune cells(8). In this study, we aim to further shed light on the factors that determine how immune cells function during allergic inflammation. Special emphasis is placed on dendritic cells and mast cells as well as cytokine signaling. Additionally, we explore the possible role of R-Ras, a small GTPase, in IgE responses. Given the huge negative impact allergic disorders have on modern societies, it is essential to strive for better understanding of the underlying mechanisms.

2 REVIEW OF THE LITERATURE

2.1 The immune system

The human defense system against invading pathogens, allergens, irritants and malignant cells can be divided into three parts or levels: the first defense line comprises of **anatomical and physiological barriers**, such as intact skin and mucosal surfaces, normal microflora, surface-deposited antimicrobial lipids and peptides, the cilia of airways and low pH in the stomach(9,10). If these barriers are breached by pathogens, the **innate immune system** is the next defender in line, rapidly mounting an inflammatory response with phagocytic and cytotoxic cells and soluble proteins of the complement(11). Innate immunity is a branch of the immune system that is shared by all living organisms. It relies on a limited repertoire of genetically coded pattern recognition receptors (PRRs) that recognize common and evolutionally conserved microbial structures and molecules that are upregulated or released during cell lysis as a consequence of inflammation or infection. These molecules are called pathogen-associated molecular patterns (PAMPs) and danger(/damage)-associated molecular patterns (DAMPs), respectively. Innate immunity employs cells of the hematopoietic lineage: myeloid cells (dendritic cells, mast cells, macrophages, monocytes, granulocytes) and innate lymphocytes (innate lymphoid cells, natural killer cells natural killer T cells and natural killer-like B cells). Some non-hematopoietic cells can also be considered belonging to innate immunity, namely the epithelial cells in the skin and respiratory, genitourinary and gastrointestinal tracts that are capable of responding to microbial and environmental insults and producing molecules that regulate the functions of innate effector cells(2,10,12).

Finally, the innate immune system activates the last line of immune defense, found exclusively in vertebrates: the **adaptive immunity**, the fundamental feature of which is antigen recognition that is generated *de novo* specifically for each antigen by B and T lymphocytes. Whereas the recognition molecules of the innate cells are germline-encoded, the antigen receptors (T cell receptor, TCR, and B cells receptor) of adaptive cells are a result of somatic rearrangements of a few hundred gene elements. This enables the formation of an enormous number of unique antigen

receptors(13). Activation of the receptor by its specific antigen leads to the proliferation of effector cell populations that direct immune responses through cytotoxicity (cytotoxic T cells), immunoglobulin (Ig) production (B cells) or cytokine production (helper T cells, Th). Of these cells, different subclasses of Th cells are in the orchestrating role of shaping the proper effector mechanisms for different types of pathogens. Their cytokine production and direct cell interactions affect both innate and adaptive immune cells(14). This reflects the close cooperation network between innate and adaptive immunity: adaptive cells are activated by innate cells, and in turn, cells of the adaptive immunity recruit more innate effector cells to the target site. Thus, close cooperation of both systems is essential for a complete and effective immune response(2,11). Overview of the immune system is summarized in Figure 1.

Although immune mechanisms display vast variety and extreme complexity, a simple and fundamental principle underlies many of them: cells that sense invaders and insults, produce cytokines that target lymphocytes, promoting them to produce another set of cytokines that further activate immune effector functions(15). The challenge posed by pathogens is not the only one the immune system faces, as it is crucial that the system is able to discriminate between self and non-self and choose correctly between tolerance and aggressive responses. The balanced operation of immune functions requires equilibrium between inflammatory and anti-inflammatory responses. Autoimmunity and allergy are the consequences of failed equilibrium(16).

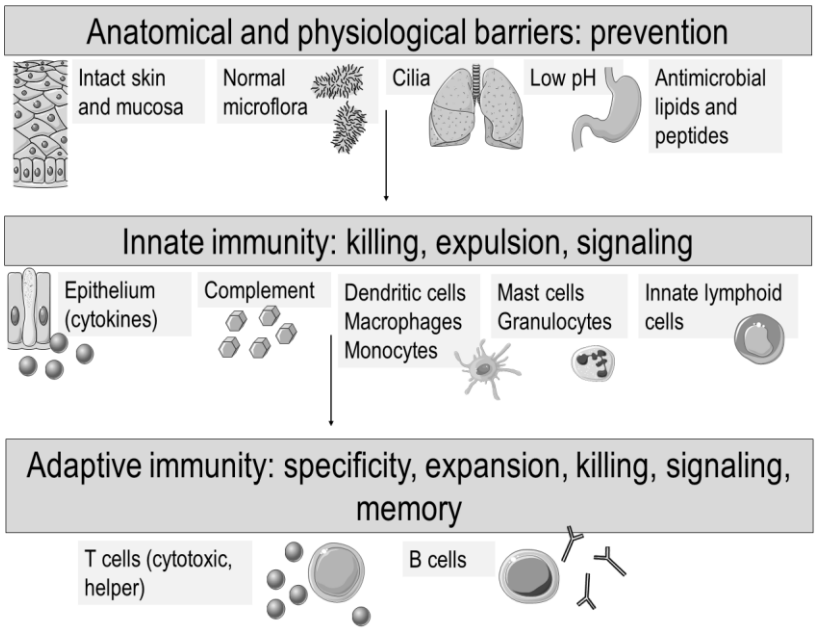

Figure 1. Overview of the immune system. First, anatomical and physical barriers block the invasion of the invading pathogen. If the barriers are breached, the innate immunity recognizes the pathogen via pattern recognition receptors. Phagocytic and cytotoxic cells destroy the pathogen or mediate its expulsion. The cells also secrete cytokines and chemokines to alert other immune cells. Finally, the adaptive immunity rises to encounter the invader, utilizing its high specificity against antigens. Cytotoxic T cells and B cells that secrete immunoglobulins act as effector cells while helper T cells secrete cytokines(10). Graphics by(17).

2.1.1 Cytokines as signaling molecules in the immune system

Cytokines are a broad category of small (generally under 50 kDa) proteins that target and affect the behavior of other cells (in a paracrine or endocrine manner) or even the cytokine-producing cell itself. They resemble hormones but whereas hormones are usually produced by certain tissues or cells, cytokines are produced by a broader range of cells. Cytokines are also more potent than hormones, provoking responses even at picomolar concentrations. Pleiotropism and, in many cases, redundancy are hallmark features of cytokines and they are widely involved in biological processes ranging from inflammation, infection and cancer to reproduction and ageing. (18,19).

Best known for their immunomodulatory functions, cytokines are produced by all the cells of the immune system and by epithelial cells, keratinocytes, fibroblasts

and certain stromal cells. Most cytokines are secreted as soluble proteins, but membrane-bound forms are also known(18,20,21). Cytokine classification is typically based in relation to their structure, receptor, function or a mixture of these(18,22). Classification is not always straightforward as many of the charachteristics and functions of cytokines are overlapping. Table 1 presents an example division of cytokines into different classes. Some cytokines belong to more than one class: for example, interleukin (IL)-4 is a common γ-chain cytokine as well as a type 2 cytokine. Likewise, IL-33 belongs to IL-1 family while also being a type 2 cytokine. If interleukins, interferons (IFN), tumor necrosis factors (TNF) and all chemokines are included, the number of different cytokines known to date is around one hundred.

Table 1. Classification of cytokines(18).

Cytokine family	Examples
IL-1 family	IL-1α, IL-1β, IL-18, IL-33
Common γ-chain family	IL-2, IL-4, IL-7, IL-9
IL-10 family	IL-10, IL-19, IL-22, IFNλ
IL-12 family	IL-12p40/p70, IL-23, IL-35
Cytokines of type 2 responses	IL-4, IL-5, IL-13, IL-25, IL-33, TSLP
Chemokines	IL-8, CC chemokines, CXC chemokines
IL-17 family	IL-17A, IL-17F, IL-25
Interferon family	IFNα, IFNβ, IFNγ, IFNλ
Other	IL-3, IL-6, TGF-β, TNF, GM-CSF

The IL-1 family has been named after the cytokine IL-1, which includes two proteins, IL-1α and IL-1β. Most of the IL-1 family members are pro-inflammatory, linked to type 1 responses during infections and autoimmune conditions. However, some are crucial in inhibiting inflammation(23). IL-33 has a role especially in allergic type 2 inflammation(24).

The common γ-chain cytokines all share the common γ-chain receptor subunit. For signaling they require an additional receptor chain. Many of them are involved in lineage-specific cell differentiation, cell growth and proliferation(25).

Cytokines of the **IL-10 family** have several different functions but they signal through receptors with similar sequence. Some of the cytokines, such as the IL-10 cytokine itself, act as a suppressor of inflammatory responses. Others mediate

protection against viruses, extracellular pathogens and enhance wound healing and remodeling of tissues(26).

The IL-12 family cytokines are heterodimers with diverse pro-inflammatory and anti-inflammatory functions. For example, they direct T helper 1 and 17 development(27).

Cytokines related to type 2 immune responses have a role in defense against large extracellular parasites, such as helminths. These cytokines drive the differentiation of T helper 2 cells(28). Type 2 cytokines are be discussed in detail below.

Chemokines, the word derived from chemotactic cytokines, are signal molecules that guide the migration and positioning of immune cells. Cell movement along a chemokine gradient, chemotaxis, is required for immune functions ranging from differentiation and homeostasis to mediating acute inflammation(29).

Cytokines that belong to the **IL-17 family** are named IL-17A-F and they are produced by both immune and non-immune cells. The most studied cytokine of the family, IL-17A, is secreted by T helper 17 cells. It mediates antimicrobial defense and is implicated in different inflammatory diseases including autoimmunity and cancer. IL-25 (IL-17E) is also a type 2 cytokine, produced by epithelial cells(30).

Interferons are related to type 1 responses against microbes. Interferon α and β are especially involved in antiviral responses whereas interferon γ is the dominating cytokine in shaping T helper 1 and cytotoxic T cell activities as well as activating macrophages(18)..

Other cytokines in this division encompass a variety of cytokines. For example, tumor growth factor (TGF)-β that is produced by regulatory T cells as an immunosuppressive cytokine has also various other functions in cell differention, proliferation and apoptosis(31). Tumor necrosis factor (TNF) is another pleiotropic cytokine that both initiates a strong inflammatory response and limits its duration. It also inhibits autoimmunity and tumorigenesis. Due to its many functions, it is one of the most important and diverse cytokines(22).

In this study, the focus will be on T helper 2 mediated immunity and the allergic inflammatory responses that arise from misguided Th2 immunity. In the following sections, the complex cellular network of adaptive and innate immunity in this context will be discussed, as well as the crucial cytokine signaling that drives Th2 cell differentiation, IgE antibody production and activation of innate cells in the establishment of allergic inflammation.

2.1.2 Overview of Th responses

Helper T cells are in the heart of adaptive immunity as drivers of effector functions of other immune cells. Their activation requires that an antigen presenting cell (APC) processes a peptide and displays it on its surface, loaded onto a major histocompatibility complex (MHC) class II molecule, and presents it to the TCR of a Th cell(32). Following antigen recognition, Th cells change their phenotype according to the encountered pathogen and signals from innate cells: **Th1 cells** are induced in response to viral, bacterial and protozoan infections upon stimulation with IL-12, IFNγ and IL-18. In response, they produce IFNγ and TNF, lymphotoxin α and IL-2. The master regulator of transcription in Th1 responses is T-bet(14,33,34). Th1 cells activate cytotoxic $CD8^+$ T cells and IgG2a producing B cells. Uncontrolled Th1 responses underlie many autoimmune conditions(14,34). **Th17 cells** are involved in fighting fungal and bacterial infections, producing IL-17A and F, IL-22, GM-CSF (granulocyte macrophage colony-stimulating factor) and TNF(35). Important cytokines for their differentiation include IL-6, tumor growth factor (TGF)-β, IL-21 and IL-1 and the transcription factor RORγ is critical in Th17 responses(36). As Th1 cells, Th17 cells are also involved in autoimmune diseases(34). Regulatory helper T cells (Tregs) control immune responses to self-antigens, preventing autoimmunity, and they suppress effector T cell responses during inflammation to limit tissue damage and immunopathology. They differentiate in the presence of IL-2 and TGF-β and their hallmark feature is the production of IL-10 and TGF-β. The master transcription factor of Tregs is FoxP3(37,38). Additionally, there are other Th subtypes: **Th9, Th22** and **follicular helper T cells** (Tfh), that produce IL-9, IL-22 and IL-21, respectively(14). Th9 cells differentiate in response to TGF-β and IL-4 and contribute to allergy and asthma (39). Tfh cells help B cells in the germinal center and secrete IL-4 that promotes IgE production during allergy and helminth infections (40).

Th2 responses, or type 2 responses, evolved to counter the threat of multicellular parasites (helminths) and tick, insect and snake bites(5,41). Although it is possible that an oxidative immunological attack, often involved in defense against microbes, could kill multicellular organisms, this would likely result in substantial collateral tissue damage as parasites cannot be phagocytosed into a single cell. Thus, Th2 cells evolved to control parasitic infections: this would involve repairing the damage the parasite had caused by harnessing the wound healing responses of innate immunity and either tolerating the invader, isolating it through granuloma formation or expelling it by gut motility, mucus production, scratching and coughing(42,43).

The prototypical type 2 response is characterized by the induction of CD4+ helper T cells, which mediate their functions by secreting cytokines IL-4, IL-5, IL-9, IL-13 and IL-25. With IL-4, Th2 cells promote the production of antigen-specific IgG1, IgG4 and IgE from B cells. The crosslinking of high-affinity IgE receptors on the surface of mast cells and basophils leads to their degranulation and release of cytokines and inflammatory mediators that induce smooth muscle constriction, vascular permeability and the recruitment and expansion of eosinophils and alternatively activated macrophages. Differentiated Th2 cells migrate into lungs or intestinal tissues and recruit eosinophils and mast cells through IL-5 and IL-9, respectively. Th2 cytokines also act directly on epithelial cells (IL-4, IL-9 and IL-13) and smooth muscle cells (IL-4 and IL-13), inducing mucus production, goblet cell hyperplasia and airway hyperresponsiveness. Macrophages, other T cells and innate lymphoid cells are also affected by the cytokines that Th2 cells produce(44-47).

Initiating and orchestrating type 2 responses is notably less understood than the mechanisms that control Th1 and Th17 responses. Likewise, the reason why type 2 responses are directed against allergens remains elusive. Although indispensable to immunity against extracellular parasites, type 2 responses also give rise to allergic disorders, such as asthma and atopic dermatitis(45,48). The helper T cell overview is summarized in Figure 2.

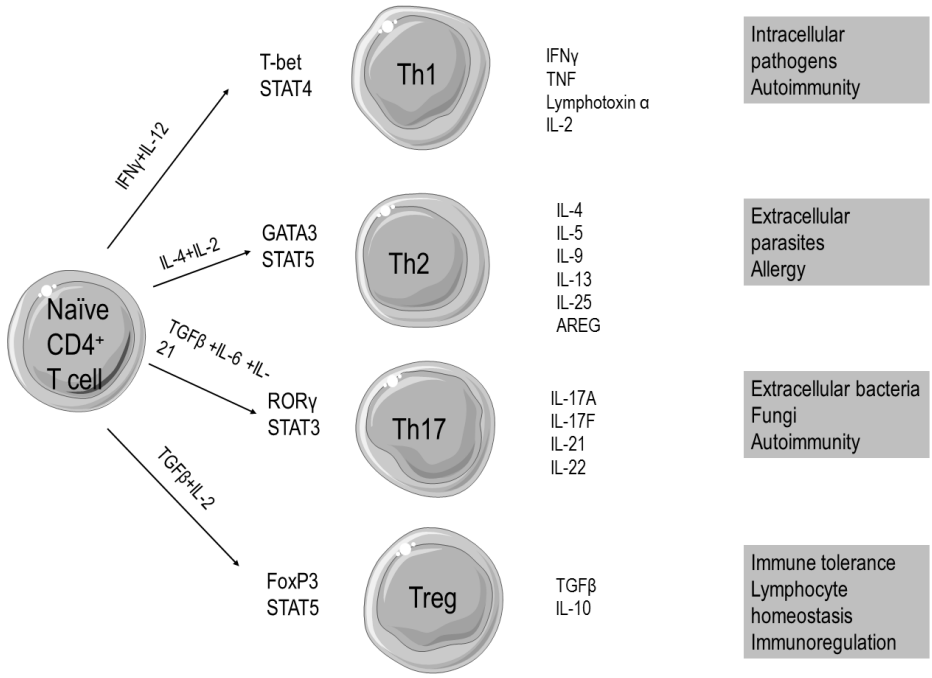

Figure 2. Overview of the most well known helper T cell subsets and function (34,49). Graphics by(17).

2.1.3 Th2 cell differentiation

In type 2 responses, IL-4 is considered the hallmark cytokine that is both produced by Th2 cells and needed for their differentiation *in vitro*(50). IL-4 upregulates its own receptor (IL-4Rα) during Th2 differentiation(51). In naïve T cells, IL-4 combined with TCR stimulation activates signal transducer and activator of transcription (STAT)6, which upregulates the Th2 master transcription factor, GATA-binding protein 3 (GATA3). GATA3 induces direct transcription activation and epigenetic changes in Th2-specific gene loci(52,53). GATA3 selectively stimulates Th2 cell growth, suppressing Th1 differentiation(54). STAT6 is necessary and sufficient for GATA3 *in vitro,* but *in vivo* it is possible to induce Th2 differentiation independently of STAT6 and IL-4, although still GATA3-dependently(55-58).

GATA3 expression can also be induced by TCR signaling(59). The strength of a TCR signal regulates Th1 and Th2 differentiation *in vitro*: strong signal leads to Th1

differentiation, while weak signal by cognate antigen preferentially stimulates Th2 differentiation. Weak stimulation of TCR activates GATA3 independently of IL-4 and stimulates the production of "early IL-4" from the T cell itself. This early IL-4 is dependent on TCR-induced IL-2 production, which activates STAT5. STAT5 combined with GATA3 results in early IL-4 secretion. Co-stimulation via CD28 is necessary for the production of IL-2.(59-61). Activation of STAT5 by IL-2 is indispensable for the production of Th2 cytokines. This might be due to maintaining GATA3 expression in differentiated cells or remodeling of chromatin in the Th2 locus(50,62).

However, it is noteworthy that Th2 differentiation *in vivo* is not nearly as well-known as it is *in vitro*. Th2 differentiation can still occur in mice with deletion in IL-4, IL-4Rα and STAT6(58). Parasitic infections can induce Th2 differentiation with and without IL-4: for example, response to *Trichurus muris* is dependent on IL-4(63), whereas *Nippostrongylus brasiliensis* is able to induce Th2 differentiation without IL-4, although IgE production is abolished in this setting(64). The fact that these responses are still GATA3-mediated and that Th2 differentiation can happen even with low concentrations of GATA3(65) and that a constitutively active STAT5 signal can drive Th2 differentiation even in Th1 conditions(66), underlines the crucial importance of STAT5 signaling.

In addition to IL-2, STAT5 is induced by thymic stromal lymphopoietin (TSLP) and IL-7. TSLP is produced by epithelial cells, dendritic cells and basophils and it may initiate type 2 responses *in vivo*(67-69), possibly by acting directly on T cells(70,71). The role of IL-7 *in vivo* is not so clear, although it seems to be able to cooperate with IL-33 in the induction of IL-13 from mouse Th2 cells(72).

In vivo, the source of the initial IL-4 is still elusive and basophils have been proposed as the producers. Basophils secrete IL-4 after crosslinking of FcεRI or activation through other receptors(57,73) and they enter lymph nodes after immunization with papain, before the appearance of Th2 cells(67,74). In this papain-induced allergy model, basophils have been shown to be important for type 2 responses and IgE production(75). Basophils can even act as APCs under some circumstances and depleting them impaired Th2 responses in a *T. muris* infection model(76). However, their role as APCs is controversial in both humans(77,78) and mice(79,80). Recently, group 2 innate lymphoid cells (ILC2s) have been an interesting candidate as early IL-4 producers in *Heligmosomoides polygyrus* infected mice, in which specific deletion of IL-4 from ILC2s *in vivo* resulted in impaired Th2 differentiation(81).

Similarly, the importance of the strength of the TCR activation *in vivo* is not as strongly established as it is *in vitro*. It is known that mutations that negatively impact the strength of TCR signaling are associated with a tendency to type 2 responses(82,83). Yet, helminth infection would not seem to produce a low antigen load and weak signals. Interestingly, Omega-1 (an RNase from *Schistosoma mansoni*) has been found to inhibit activation of dendritic cells (DCs), weaken the strength of TCR signals, inhibit Th1 differentiation and induce Th2 cells *in vivo* independently of IL-4(84-86). If such a mechanism exists in other parasitic infections, it could explain the perceived controversy. Others argue that intracellular pathogens would offer a more efficient source of antigens for uptake, as antigens from large parasites would mainly have to be retrieved from the particles shed from the parasites themselves. Lastly, type 2 inflammation typically takes place at sites populated by basophils and ILC2s and as they express MHCII weakly, this might result in low-dose priming of Th2 cells and subsequent local type 2 responses(28).

2.1.4 IL-4 vs. IL-13

As discussed above, IL-4 plays a pivotal role in Th2 cell differentiation and cytokine production as well as IgE production from B cells. IL-13 is a similar cytokine that was originally thought to play a redundant role with IL-4. However, IL-13 is especially involved in mucus secretion, airway hyperresponsiveness and subepithelial fibrosis during allergic inflammation and thus, the two cytokines have distinct functions(87,88). IL-4 and IL-13 share receptor chains: IL-4 signals through a heterodimer consisting of IL-4Rα and γc subunits (type I receptor) or IL-4Rα and IL-13Rα1 (type II receptor). IL-13 signals through this type II receptor, and additionally, it binds to a second receptor chain, IL-13Rα2(87,89,90). Signaling through type I and type II receptors results in STAT6 signaling, but the insulin receptor substrate (IRS)-2 pathway is activated only after type I receptor activation(91). The IL-13Rα2 chain has previously been thought to act as a decoy receptor, blocking IL-13 effector functions(92), but signaling through it might result in the production of TGF-β(93). IL-4 and IL-13 signaling is summarized in Figure 3.

The expression of the receptor chains on different cell types partially explains the different functions of IL-4 and IL-13. In T cells of mice and humans and B cells of mice, type I receptor, but not type II receptor, is present. Type II receptor is expressed mainly on non-hematopoietic cells and myeloid cells express both(94).

This is likely mirrored in the important role of IL-4, rather than IL-13, in the initiation of allergic responses.

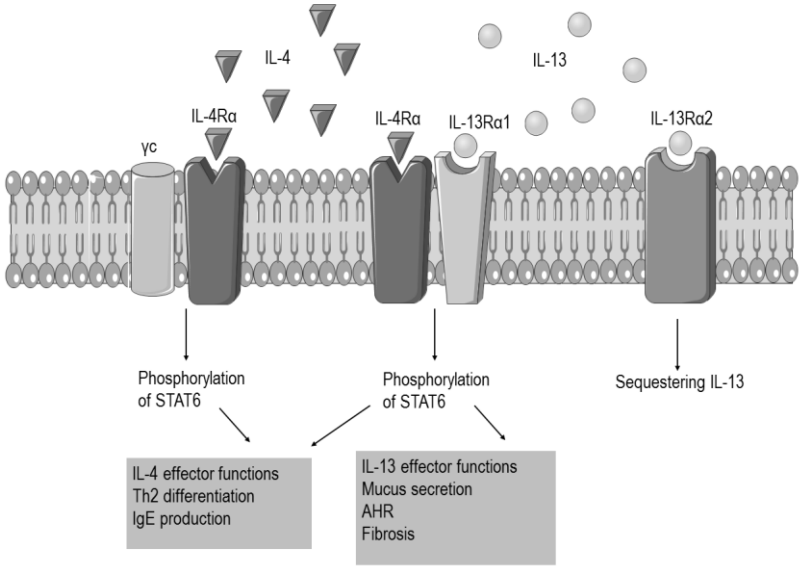

Figure 3. IL-4 and IL-13 receptor assembly and signaling (88,95). Graphics by(17).

Studying the distinct functions of IL-4 and IL-13 is challenging due to the utilization of the same type II receptor. Experiments with IL-13Rα1-deficient mice showed the absence of AHR, mucus production and fibrosis after challenge with antigen, IL-4 and IL-13. However, eosinophilic infiltration was independent of the type II receptor and the induction of alternatively activated macrophages needed both receptor types (96-98).

There are also several other possible explanations for the different functions of IL-4 and IL-13. One puzzling feature of the cytokines has been the difference in their quantities: IL-13 is often elevated in inflamed tissues when compared to IL-4. Recently it has been shown that their expression varies depending on the cell type in the inflamed setting. It would seem that IL-4 is expressed mainly by basophils and Tfh cells in the lymph nodes during airway inflammation, while tissue Th2 cells express both cytokines(99) and IL-13 is mainly produced by ILC2s(100). As ILC2s

are thought to secrete large quantities of IL-13 rather than IL-4 after stimulation from IL-25 and IL-33, it would explain the quantity and timing of IL-13.

The difference in the amounts of the cytokines could also be explained by the higher affinity of the IL-4Rα for IL-4 in comparison to the affinity of IL-13 to the IL-13Rα1 subunit. Due to the high affinity of IL-4Rα subunit, signaling would require only low concentrations of the cytokine. High expression of IL-13Rα1 would allow high amounts of IL-13 to signal more effectively than IL-4(101,102). Thus, signaling is influenced by cytokine and receptor subunit expression.

An interesting possibility is that via the type I receptor, IL-4 might inhibit the pro-inflammatory pathways mediated by the type II receptor. Signaling through the type I receptor inhibits IL-13-specific genes and upregulates IFNγ and IL-12 in the mouse lung, activating immunoregulatory mechanisms(103,104). Furthermore, mice deficient in γc show exacerbated allergic lung inflammation with higher levels of Th2 cytokines and less IFNγ in the bronchoalveolar lavage (BAL) fluid(105).

Lastly, it cannot be excluded that the reason why IL-4 is found in much lesser quantities in inflamed tissues is the possible toxicity of IL-4 compared to IL-13. Supporting this view, clinical trials in the 1990's, where large intravenous doses of IL-4 were given to cancer patients in an attempt to evoke T cell or innate immunity responses resulted in vascular leak syndrome on two thirds of the patients(106). Additionally, a study using an adenovirus vector showed that IL-4 was directly cytotoxic to hepatocytes (107) and transgenic mice that constitutively produce large quantities of IL-4 develop diseases and die at an early age (108).

2.1.5 The alarmins TSLP, IL-33 and IL-25 in type 2 responses

In addition to the classic Th2 cytokines (IL-4, IL-5, IL-9 and IL-13), epithelial cytokines TSLP, IL-33 and IL-25 have recently been the focus of many studies in the context of type 2 responses and allergic inflammation.

2.1.5.1 TSLP

TSLP, originally discovered in thymic stromal cell line supernatant and studied as a B cell growth factor, is a paralog of IL-7. Both are able to stimulate thymocytes and B cell lymphopoiesis(109,110). TSLP shares the IL-7Rα receptor subunit with IL-7, but IL-7 signaling requires subunit dimerization with common gamma chain (γc) whereas TSLP utilizes TSLPR subunit. Binding of IL-7 or TSLP to their receptor

complexes activates STAT5 via Jak1/Jak3 (in case of IL-7) or Jak1/Jak2 (TSLP)(111). In human peripheral blood DCs, activation of STAT1, 3, 4, 5 and 6 have been reported by TSLP(112). The IL-7 and TSLP receptor assembly and signaling is summarized in Figure 4.

Epithelial cells, keratinocytes and stromal cells produce TSLP during allergic inflammation, pathogen recognition and tissue damage(113-116). Although barrier ECs are considered the main sources of TSLP, also basophils(75), mast cells(117) and DCs(118) have been reported to produce it. IL-7 plays a role in ILC development and TSLP activates ILC2s to produce Th2 cytokines(119). However, in contrast to TSLP, IL-7 is mainly produced by stromal cells in secondary lymphoid organs(120), which suggests a functional specialization between the two cytokines. A variety of cells produce the functional receptor subunit TSLPR, including NK cells, monocytes, eosinophils, basophils, DCs, T cells, B cells and ECs(121,122).

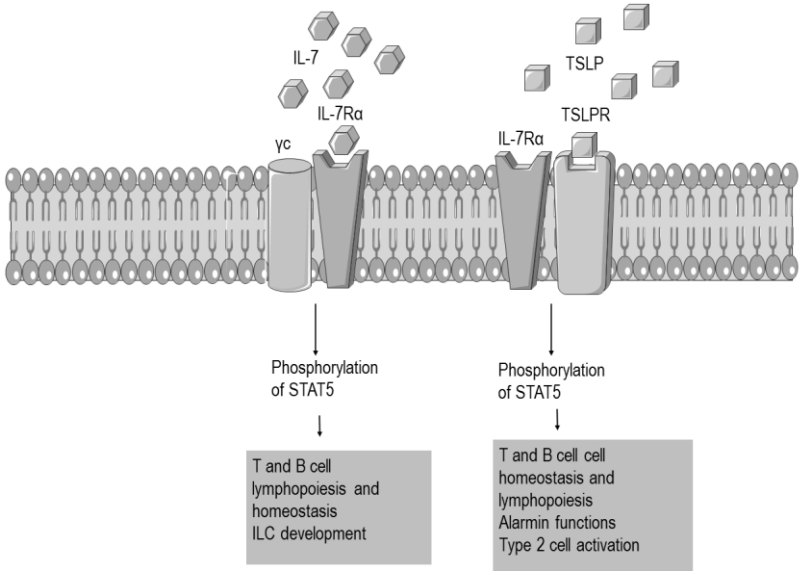

Figure 4. TSLP and IL-7 receptor assembly and signaling(5,111,119). Graphics by(17).

Multiple cell types have been shown to be the targets of TSLP and several functions for it have been identified. Recently, especially DCs have been implicated as important targets of TSLP(68,69,113,114). DCs stimulated with TSLP prime $CD4^+$ T cells in an allogeneic culture to produce TNF, IL-4, IL-5 and IL-13. They

also produced Th2-attracting chemokines CCL17 and CCL26(113). Driving Th2 differentiation was dependent on OX40/Ox40L interactions in the absence of IL-12(68). TSLP-activated DCs were also shown to maintain and further polarize Th2 effector memory cells(123).

In $CD4^+$ T cells, TSLP induced IL-4 expression in the presence of TCR stimulation, promoting subsequent Th2 differentiation and TSLPR receptor upregulation, which enabled a positive feedback loop(71,124). Mouse effector Th2 cell expressed higher TSLPR levels than Th1 or Th17 cells, which was mirrored in its capacity to induce the proliferation of Th2 cells(125). In a mouse model of allergic asthma, *TSLPR*$^{-/-}$ mice develop Th1 responses in response to an inhaled allergen, but the transfer of wild type $CD4^+$ T cells restores the Th2 response(126). In a Th2-mediated skin inflammation model, TSLPR-deficient mice showed decreased infiltration of eosinophils and production of Th2 cytokines, although DC maturation Th2 cell development was not affected, implying that TSLP acts on differentiated Th2 cells(70).

Lastly, TSLP promotes Th2 cytokine production from mast cells(127), NKT cells(128), and ILC2s(129). TSLP also promotes IL-4 production, differentiation and proliferation of basophils independently of IL-3 and causes basophil degranulation independently of IgE(130).

Numerous studies have shown a role for TSLP in allergic diseases of the airway, atopic eczema and food allergies (reviewed in (131)). To name a few findings, TSLP is highly expressed in the lesions of atopic dermatitis(113) and overexpression of TSLP in mouse skin caused a disease phenotype of atopic eczema(132). Allergen challenge of the skin rapidly induced the production of TSLP and subsequent recruitment and activation of DCs(133). In an allergic tape-stripping model, TSLP was needed for the development of inflammation(70).

TSLP may be a contributing factor during atopic march (the progression of atopic eczema to asthma and allergic rhinitis), as induction of TSLP in mouse keratinocytes result in subsequent airway inflammation after intranasal immunization(134). TSLP expression is increased in lungs of mice that have airway inflammation, and the overexpression of TSLP in the lungs induces airway inflammation via Th2 cells(69). In human asthma, TSLP levels are higher in the lungs of asthmatics than healthy controls(135).

However, TSLP is dispensable in the development of some helminth infections, such as *H. polygyrus* and *N. brasiliensis*, as TSLPR-deficient mice develop normal protective immunity when infected with these parasites. Interestingly, in *T. muris* infection TSLP is required for type 2 responses. It is possible that TSLP mediates its

actions by inhibiting the expression of Th1 cytokines, such as IFNγ and IL-12p70, as blocking these cytokines with antibodies restores type 2 responses in *TSLPR*$^{-/-}$ mice(136).

Finally, it is worth mentioning that a recent report by West et al. described a surprising role for TSLP in enhancing neutrophil efficiency in killing methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes*, both of which are common causes of human skin infections (137). The effect was dependent on the complement and neutrophil ROS production. However, the authors did not show the expression of IL-7Rα subunit on neutrophils. Previously, neutrophils have been shown to lack the receptor subunit expression (138).

2.1.5.2 IL-25

IL-25, also known as IL-17E, is another epithelial cell-derived cytokine that is known to induce type 2 responses, although originally it was identified as a product of Th2 cells(139,140). Administration of recombinant IL-25 increased serum IgE and induced eosinophilia and the production of IL-4, IL-5 and IL-13 in naïve mice. In Rag-knockout mice, IL-25 induced eosinophilia and histopathological changes mediated by IL-5 and IL-13(139). Transgenic overexpression of IL-25 had similar effects(141). IL-25 is expressed by human and mouse lung epithelial cell lines and allergen-activated mouse lung ECs(142,143) and colon ECs (144). IL-25 mRNA has been detected also in macrophages(145), mast cells(146), microglia(147) and intestinal tuft cells(148) whereas bioactive IL-25 protein was found to be expressed by activated eosinophils and basophils(149). In addition to these, multiple other studies have linked IL-25 with allergic pathologies(150-154) and helminth immunity(155-157). IL-25 is constitutively expressed in resting normal human bronchial ECs and stored in cellular compartments, from which it is released upon exposure to proteases, such as papain or allergen proteases from house dust mite extract(158).

IL-25 acts through its cognate receptor IL-17Rβ(159), which is expressed on ECs(154,157) but also in type 2 cell populations such as Th2 T cells(143) and DCs(149). A special notion should be made of ILC2s that were identified as targets of IL-25 before the innate lymphoid cell populations were actually characterized (ILC2s reviewed in (160) and (5)). Interestingly, a recent paper demonstrated that Th2 cells require exposure to IL-25 (as well as TSLP and IL-33) at the site of the inflammation in order to be able to fully acquire their effector functions(161).

2.1.5.3 IL-33

IL-33, a cytokine of the IL-1 family(162) was first thought to act as a Th2-biasing signal molecule as its ligand-binding receptor subunit ST2 (IL-2R4) was expressed on Th2 cells and mast cells(163,164), and disruption of the receptor function in studies implied a role in type 2 immunity(165,166). In the first study describing IL-33, recombinant IL-33 caused type 2 cytokine production and pathological changes especially in mucosal tissues in mice(162). Since then, the knowledge of the function of IL-33 in multiple pathologies has expanded (reviewed in (24)) and a role for IL-33 has been established in infectious diseases, including helminth, protozoan, bacterial, viral and fungal infections, as well as in inflammatory conditions such allergies, cardiovascular and musculoskeletal diseases, inflammatory bowel disease, and also in cancer.

IL-33 is constitutively produced by quiescent endothelial and epithelial cells at barrier surfaces(167-169). Mouse endothelial cells induce IL-33 expression only under inflammation(170), whereas constitutive expression by ECs is lost upon acute inflammation. In human skin keratinocytes, the opposite is true: steady state expression of IL-33 is weak but IFNγ-induced inflammation upregulated IL-33(171). Constitutive IL-33 expression can be further induced, for example, during chronic obstructive pulmonary disease(172) and atopic dermatitis(173) and in the mouse lung after nematode infection(174) or allergen challenge(175). During steady state, IL-33 is stored in the nucleus where it is associated with chromatin(170,176). If the nuclear localization sequence from the protein is deleted, IL-33 is released in large quantities, leading to profound Th2 inflammation and multiorgan failure(177).

IL-33 is not secreted as it lacks the signal sequence, but rather is released after mechanical injury or necrotic cell death as an alarmin to alert the immune system of infection or damage(178-180). It is biologically active already in its full-length form(178) but cleavage by proteases from sources at the site of inflammation, such as neutrophils and mast cells, can process it to a truncated form with up to 30-fold higher activity(181,182). Although barrier epithelial and endothelial cells are the main source of IL-33, also activated leukocytes have been reported as producers of IL-33 after stimulation with TLRs or cytokines(183,184).

The minimal receptor complex for IL-33 consists of the ligand-binding subunit ST2 (IL-1R4) and IL-1RAcP (IL1R3). IL-33 first binds the ST2 subunit, after which IL-1RAcP is recruited(185). Receptor complex formation is followed by signal transduction involving MyD88-IRAK-TRAF6 signaling module and the activation of MAPKs and NF-κB(186). In mast cells, activation of c-Kit by stem cell factor synergizes with IL-33 signaling, leading to the production of proinflammatory

cytokines(187). ST2 expression is dependent on GATA3 and STAT5(188). As mentioned above, ST2 is expressed on mast cells and Th2 cells, but ILC2s are also an important target for the cytokine: upon stimulation with IL-33 ILC2s and other innate immune target cells release type 2 cytokines, especially IL-13 and IL-5, to initiate the type 2 inflammatory cascade(5,189).

Numerous studies link IL-33 to type 2 responses and allergic inflammation. For example, a study with IL-33 deficient mice showed that the hookworm *N. brasiliens* expulsion was impaired due to selective defect in ILC2-derived IL-13(190). In an infection model involving the nematode *S. venezuelensis* or administration of chitin, endogenous IL-33 was important for lung eosinophilia, caused by IL-5 from ILC2s(174,191). Another study with IL-33 deficient mice demonstrated that airway inflammation caused by papain and ovalbumin was dependent on IL-33(192,193) – via the action of ILC2s(194,195). In inflammatory models as these, parasite and allergen-derived proteases cause tissue damage, which results in rapid release of IL-33 into BAL fluids(196,197). In humans, ILC2s responsive to IL-33 have been shown to be enriched in lesions of patients with atopic dermatitis; the same study demonstrated that the absence of IL-33 attenuated the inflammation of the skin in a mouse AD model(198). In human asthmatic patients, lung smooth muscle cells and epithelia cells show higher levels of IL-33 than healthy controls(199,200). It is noteworthy that in asthma, the genes encoding for IL-33 and ST2/IL- 1RAcP have been identified as susceptibility loci(201).

2.1.6 Sensing the pathogen: PAMPs, DAMPs and PRRs

Orchestrating type 2 responses begins at the level of epithelial cells of the skin and mucosal surfaces of lungs and gut, for example. These are the first cells to encounter the parasite or allergen and their products(45). Next, innate cells react to the invasion. Innate immune cells use PRRs to sense structurally conserved PAMPs that include a wide range of proteins, lipids, lipoproteins, glycans and nucleic acids, and DAMPs that are released from damaged host cells. For example, bacterial lipopolysaccharide (LPS), single strand and double strand RNA and flagellin are known PAMPs(202). DAMPs are associated with trauma and tissue damage and their levels in serum are increased in inflammatory diseases such as cancer, atherosclerosis, arthritis and sepsis. Typical examples include uric acid, ATP and chromatin-associated protein HMGB1(203). As discussed above, also IL-33, a well-known enhancer of allergic inflammation, can act as a DAMP or an alarmin upon tissue injury(204).

PRRs are found on the surface of cells and in cellular compartments and their main categories include Toll-like receptors (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs)(205). In addition to innate cells, TLRs are expressed in non-immune cells, such as ECs and fibroblasts.

In type 2 responses, PRRs have known roles. TLRs, NLRs and CLRs expressed by DCs take part in the recognition of type 2 provoking signals. For example, helminth extracts, Porphyromonas gingivalis, LPS, and synthetic TLR2 ligands induce type 2 responses via DCs. Likewise, bacterial peptidoglycans also induce Th2 responses through NLRs or C-type lectin DC-SIGN(206). Allergens can be sensed by PRRs: house dust mite (HDM) allergens signal through TLR4 – which is best known for its role in sensing LPS- of airway epithelia cells, inducing the release of TSLP, GM-CSF, IL-25 and IL-33(115). It has been suggested that type 2 responses are provoked by low doses of LPS, whereas type 1 responses are a consequence of exposure to high doses of LPS(207). As HDM extract contains LPS(208), the resulting Th2 response might reflect this(115). In fact, the HDM allergen Der p2 is structurally homologous to the LPS-binding part of TLR4(209). TLR4 is also involved in type 2 responses elicited by papain(74). DCs from patients with HDM allergy express more mannose receptors – a C-type lectin receptor expressed by myeloid cells(210) – than healthy individuals and they show increased allergen uptake(211). DCs from the BAL of asthmatics also express more mannose receptors when compared to healthy controls(212). Mannose receptors have been shown by several other studies to be important in recognition of allergens such as HDM, cockroach, dog, peanut, cat(213,214) and omega-1, the RNase from S. mansoni eggs(86).

As mentioned in the section discussing epithelial alarmins, mediators that are released from the cells during cell death are able to drive type 2 responses(43). For instance, aluminium sulfate (alum) is often used as an adjuvant in vaccines, and it is able to elicit type 2 responses independent of any known PRR activation(206,215). Instead, it relies on tissue damage and the subsequent release of uric acid, which induces a type 2 response driven by DCs(216). In a papain-immunization model, papain has been shown to induce ROS in ECs. ROS-mediated induction of type 2 responses relied, in part, on the oxidation of lipids that caused the release of TSLP from ECs. The release was mediated by TLR4(74).

As parasites cause tissue damage, the recruitment of type 2 responses seems logical, as they initiate the rapid repair of the tissues. Indeed, after helminth infection, the wound repair mechanism involving the synthesis of collagen I and III is dependent on IL-4 and IL-13. (43). Additionally, in mice infected with *N. brasiliens*,

signaling through the IL-4 receptor suppressed the Th17-mediated inflammation and led to the development of M2 macrophage, resulting in resolution of tissue damage(217).

A special notion should be made, that the innate system does not recognize merely pathogens but instead seems to make no difference between harmful and commensal microbes as both express PAMPs that are detectable by PRRs. However, the responses elicited by commensal bacteria are very different from those that arise from contact with pathogens, as innocuous bacteria are not only tolerated but required for the normal development of the immune system(218). Likewise, the structural features and diverse biological functions of numerous allergens from different sources are overlapping with nonallergens(48).

2.1.6.1 Recognition by functional features

Apart from the recognition of patterns and microbial structures, the innate system is able to detect pathogens by recognizing features associated with the functionality of the pathogen, such as enzymatic activity or membrane pore formation(219). For example, the NLRP3 inflammasome is activated by bacterial exotoxins and viroporins that form pores and ion channels in cell organelles(220,221). Also, allergens are sometimes sensed because of their enzymatic activity, a mechanism that is likely shared with multicellular parasites(222). There are four known protease activated receptors (PARs), three of which are expressed by vascular cells and that are involved in thrombosis and homeostasis and one (PAR2) that is found in endothelial and epithelial cells, fibroblasts and multiple immune cells, including T cells, mast cells, neutrophils, eosinophils, macrophages and dendritic cells(223,224). PAR2 is activated by serine proteases followed by proteolytic cleavage of its extracellular N-terminal domain. Because of this, PAR-2 can be activated by several proteolytic allergens, such as house dust mite, pollen and mold(224,225). Studies have shown that in airway ECs, allergen-induced cytokine production depends strongly on the protease activity of the allergen(226-228). Experiments with papain and other protease allergen have demonstrated that protease activity is needed for the induction of TSLP, IL-25 and IL-33(195,228). This kind of detection strategy lessens the need for a large repertoire of specific receptors, as many agents share the common activity and, coupled to PRR recognition, this functionality can aid in separating pathogens from innocuous flora. It is possible that the recognition of functional features without PRR recognition acts as a signal to begin Type 2

responses, because helminths do not possess structures that are sensed by PRRs, but instead cause tissue damage(15).

2.1.7 Innate cells in type 2 responses and allergic inflammation

Although this study focuses on mast cells, basophils and dendritic cells, several other innate cell types are also discussed here as the cells form a solid network of cooperation. The initiation of type 2 responses and the role of innate cells is summarized in Figure 5.

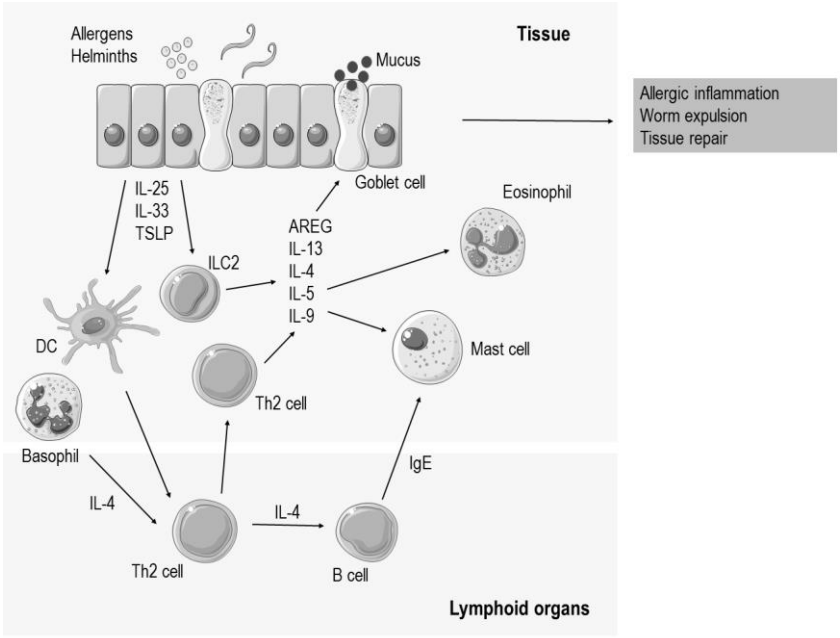

Figure 5. Initiation of type 2 responses(67,229). Graphics by(17), modified from original.

2.1.7.1 Epithelial cells

Epithelial cell layer is the first barrier a pathogen must breach in order to gain access to the host organism and epithelial barriers begin the type 2 response. As discussed above, many pathogens that induce type 2 responses use proteases for penetrating the rather impermeable mucus layers and tight junctions that connect the epidermal

cells. When damaged, the cells produce DAMPs or alarmins such as ATP, uric acid, HMGB1, TSLP, IL-25, and IL-33. These alarmins can be released upon cell death or they can be actively secreted(5,48). ECs also express a wide range of PRRs that can sense DAMPs and PAMPs such as TLRs, CLRs and PARs. Activation through these receptors triggers the production of chemokines and cytokines that activate and attract immune cells. For instance, immunity to HDM and development of asthmatic features requires recognition through TLR4(115,230) by ECs. Stimulation by HDM via TLR4 elicits the release of chemokines CCL2 and CCL20 and alarmins TSLP IL-25, and IL-33 from the barrier cells(115,216). Release of TSLP by ECs has also been reported after stimulation through TLR3, TLR5 and TLR2-TLR6(231). PAR2 is expressed by nearly all skin cell types and high amounts of PAR2 have been observed in the lesions of AD patients(232). PAR2 activation has also been connected with proteolysis by the fungus *Alternaria alternata*(233), cockroach(234) and mold(235) in the lung.

In the stereotypical response of epithelial cells to insults, the cells secrete chemokines such as CCL17 and CCL22 that act on innate lymphoid cells, Th2 cells, Tregs and basophils and CCL11, CCL24 and CCL26 that recruit eosinophils and Th2 cells(5). Additionally, ECs produce prostaglandin D2 that takes part in the recruitment of Th2 cells, mast cells, basophils and ILC2s. Interestingly, ILC2s have a positive feedback loop in which stimulation by prostaglandin D2 induces further production of CCL17, CCL22 and prostaglandin D2 from the cells themselves(236). IL-4 and IL-13 enhance the production of CCL11, CCL17 and CCL22 from ECs via STAT6, while IFNγ inhibits it(237). The prototypical response includes also the release of TSLP, IL-25 and IL-33, which have already been covered in detail. Their target cells include ILC2s, DCs, eosinophils, basophils and mast cells and the crosstalk between ECs and these cell types will be discussed in the next sections.

2.1.7.2 Innate lymphoid cells

ILCs are recently discovered cells of the innate immunity that are especially enriched on mucosal surfaces and epithelial barriers, although they are found within nearly all tissues that have been studied. Because of this location, ILCs are one of the first-line responders during pathogen entry, shaping subsequent immune responses(238). There are cytotoxic ILCs (NK cells), and three classes of non-cytotoxic ILCs, one for each helper T cell subset (excluding Tregs): ILC1s, that are T-bet$^+$, IFNγ producing cells involved in type 1 immunity against intracellular pathogens(239); GATA-3$^+$ RORα ILC2s that produce (IL-4), IL-5, IL-9, IL-13 and amphiregulin

(AREG) and work in type 2 responses with Th2 cells against helminths, responding to IL-25, IL-33 and TSLP(240,241); and lastly, ILC3s of the type 17 immunity, expressing RORγt, IL-17 and IL-22 and protecting against extracellular bacteria(242). ILCs develop from bone marrow precursors and they lack the antigen receptors of adaptive lymphocytes and leukocyte lineage markers. Mouse ILCs also lack PRRs, but in humans, it is known that at least ILC3s express TLRs(238,243).

ILC2s have been implicated in allergic inflammation, especially in the lungs. Many studies have concentrated to the role of ILC2s in RAG-1-deficient mice that lack B and T cells(244). Intranasal administration of IL-25 or IL-33 induces allergic inflammation in this mouse model, suggesting a role for ILC2s(245). Protease allergens papain and HDM allergen cause lung inflammation in the absence of T cells, with ILC2s being the producers of IL-5 and IL-13. Restoring ILC2s into mice deficient in T cells and ILC2s triggers the inflammatory condition(192,246). ILC2s are also shown to induce the cardinal feature of asthma, AHR, via IL-13 in the absence of T and B cells(247). In human and mouse AD model, skin lesions show enrichment of ILC2s. Interestingly, Kim et al. concluded that these cells respond to TSLP rather than IL-33 or IL-25(129), while Salimi et al. found that all three epithelia cytokines could stimulate skin ILC2s(198). In T cell dependent asthma models, ILC2s seem to produce considerable amounts of IL-13 and IL-5, even exceeding that of T cells(248). IL-4 does not seem to be expressed by ILC2s in lung inflammation model in great quantities (100,248,249), although it might play a role in certain parasitic infections(81,250).

Recently, studies have shown that PGD2, which binds to CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), potently induces ILC2s in humans. PGD2 increases type 2 cytokine production from ILC2s, but also GM-CSF and IL-3, in addition to functioning as a chemoattractant to ILC2s(251,252). This implicates cooperation with mast cells, as mast cells are known producers of PDG2 during allergic responses(253). In mouse lung ILC2s, leukotriene D4 also stimulates the production of type 2 cytokines (254). It is also shown that ILC2S can present antigens to $CD4^+$ T cells via MHCII(255) and this crosstalk is needed for *N. brasiliensis* expulsion(256).

2.1.7.3 Dendritic cells

Dendritic cells are a heterogeneous, hematopoietic population of cells that are involved in initiating and modulating immune responses especially in the role of antigen presenting cells. Situated in lymphoid tissue and in the tissues of lung, liver,

skin and gut, they act as sentinels of the immune system, linking adaptive and innate immunity. After exiting the bone marrow, they differentiate into resident and migratory DCs *in situ*: Resident DCs take up antigens in lymphoid tissues and present it to T cells, whereas migratory DCs in non-lymphoid tissues upregulate chemokine receptor CCR7 and constantly migrate to lymph nodes for subsequent antigen presentation(257,258). Migration results in the change of DC phenotype to a mature state, in which MHCII and co-stimulatory molecules are upregulated. Migration happens during steady state and inflammation, but only in inflammatory settings this process involves the production of inflammatory cytokines from DCs(259). The driving of T cell subset differentiation by antigen presentation is accomplished with the aid of co-stimulatory molecules, such as CD40, CD80 and CD86, and secretion of cytokines (including IL-1, IL-6, IL-10, IL-12, IL-23, IL-27 and TNF) from DCs themselves. In addition to antigen uptake, DC functions are regulated by signals from PRRs and other cells through cytokine signaling or direct cell contact(14).

DCs can be divided into plasmacytoid (pDC), classical or conventional DCs (cDC) and Langerhans cells (LC). Plasmacytoid DCs have a plasma cell-like morphology and they are found in the blood and lymph nodes. They seem to originate from common myeloid precursors and partially from common lymphoid precursors(260). pDCs recognize nucleic acids from viruses, bacteria and dead cells and produce large quantities of type I IFN during viral infections. When activated, pDCs become efficient APCs that drive $CD4^+$ cell differentiation(261-263). LCs are the only DC population in the epidermis and after activation and antigen uptake, they migrate to LN to secrete chemokines that attract T cells. (264).

Conventional DCs are located in lymphoid and non-lymphoid tissues so that they are constantly scanning for tissue and blood antigens. They have a superior capability to present foreign and self-antigens on MHCII and migrate to lymph nodes(265). Two subsets with differing functions are known to exist: cDC1 and cDC2(266). In mouse, cDC1 are involved mainly in Th1 responses(267,268), but in humans, both cDC1 and cDC2 subsets induce Th1 and Th2 responses(269). cDC2 subset promotes $CD4^+$ and $CD8^+$ T cell proliferation and induce Th2, Th17 and Treg responses and are likely to be superior at trafficking to lymph nodes(266).

In type 2 responses, dendritic cells act in collaboration with ILC2s and basophils in the beginning stages of type 2 inflammation(270). DCs have been shown to react to type 2 cytokines: for example, IL-33 promotes DCs to induce $CD4^+$ T cells(271,272) and TSLP increases the expression of the co-stimulatory molecule OX40L as well as secretion of basophil-attracting chemokines from DCs(68,74). Intrestingly, DCs themselves do not produce the key Th2 cytokine, IL-4(273), which

has been obscuring the role of DCs in Th2 models. Several studies confirm the key role for DCs in type 2 cell responses: depleting DCs in transgenic mice abrogated the features associated with asthma, such as AHR, eosinophilia and goblet cell hyperplasia(274,275). It has been reported that DCs are sufficient and indisposable for for type 2 responses against HDM, *S. mansoni* and also papain, depending on the experimental setting(80,276,277), but the exact mechanisms that DCs employ to induce type 2 immunity is somewhat poorly defined. Some studies indicate that basophils are needed in addition to DCs in order to establish an optimal type 2 response after exposure to allergens or helminths(74,75,278).

When discussing and studying dendritic cells, it should be noted that they share many functions and phenotypic markers between macrophages and monocytes and, especially during inflammation, classification of APC subsets is challenging. APCs can upregulate markers, such as CD11b, CD14, CD16 and CD141, that are normally used in defining lineage. The differentiation of blood monocytes into dendritic cells and macrophages in inflammatory settings further complicates the separation of subpopulations(257).

2.1.7.4 Eosinophils

Eosinophils develop in the bone marrow and are released into the bloodstream in a mature state, from which they migrate to thymus or gastrointestinal tract to reside under homeostasis(279). Relatively few eosinophils are found in the circulation as compared to tissues that are exposed to the environment (such as the gut). During inflammation, eosinophils are activated and they accumulate in peripheral tissues. Normally eosinophil half-life is only 8-18 hours, but under inflammatory conditions their survival is considerably prolonged(280). Historically, eosinophils have been thought to aid in defense against helminths, but this view has been challenged as in some studies eosinophils have been found to promote parasite survival(281,282) or aggravate parasite-associated tissue damage(283). Key factors that contribute to eosinophil development, activation and survival include IL-5 and CC-chemokine ligand 11 (CCL11, eotaxin) which promotes eosinophilia in cooperation with IL-5 and through an independent mechanism(284,285). Recently, it has been shown that TSLP, IL-25 and IL-33 from epithelial cells can induce eosinophilia by promoting IL-5 production(100,286). A prominent eosinophil feature is their degranulation, in which they release toxic proteins, leukotrienes, prostaglandins, cytokines and growth factors, among other mediators of inflammation(287). Eosinophils interact with several other cell types: For example, they are able to promote both Th1 and Th2

responses(288). In allergic settings, they recruit Th2 cells and regulate DCs(289,290). Additionally, eosinophils are involved in the production of IgM from B cells and supporting plasma cell survival(291,292).

2.1.7.5 Basophils

Basophils can be characterized as strongly type 2 response -oriented cells that contribute to protection against helminths and ticks. Basophils are the rarest type of granulocytes and represent less than 1% of blood circulating leukocytes(293) and they are very short-lived (approximately 1-3 days) cells(294). Basophil function overlaps with mast cells as both express different proteases, vasodilating substances, pro-inflammatory chemokines and lipid mediators as well as cytokines(295). Possibly the best known functional feature of basophils is their activation through FcεRIα and IgE crosslinking, which results in the release of histamines and leukotrienes(296,297). Additionally, basophils can be activated by IgG and IgD, the latter inducing production of antimicrobial peptides. During type 2 responses, peripheral basophilia is typical and IL-3 signaling has a crucial role in basophil development and expansion during *Nippostrongylus brasiliensis* or *Strongyloides venezuelensis infection*(298-300). IL-3 promotes IL-4 and IL-13 production following IgE-mediated activation(301). TSLP is also an important cytokine in basophil responses, in cooperation with IL-3 or without it. TSLP-elicited basophils are unable to degranulate after IgE-FcεRIα signaling but produce efficiently IL-4 in response to IL-3, IL-18 or IL-33, unlike IL-3 stimulated basophils(130). Basophils respond directly to protease activation. For example, Derp 1 (house dust mite protease) and cysteine protease papain promote the production of IL-4, IL-5 and IL-13 from basophils(67,302).

Basophils have been proposed as the source of the "early" IL-4 during type 2 responses as they are recruited into the lymph nodes in the beginning of the response, where they produce IL-4 and polarize DCs. Additionally, they can act as antigen presenting cells to T cells (67,75,80). Their importance in type 2 responses is shown in the murine *Trichurus muris* infection model, in which type 2 responses were impaired when basophils were depleted(76). However, the dependence of type 2 responses on basophils varies from experimental setting to other. For example, in studies using genetic tools that deplete the majority of basophils, type 2 responses were still able to develop (303,304).

2.1.7.6 Mast cells

Mast cells resemble basophils but they are not normally found in the bloodstream: mast cell progenitors migrate from the bone marrow into tissues where they differentiate into mature cells. They are found in mucosal and epithelia tissues and below the epithelium in the connective tissue in nearly all vascularized tissues(295,305). Mast cells are long-lived and their tissue distribution, expansion and phenotypic qualities are a subject to change during infection and especially persistent inflammation and tissue remodeling. Cytokines that are of special importance to mast cells include stem cell factor (SCF, the ligand for c-Kit), IL-3 and Th2 cytokines(306). Mast cells are key players in type 2 responses against parasites but they also display protective functions against bacteria, viruses and fungi(307,308). They are well known for their contribution to allergic reactions. As with basophils, the prominent feature of mast cells is their ability to degranulate in response to antigens crosslinking IgE receptors, FcεRI, that are situated on the surface of the mast cell. Degranulation releases multiple types of inflammatory mediators, such as chemokines, cytokines, histamine, heparin, tryptase, chymase, serotonin and prostaglandins(309,310). In both mouse(311) and human(312,313), two subsets of mast cells exist: mucosal and connective tissue mast cells. The two subtypes differ by the expression of their proteases. Mediator release has different effects on different organs: in the gastrointestinal tract, it triggers fluid secretion and increased peristalsis; in the skin, it causes urticarial reactions and angioedema and in the respiratory system, the activation results in mucous production, airway constriction and cough(305). In addition to to FcεRI-mediated activation, mast cells degranulate or secrete cytokines or chemokines in response to a wide range of stimuli: for example, PRRs, IgG, complement proteins, and cytokines. Some activating stimuli further potentiate FcεRI-mediated degranulation(309). Cells of the innate and adaptive immunity communicate with mast cells and a role in the modulation of type 2 responses has been implicated for mast cells. It was shown that mast cell mediator release regulates TSLP, IL-25 and IL-33 release from epithelial cells during helminth infection(314). Mast cells are able to regulate DC activation and migration to lymph nodes through the release of TNF and IL-1β along with other mediators, and they modify DC capability to induce Th2 differentiation(306). TSLP and IL-25 derived from mast cells themselves may also contribute to the regulation of DC function(113). Human mast cells express CD40L that interacts with CD40 on B cells and mast cells have been shown to induce class switch to IgE via CD40/CD40L *in vitro*(315) but in vivo they seem to be dispensable in IgE responses to helmiths(316). Lastly, mast cells are involved in the mediation of immune tolerance via IL-10 and

TGF-β that downregulate FcεRI, limit degranulation and leukocyte infiltration and inflammation(317-319). Moreover, differentiation of immunosuppressive regulatory T cells have been shown to rely on mast cell derived cytokines(320,321).

2.1.8 Papain-induced allegy model

In allergy research, several animal models of type 2 inflammation in the respiratory system, skin, mucosal surfaces and gastrointestinal tract are widely used. Although other animals, such as guinea pigs, sheep and dogs are sometimes used, mouse is the predominant laboratory animal in allergy studies(322). The most often used strain is the BALB/c strain as they develop a robust type 2 response(323), but the C57BL/6 strain(324) has also been used successfully. The route of allergen challenge varies according to the allergic pathology the model is simulating. For example, in asthma models, the allergen is typically inhaled or administered in an aqueous formulation intranasally or intratracheally(325), while in atopic dermatitis models an epicutaneous approach is usually employed(326). Subcutaneous, oral or intraperitoneal administration routes are also used(322,327). The most often used allergens include chicken egg ovalbulmin(324), house dust mite and cockroach extracts(325), haptens(328), *Alternaria* and *Aspergillus* extracts(329), chitin(330) and papain(331), to name a few.

In our study (III), the papain model was chosen. Papain from *Carica papaya* is an prototypical cysteine protease that is a common occupational allergen, causing urticaria, rhinitis and asthma(332,333). Because of its proteolytic activity that is shared by many other clinically significant allergens and helminths, papain has been used in multiple other experimental allergy setups.

The exact mechanism how papain induced type 2 responses is not known, and experimental conditions seem to give somewhat varying results. Enzymatically active papain induced mast cell degranulation, IgE and IgG1 production, but when papain was inactivated by E-64, mice appeared to become desensitized to the active enzyme(331). In a subcutaneous immunization experiment by Sokol et al. papain was shown to act independently of TLRs and MyD88, and while papain did induce mast cell degranulation, depletion of mast cells did not affect the production of IgE. In this report, papain induced Th2 differentiation and it directly activated basophils to produce type 2 cytokines(67). Later, the same group showed that basophils are able to present antigen to T cells after papain immunization and that DCs were not required in this setting(75). However, in another experiment, both basophils and DCs alone were insufficient for polarizing the Th2 response. Instead, basophils and

DCs cooperated via ROS-mediated signaling. Responses to papain were also dependent of TLR4(74). The controversy over the role of basophils and DCs was seen also in a report by Ohnmacht et al. as basophils were dispensable in their experiments(276). When papain was administered intranasally, eosinophilia and IgE/IgG1 response was elicited, but while papain-stimulated basophils expressed IL-4, depleting them or mast cells did not lead to attenuation of *in vivo* responses to papain. The type 2 responses were shown to mediated by IL-33(195). Interestingly, in the skin the results were opposite: sensitization was dependent on mast cells but not IL-33(334). One study administered papain via the genital tract and in this setting, IL-33 was released from the mucosa, leading to activation of ILC2s. MyD88 signaling pathway was shown to be critical for the development of type 2 immune response, and the response required a subset of DCs(335). A role for ILC2s was also implicated in papain-induced type 2 responses in the lung in a study by Halim et al. In this study, IL-4 was dispensable but IL-13 was needed for the migration of DCs into draining lymph nodes, where they primed T cells(194).

2.2 R-Ras in the immune system

2.2.1 Ras proteins

The proteins of the Ras superfamily are small, monomeric GTPases that operate as molecular switches that are inactive when GDP is bound and active when GTP is bound, transducing signals in turn to their downstream effector molecules(336). They modulate a wide range of cellular processes including cell cycle, growth, migration, cytoskeletal changes, survival and senescence via signaling pathways and networks(337,338). The superfamily is divided into five major branches based on their sequence and function: Ras, Rho, Rab, Ran and Arf(338). Originally identified as retroviral oncogenes, several Ras proteins are well-known for their activating mutations in tumor development and it is estimated that in 20% of all human tumors an activating Ras mutation is present(339). In human cancer and leukemia, p21 Ras proteins H-Ras, N-Ras, K-Ras4A and 4B are often mutated. In addition to these, the Ras-family has non-p21 proteins R-Ras, TC21 (R-Ras2), M-Ras (R-Ras3), Rap1A and B, Rap2A and B and RalA and B. The different proteins are very similar in their amino acid identities and there is overlap in their mechanisms of regulation and effector functions(336).

2.2.2 R-Ras

R-Ras is structurally somewhat similar to prototypic Ras proteins (H-Ras, K-Ras and N-Ras), showing 55-60% amino acid identity with them(340). However, functionally R-Ras is distinct from these proteins. Mutations in R-Ras are not known to be able to induce transformative activity(340) and despite the role of other Ras proteins in human cancers, no activating mutations have been reported for R-Ras. Quite the contrary, R-Ras appears to inhibit tumor proliferation and invasion in breast cancer cells *in vitro*(341) and during tumor angiogenesis and arterial injury R-Ras promotes vascular quiescence and integrity(342,343). Normally, the expression of R-Ras is restricted to endothelial and vascular smooth muscle cells(342) and cells of the immune system, including T cells, B cells and dendritic cells(344).

R-ras has several known roles in cellular processes, many of them involving the activation of integrin ligand binding and thus, cellular adhesion, spreading and migration. R-Ras enhances integrin affinity to their ligands and cell adhesion to the extracellular matrix(345). It localizes to the leading edge of migrating cells, regulating integrin trafficking by affecting membrane protrusion, ruffling and endocytosis(346,347). Especially Rac activity is upregulated at the leading edge, and R-Ras has been shown to regulate Rac(348,349). A key requirement for cell spreading is actin cytoskeleton organization and indeed, R-Ras has been reported to regulate the actin organization and following membrane protrusions via phospholipase Cε(350) and afadin, which promotes neural axon branching(351). Another protein that R-Ras interacts with is Filamin A, an actin binding scaffold protein that additionally binds to integrins and mediates cell migration(352).

2.2.3 The role of R-Ras in the immune cells

Perhaps not surprisingly, R-Ras also affects leukocyte functions that involve cell adhesion, migration, homing and mobilization: R-Ras downregulates Rac, the activation of which is needed for maintenance of hematopoietic progenitor cells (HPCs). Elimination of R-Ras caused increased directional migration, HPC mobilization and decreased bone marrow homing(353). In macrophages, R-Ras is able to functionally activate the αMβ2-integrin (complement receptor 3), leading to phagocytosis of opsonized targets(354). A recent study showed that CD4+ and CD8+ T cells from *Rras*$^{-/-}$ mice had a lowered expression of L-selectin (CD62L), which is critical for the adhesion of T cells on the high endothelial venules when T cells are entering lymph nodes. The trafficking and proliferation of T cells from these

mice was impaired. R-Ras was also found to be activated by the chemokine CCL21 and *Rras*$^{-/-}$ T cells showed attenuated binding to ICAM-1 (intercellular adhesion molecule 1) when stimulated with CCL21(355).

R-Ras has been implicated in autoimmunity. In biliary atresia, human *RRAS* gene was identified to correlate with negative prognosis(356) and in mouse experimental autoimmune encephalomyelitis (EAE), *Rras*$^{-/-}$ mice exhibited attenuated EAE with increased numbers of tolerogenic dendritic cells and regulatory T cells. Additionally, in the central nervous system of the *Rras*$^{-/-}$ mice, the absolute number of immune cells was reduced significantly(357). Interestingly, a previous study from the same authors had discovered that DCs from *Rras*$^{-/-}$ mice were defective in their ability to prime allogeneic and antigen-specific T cell responses and their formation of immune synapses with T cells was impaired, due to failure in actin cytoskeleton functions(344). However, the reduced EAE severity was not caused by a deficiency in peripheral CD4+ T cell priming in this disease model(357).

Finally, in an experimental skin tumorigenesis model *Rras*$^{-/-}$ mice showed resistance to skin tumor formation upon DMBA/TPA induction. The model is highly dependent on inflammation, and in R-Ras KO mice the mRNA expression of pro-inflammatory cytokines (IL-1α, IL-6 and IL-17A) and leukocyte infiltration was reduced. It is possible that the defect in leukocyte extravasation could be caused by the lack of endothelial R-Ras, but the reduced cytokine mRNA expression was already observed before the extravasation took place. This suggests that the immune cells already present in the untreated skin of R-Ras KO mice have an impaired ability to produce inflammatory cytokines(358).

2.3 The differences of mouse and human in immunological research

Many of the immunological findings cited in this work have been made in the mouse *in vivo* or *in vitro* model. As the dominating laboratory animal species, the mouse has enabled several scientific discoveries and advances. In immunology, the mouse model has been critical since the mid-twentieth century, vastly aiding in the understanding of antibody interactions with antigens, pathogen response, lymphoid differentiation and MHC genes(359). However, even though mice and humans share over 90% of genes and the genetic content between the two species is very homologous(360), there are considerable differences spanning the whole immune system and its functions between the two species. In the context of immunological research, it should be kept in mind that what is true in one species might not apply to the other at all. In addition to this, laboratory mice are heavily inbred and certain

traits might have enriched in different strains, further complicating any generalizations and the interpretation of study results. In this last chapter of the literature review, some of these key differences are briefly discussed, along with problems arising from the attempts to translate data from mouse disease models to human.

2.3.1 The mouse and human immune systems

The general characteristics of human and mouse immune systems are rather similar with a few notable differences. For example, the human and mouse neutrophil-lymphocyte balance is strikingly different, with human leukocytes being dominated by neutrophils (50-70%) and less by lymphocytes (30-50%), while mouse leukocytes are mostly lymphocytes (75-90%). As another example, mouse bronchus associated lymphoid tissue is considerably larger than that of humans(361).

More differences are apparent when cells and their functions are inspected. To name a few in the innate system, human neutrophils are rich in defensins while in mouse they are expressed by Paneth cells of the small intestine. Mouse neutrophils are also less abundant in other antimicrobial molecules(362). Paradoxically, despite the relative sparseness of neutrophils in mouse blood, delayed-type hypersensitivity reactions in mice are dominated by neutrophils, while in humans they are dominated by T cells and macrophages(363). In humans, the chemokine eotaxin-3 has an important role in eosinophil migration during asthma, but eotaxin-3 is only present as a pseudogene in mice and has no effect on eosinophils (364,365). The metabolism of arginine is very different in mouse and human macrophages: inducible NO synthase is strongly upregulated in murine macrophages upon stimulation with LPS and IFNγ(366), but the same does not apply to human macrophages. Instead, human macrophages produce iNOS under certain disease conditions, possibly due to the effect of IFNα and β. Additionally, in mice only, alternatively activated macrophages produce arginase type-1, antagonizing the iNOS and proinflammatory responses. IL-4 and IL-13 upregulate arginase type-1 in mice, but in humans such is not observed(362,367). In murine mast cells, serotonin is the dominant mediator of anaphylactic reactions, whereas in humans especially histamine has an important role(361). Murine mast cells are closer to basophils, producing large quantities of TNF, while human mast cells are important producers of IL-5. Unlike mouse mast cells, human mast cells react strongly to IL-3(368).

Reaction elicited by TLR agonists are different in murine and human immune systems. Human TLR ligands cause inflammatory responses in infectious and

noninfectious settings, whereas tolerance strategies are preferred in mouse. A striking demonstration of this is the response to the TLR4 agonist LPS in human and mouse: exposure to several orders of magnitude of LPS is needed in mouse to induce the same effect (fever, cytokine production, shock) as in humans(369). As might be expected, not all TLRs are shared by mice and humans nor are they expressed by the same cells(363).

2.3.2 Mouse disease models

Translating the data from mouse disease models is a controversial topic. In addition to human and mouse immune systems being fundamentally different in some aspects, many disease models rely on artificially inducing and hastening diseases that would not naturally occur in mice. On many instances, research has created cures for induced mouse diseases that have failed in humans. At the same time, some models have produced success stories. For example, cancer research often relies on transplanted xenografts into the mouse and many times the resulting tumors do not represent human ones(362). Yet, mouse studies led to the discovery of inhibitory receptors and checkpoint blockade, now utilized in immunotherapy (370). Multiple sclerosis provides another example: in a mouse EAE (experimental autoimmune encephalomyelitis) model, IFNγ has a protective effect(371). However, human trials had to be stopped because IFNγ seemed to worsen the disease(372). On the other hand, blocking VLA-4 ($\alpha_4\beta_1$integrin)-VCAM-1 interactions proved successful in the treatment of MS in both species(373,374). In some cases, mouse models are under special criticism. It has been argued that the transcriptional inflammatory response in mice does not correspond to that of humans very well (375-377). In a 2013 study, animal model based drugs to treat acute inflammation and sepsis had failed every time(375) – compared to a typical 90% fail rate of all drugs in human clinical trials(378). A special highlight might be the regulation of TLRs – in humans they are broadly upregulated in different inflammation settings, while in mice the expression levels vary(375), possibly indicating a reason for differences in inflammatory responses between the species.

Lastly, it is worth considering the impact of mouse housing conditions on the disease models and experimental results. It has been reported that the housing the animals in human room temperature subjects them to chronic cold stress, introducing alterations to several physiological parameters in comparison to mice living in a more natural nesting temperature of 30-32°C. (379,380). Responses to LPS, infection and cancer have been shown to be affected by the mouse housing

temperature(379). Maybe more importantly, the microbial environment plays a key role in many disease models and experiment results. Understandably, researchers have strived to minimize the unpredictable effect of pathogens and other "wild card" micro-organisms in their experiments by establishing specific pathogen free (SPF) housing for the animals. SPF animal facilities are controlled for a certain list of micro-organisms. However, this has led to the laboratory animals being quite different from their wild counterparts in terms of infectious history(359). Recently it has become clear that the microbial environment and commensal microbiota has a profound effect on the host immune responses(381,382). Some mouse strains, such as the non-obese diabetic mouse, show very different disease occurrence when housed in clean versus "dirty" environment(383). Even animals from different vendors show different gut microbiota and thus, have different presence of Th17 cells in their gut(384). Exposing SPF animals to pathogens had significant effects on, for example, adaptive immune responses and infection resistance(385,386). A very intriguing finding was that feral mice and mice from pet shops had elevated numbers of $CD8^+$ memory t cells, $CD4^+$ T cell subsets, ILCs and lymphoid cells when compared to laboratory mice – immunologically resembling adult humans. Importantly, the "dirty" mice were able to induce similar immunological changes in laboratory animals during cohousing(387).

3 AIMS OF THE STUDY

Allergy is a result of complex co-operation of cells of the adaptive and innate immunity, driven by cytokine signaling. It is an immune dysfunction, in which the responses that have evolved to fight parasitic infections are instead evoked by innocuous antigens. The purpose of this study was to investigate the underlying mechanisms of allergic inflammation, especially from the point of view of cytokine environment. Additionally, the possible role of the protein R-Ras in allergic responses was studied. The main focus in this thesis was on mast cells, basophils and dendritic cells.

The detailed aims of the study were the following:

1. To study cytokine-induced production of interleukin 13 by mouse mast cells and basophils
2. To examine how interleukin 7 receptor regulates the sensitivity of dendritic cells to thymic stromal lymphopoietin
3. To investigate the effect of R-Ras in the development of allergic inflammation

4 MATERIALS AND METHODS

4.1 Mice (I-III)

4.1.1 Wild type, *TSLPR*$^{-/-}$ and *MyD88*$^{-/-}$ mice (I-III)

Wild type (WT) C57BL/6 mice were from Jackson Laboratories (Bar Harbor, ME) or Taconic Farms (Hudson, NY) and housed in the NIAID pathogen-free animal facility (publications I-II), or from Harlan Sprague Dawley Inc (Indianapolis, IN) and housed at the SPF animal facility of School of Medicine, University of Tampere (publications I-III). For studies I and II, B6 *MyD88*$^{-/-}$ mice were provided by Dr. R. Caspi (National Eye Institute, Betsheda, MD) with permission from professor S. Akira (Osaka University, Osaka, Japan)(388). TSLPR$^{-/-}$ mice were provided by Dr. W.J. Leonard (National Heart, Lung and Blood Institute, National Institutes of Health, Maryland, MD) for study II.

4.1.2 DsRed transgenic mice (I)

120-kbp bacterial artificial chromosome (BAC172/pBACBelo11) construct was provided by Richard Flavell and Gap Lee (Yale School of Medicine, New Haven, CT, USA). The cassette contained *Il4*, *Il13* and *Il5* genes and the *Il4*/*Il13* locus control region (I, Fig. 1A). A DsRed-encoding construct (Clontech, Mountain View, CA) was inserted after the ATG of the *Il13* gene using *galK*-selection(389): Shortly, the Il13 translation-initiating ATG in the BAC172 clone was targeted with a *galK*-construct with *Bam*HI restriction sites and homology arms at the 5' and 3' ends of the *galK* gene. The DsRed construct was used to target the *galK* in turn. The resulting BAC172 construct was fully sequenced between the homology regions and the construct was microinjected into oocytes of B6 mice. These oocytes were transferred into pseudopregnant foster mothers. *Bam*HI was used to digest the genomic DNA of 121 transgenic pups for southern plotting: the DNA was separated on 0.8% agarose gel and a 916-bp PCR fragment spanning the 5' and 3' homology arms was used after the DNA was transferred to a nylon membrane.

4.1.3 *Rras*$^{-/-}$ mice (III)

R-Ras KO mice (OST24882) were generated as described previously(342,390) by inactivating *Rras* with the insertion of a gene-trap vector VICTR20 between exons 4 and 5 of *Rras* on chromosome 7. Mice were backcrossed with C57BL/6 strain.

4.2 Human subjects (II)

For study II, 60 ml of blood was collected into EDTA tubes (BD) from healthy male volunteers aged 30-50 years.

4.3 *In vivo* experiments (II-III)

4.3.1 Intraperitoneal IL-4 administration (II)

WT C57BL/6 mice received two intraperitoneal injections (8 hours apart) of 6 μg of IL-4 (Peprotech, Rocky Hill, NJ) in PBS, complexed with 30 μg of anti-IL-4 (Thermo Fisher Scientific, Carlsbad, CA). Alternatively, the mice were injected twice with 30 μg of anti-IL-4 alone. Complexing the cytokine with the antibody prolongs its half-life, as described by Morris et al(391). After 12 hours from the last dose, mice were euthanized and their spleens collected.

4.3.2 Papain immunization (III)

0.5 mg of papain (from *Carica papaya*, Merc Millipore, Darmstadt, Germany) in PBS or PBS only was injected subcutaneously under the scruff of WT and R-Ras KO mice. The injections were given 14 days apart. On day 0, blood samples for ELISA were collected from the tail vein and terminal bleeding was done on day 16 or 21 by cardiac puncture. Serum blood tubes (BD, Franklin Lakes, NJ) were used according to manufacturer's instructions. Additionally, spleens and lymph nodes (cervical, axillary, brachial, mesenteric and inguinal) were harvested for subsequent cell culture and flow cytometric analysis.

4.4 Cell culture and stimulation (I-III)

4.4.1 Study I

To reveal the correlation of DsRed expression with simultaneous IL-4 expression, CD4+ T cells from mice born from the founders were cultured under Th2 conditions(392) for 3 days and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours, after which the cells were stained for CD4 and IL-4.

Peritoneal mast cells were obtained by flushing the peritoneal cavities of WT and DsRed transgenic mice with 2 mmol EDTA in PBS. The cells were plated and cultured at 37˚C with 5% CO_2 in RPMI containing 2% FBS, penicillin/streptomycin and 2mmol L-glutamine. For DsRed expression experiments with IL-1-family cytokines and IL-3, cells were either left unstimulated or stimulated for 16 hours with 10 ng/ml of IL-1, IL-33 (Peprotech) or IL-18 (MBL International, Woburn, MA) with or without IL-3 (Peprotech), or IL-3 alone. For DsRed and pSTAT5 experiments with STAT5 inducers, cells were stimulated with 20 ng/ml of IL-33 alone or together with 20 ng/ml of IL-5, GM-CSF (Peprotech), TSLP or IL-7 (R&D Systems, Minneapolis, MN) for 16 hours. In the IL-33 receptor ST2 expression analyses, peritoneal mast cells or BMMCs were stimulated for 16 hours with IL-3, IL-33 or a combination of them.

Bone marrows of DsRed, WT and *MyD88*$^{-/-}$ (where indicated) mice were flushed and red blood cells depleted. The cells were grown as described above with the addition of continuous 20 ng/ml of IL-3. The culturing was continued for 10-12 days in order to obtain basophils and up to 40 days for bone marrow derived mast cells (BMMCs). To establish a correlation with DsRed and IL-13 protein and RNA expression, BMMCs were stimulated with 20 ng/ml of IL-33 for 4 hours on day 40 or 5 hours on day 21, respectively. After sorting the BMMCs into DsRed low, intermediate or high cells based on their DsRed expression, the cultures were continued separately overnight for each cell fraction. For cytokine stimulation/DsRed expression study, at 10-12 days (basophils) and 40 days (BMMCs) the cells were washed twice with PBS and stimulated with IL-3 and IL-1 family cytokines in the same manner as peritoneal mast cells. Unlabeled ST2 antibody (MD Biosciences St. Paul, MN) was used in the receptor blocking experiment at a concentration of 10 μg/ml. To test the impact of continuous IL-3 in the growth medium, BMMCs were washed three times with PBS and cultured for additional 32 hours with IL-3 or IL-4 (Peprotech)(10 ng/ml each) or for 48 hours in

IL-3 or SCF (Peprotech)(10 ng/ml each). After this, cells were washed and stimulated with IL-3, IL-33 or both for 16 hours. WT and *MyD88*$^{-/-}$ BMMCs and basophils were stimulated for 5 hours with 10 ng/ml IL-3 or IL-33 or both and then stained for IL-13.

To study the effect of IL-1 family cytokines on DsRed expression in mature splenic basophils, spleens from wild type or DsRed transgenic mice were collected and minced. Cells were filtered through a 40 μm strainer and red blood cells were lysed with 30 s ACK (Lonza, Basel, Switzerland) treatment before proceeding to flow cytometric staining and analysis.

4.4.2 Study II

Lymph nodes and spleens from female C57BL/6 mice aged 6-10 weeks were collected and minced, followed by a 30 minute incubation at 37°C in HBSS containing 75 μg/ml Liberase DL and 10 U/ml DNase (both Roche, Basel, Switzerland). After filtering the cells through a 40 μm cell strainer, red blood cells were lysed using ACK lysis buffer (Lonza) for 30 seconds. Dendritic cells were enriched with MACS Pan Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Cells were used immediately or cultured overnight (16 hours) at 37°C and 5% CO_2 in RPMI with 10% FBS, L-glutamine and penicillin-streptomycin (all Lonza). For IL-7Rα analyses in lymph node dendritic cells, cells were used either freshly *ex vivo* or stimulated for 16 hours with 20 ng/ml of IL-4 or 40 ng/ml of IL-7 (both Peprotech) or left unstimulated. Splenic dendritic cells were treated in the same manner, but in some experiments additional 1 μg/ml of LPS (InvivoGen, San Diego, CA) was given with or without IL-4. For pSTAT5 staining, cultured cells were first starved for 2 hours in RPMI containing 1% FBS, L-glutamine and penicillin-streptomycin at 37°C. After this, cells were stimulated for 15 minutes with 100 ng/ml of IL-7, GM-CSF (Peprotech) or TSLP (R&D).

Human DCs were enriched from 60 ml of peripheral blood collected into EDTA tubes. Mononuclear cells were isolated with a 40 minute gradient centrifugation at 400g, RT, using Ficoll-Paque® Plus (GE Healthcare, Helsinki, Finland). Platelets were further removed by washing the mononuclear cells three times with 2mM EDTA in PBS. Dendritic cells were enriched using Human Pan-DC Enrichment Kit (Miltenyi Biotec) according to manufacturer's instructions. DCs were analyzed immediately or after 16 hours of stimulation with medium only or 20 ng/ml of IL-4 or TSLP (Peprotech).

4.4.3 Study III

Spleens and pooled lymph nodes of R-Ras KO and WT mice were collected and dendritic cells were enriched as in study II. For determining the numbers of T and B cells, the Liberase step was left out as well as the magnetic separation, and total splenocytes were stained. In some experiments, total splenocytes were gradient centrifuged with Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO) at RT, 2600 rpm, 20 min., and the middle layer was collected for subsequent DC analyses. Cells were used either freshly ex vivo or after 16 hours of culturing in either medium (RPMI with 10% FBS, L-glutamine, penicillin-streptomycin and β-mercaptoethanol (1:250 000, Sigma-Aldrich)) alone or with 20 ng/ml IL-4, 1 μg/ml LPS or 20 μ/ml Poly(I:C) (InvivoGen).

4.5 Flow cytometry (I-III)

All the anti-mouse antibodies used in flow cytometric analyses are listed in Tables 2 and 3 and anti-human antibodies in Table 4. In study I, mast cells were surface stained for c-Kit and FcεRI and basophils were identified based on their positivity for FcεRI and negativity for c-Kit, or in case of splenic basophils, their positivity for FcεRI and negativity for B220. ST2 was stained in mast cells where mentioned. T cells were stained for CD4. Cells were washed with 0.1% BSA in PBS and FcγR II and III were blocked with antibody 2.4G2 and 0.1% mouse serum. Staining with appropriate cytokines was performed at 4˚C for 20 minutes. The cells were washed twice with 0.1% BSA in PBS and analyzed with FACSCalibur (Becton Dickinson, San Jose, CA) STAT5 phosphorylation assays were done as described previously(393). Intracellular staining of IL-13 in *MyD88*$^{-/-}$ BMMCs and basophils were performed with 0.5% TritonX in the staining buffer. Cell sorting was done with FACSDiva software (BD) and analysis with FlowJo (FlowJo, LLC, Ashland, OR).

In study II, analyses were carried out with FACS Canto II flow cytometer (BD) and staining protocol was the same as in study I. Dendritic cells from spleen and lymph nodes were gated as lineage (B220, CD3, F4/80) negative and positive for CD11c and MHCII. IL-7Rα or isotype control, γc, TSLPR or isotype control, CD4, CD8 and activation markers CD80 and CD86 were stained where indicated. In pSTAT5 assays, lineage markers were excluded as they did not survive the methanol treatment of intracellular staining. In some experiments, dendritic cells from total splenocytes were identified for their expression of CD11c and lack of CD49/Dx5. Human DCs were stained in the same manner as lineage (CD3, CD14, CD19, CD56)

negative and HLA-DR and CD11c positive cells. These cells were further stained for IL7Rα and CD80. Human cells were blocked with BD Fc Block™ (BD).

Flow cytometry in study III was done as in study I, but cells were Fc-blocked with BD Pharmingen Fc Block™ (BD) prior to staining. Where indicated, DCs were identified as in study II as lineage negative, MHCII+ CD11c+ cells. Activation was determined by staining markers CD80, CD86 and OX40L. In some experiments, total splenocytes were stained with CD3, CD4, and CD8 in order to characterize T cells and with B220 to analyze B cells.

Table 2. Anti-mouse antibodies used in studies I and II in flow cytometric analysis.

Study I			
Name	**Fluorochrome**	**Clone**	**Provider**
B220	FITC	RA3-62B	BD
CD4	FITC	GK1.5	eBioscience
c-Kit	APC/FITC	2B8	eBioscience
FcεRI	FITC/PE	MAR-1	eBioscience
IL-6	PE	MQ2-13A5	eBioscience
IL-13	PE	eBio13A	eBioscience
IL-18Rα	AlexaFluor647	BG/IL18Ra	Biolegend
IL-33R/ST2	FITC	DJ8	MD Biosciences
pSTAT5 (pY694)	PE	47/Stat5(pY694)	BD
Study II			
Name	**Fluorochrome**	**Clone**	**Provider**
γc	PE	4G3	BD
γc, isotype control	PE	R35-95	BD
B220	APC-eFluor780	RA3-62B	eBioscience
CD3	APC-eFluor780	UCHT1	eBioscience
CD4	FITC	GK1.5	eBioscience
CD8	PerCP-Cy5.5	53-6.7	eBioscience
CD11c	PE-Cy7	N419	eBioscience
CD49b	PE/APC	DX5	eBioscience
CD80	PerCP-eFluor710	16-10A1	eBioscience
CD86	APC	GL1	eBioscience
F4/80	APC-eFluor780	BM8	eBioscience
IL-7Rα	APC/eFluor660	A7R34	eBioscience
IL-7Rα, isotype control	eFluor660	eBR2a	eBioscience
MHCII	FITC	M5/114.15.2	eBioscience
TSLPR	PE	Polyclonal	R&D
TSLPR, isotype control	PE	Polyclonal	R&D

Table 3. Anti-mouse antibodies used in study III in flow cytometric analysis.

Study III			
Name	**Fluorochrome**	**Clone**	**Provider**
B220	APC-eFluor780/PE-Cy7	RA3-6B2	eBioscience
CD3	APC/APC-eFluor780	145-2C11	eBioscience
CD4	FITC/APC-eFluor780	GK1.5	eBioscience
CD8	PerCP-Cy5.5/APC-H7	53-6.7	eBioscience
CD11c	PE-Cy7	N419	eBioscience
CD80	PerCP-eFluor710	16-10A1	eBioscience
CD86	APC	GL1	eBioscience
F4/80	APC-eFluor780	BM8	eBioscience
MHCII	FITC	M5/114.15.2	eBioscience
OX40L	PE	RM134L	eBioscience

Table 4. Anti-human antibodies used in flow cytometric analysis.

Study II			
Name	**Fluorochrome**	**Clone**	**Provider**
CD11c	PE-Cy7	3.9	eBioscience
CD14	PerCP-Cy5.5	61D3	eBioscience
CD19	PerCP-Cy5.5	HIB19	eBioscience
CD56	PerCP-eFluor710	CMSSB	eBioscience
CD80	APC-H7	L307.4	BD
HLA-DR	FITC	L243	eBioscience
IL7-Rα	APC	R34-34	Thermo Fisher Scientific
IL-7Rα, isotype control	APC	-	Thermo Fisher Scientific

4.6 Quantitative PCR (I, III)

All probes are listed in Table 5. For study I, DsRed and IL-13 mRNA was analyzed from sorted cells by isolating RNA with RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribing the RNA with SuperScript II First Strand Synthesis System (Invitrogen, Carlsbad, CA). The analysis was performed with 7900HT sequence detection system (Applied Biosystems, Foster City, CA). TaqMan probes for cytokines were FAM-MGB labeled and VIC-MGB-labeled for eucaryotic 18S Ribosomal RNA (all from Applied Biosystems), which was used as a reference. Results were calculated with the double delta method.

In study III, spleen samples were stored in RNAlater RNA Stabilization Reagent (Qiagen). Homogenization was done with Precellys® bead tubes and homogenizer at 4˚C. mRNA was isolated with Invitrogen Trizol Reagent (Life Technologies, Carlsbad, CA) and reverse transcription was performed using Thermo Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Life Tehcnologies) according to the manufacturer's instructions. Expression of Th1, Th2, Th17 and Treg transcription factors and cytokines was determined with Biorad CFX96™ Real-Time PCR Detection System (Biorad Laboratories Inc., Hercules, CA) using FAM-labeled TaqMan probes (Applied Biosystems) and iQ™ Supermix (Biorad). Universal cycling conditions were applied: 3 min at 95°C, followed by 39 cycles of 15 s at 95°C and 1 min at 60°C. VIC-labeled Eucaryotic 18S RNA (Applied Biosystems) was used as a reference and the results were calculated using the double delta method.

Table 5. Probes used in quantitative PCR.

Study I			
Gene	**ID**	**Probe**	**Provider**
IL-13	Mm00434204_m1	FAM-MGB	Applied Biosystems
18S ribosomal RNA	Hs99999901_s1	VIC-MGB	Applied Biosystems
Study III			
Gene	**ID**	**Probe**	**Provider**
GATA 3	Mm00484683_m1	FAM-MGB	Applied Biosystems
FOXP3	Mm00475162_m1	FAM-MGB	Applied Biosystems
IFNγ	Mm01168134_m1	FAM-MGB	Applied Biosystems
IL-10	Mm01288386_m1	FAM-MGB	Applied Biosystems
IL-13	Mm00434204_m1	FAM-MGB	Applied Biosystems
T-BET	Mm00450960_m1	FAM-MGB	Applied Biosystems
RORγ	Mm01261022_m1	FAM-MGB	Applied Biosystems
18S ribosomal RNA	Hs99999901_s1	VIC-MGM	Applied Biosystems

4.7 ELISA (I, III)

In study I, IL-13 protein was measured from the supernatants of sorted DsRed mouse cells in order to compare IL-13 protein expression to DsRed brightness. To this end, IL-13 ELISA (R&D) was used.

In study III, serum from WT and R-Ras KO mouse blood samples was used in IgE Ready-SET-Go!® ELISA (eBioscience, Santa Clara, CA) for determining total blood IgE levels.

4.8 Statistical analysis

All statistical analysis on studies II and III was done with Prism software (GraphPad Software Inc., La Jolla, CA) except for ELISA, for which R(394,395) was used. P-values in study II were calculated with two-tailed, paired student's t-tests and in study III two-tailed, unpaired student's t-tests were used.

4.9 Ethical considerations

For studies I and II, all animal studies were done under a protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee. Part of the experiments in study II and all in study III were performed in accordance of with protocols approved by the National Animal Experiment Board (ESAVI permit number ESAVI/4738/04.10.07/2014).

5 SUMMARY OF RESULTS

5.1 Mouse mast cells require IL-3 and IL-33 for efficient production of IL-13 (I)

Mast cells are best known for their release of inflammatory mediators, such as histamine and heparin, from cytoplasmic granules upon cross-linkage of FcεRI(4). However, cross-linking, and importantly, cytokine stimulation, also leads to the production of IL-13 and to some degree, IL-4, both crucial cytokines in the initiation and amplification of allergic responses (396,397). Additionally, basophils express IL-13 and IL-4 in response to cytokines (245,398). In Th cells, a two-step cytokine-induced cytokine production is common and in this model, the other cytokine is an IL-1 family cytokine and the other a STAT-activator(399). IL-33 has been shown to regulate mast cell functions in the setting of allergic inflammation (245,400). In this study, we demonstrated that mouse peritoneal mast cells and basophils produce IL-13 in response to combination of two cytokine signals: IL-33 (an IL-1 family cytokine) and IL-3 (a STAT5 activator), thus following the two-step induction model. In basophils, IL-18 also is able to induce IL-13 when administered together with IL-3.

5.1.1 Generation and characterization of IL-13 DsRed reporter mouse

Analysis by southern blotting was used to screen mice that contained the BAC (I, Fig. 1B). When CD4+ T cells from separate founder lines were cultured under Th2 conditions, it was found that the production of IL-4 and DsRed correlated well after restimulation with PMA and ionomycin (I, Fig. E1, online supplementary). A line that had approximately five copies of BAC was chosen for further studies. BMMCs from three mice were stimulated with IL-33 on day 40 of the culture and cells were sorted based on their expression of DsRed. Separate cultures were incubated overnight and the level of IL-13 was measured on the supernatant and compared to DsRed brightness. Additionally, DsRed mRNA expression was compared to IL-13 mRNA from two mice after IL-33 stimulation on day 21 of culture (I, Fig. C-D).

Based on the good correlation between DsRed brightness and IL-13 protein, as well as DeRed mRNA to IL-13 mRNA, DsRed expression was considered a good surrogate for the expression of IL-13.

5.1.2 Peritoneal mast cells require IL-3 and IL-33 for IL-13 production

We chose to study peritoneal c-Kit+FcεRI+ mast cells freshly *ex vivo* (see gating strategy in I, Fig. E2), as bone marrow derived mast cells are cultured in the continuous presence of IL-3, a STAT5 activator that could synergize with IL-33 in the production of IL-13. After 16 hours of stimulation with IL-1, IL-18 or IL-33 with or without IL-3 the expression of DsRed was measured with flow cytometry (I, Fig. 2A). No DsRed expression was observed in WT mice or in unstimulated transgenic (TG) mice (background 1.3%±0.3% positive cells). Neither IL-1 or IL-18 (n=2) induced any IL-13 above the background, whereas IL-3 (n=2) and IL-33 (n=3) alone induced only a very small increase in DsRed expression (approximately 5-6%). IL-3+IL-18 (n=2) induced about 5% cells to become positive with DsRed and IL+1-IL-3 (n=2) raised the proportion of positive cells to 10%. When IL-3 was combined with IL-33 (n=3), nearly half of the cells expressed DsRed (46.7%±4.8%).

We next cultured peritoneal mast cells in the presence of IL-33 and other STAT5 activators: IL-5, GM-CSF, TSLP and IL-7 (I, Fig. 2B). The addition of IL-7 and GM-CSF induced a positive response in 11 and 13% of the cells, respectively, whereas IL-33, IL-5 and TSLP had no effect. The effect of IL-3 was paralleled in pSTAT5 phosphorylation measurements (n=2), as only IL-3 was able to induce a considerable pSTAT5 response (I, Fig. 2C).

5.1.3 Bone marrow derived mast cells and basophils respond to IL-33 by producing IL-13

Bone marrow cells were cultured for 10-12 (n=3) days and mast cells (c-Kit+FcεRI+) and basophils (c-Kit-FcεRI+) were analyzed by flow cytometry after 16-hour stimulations with IL-1, IL-18 or IL-33 with or without IL-3. In mast cells (Fig. 3A), there was a basal level of DsRed expression of approximately 11% (cells positive for DsRed) and only stimulation with IL-33 induced a strong expression of DsRed (69%±9%). When these cells were cultured for 40 days (n=3), the background level decreased to approximately 5% and IL-1 and IL-18 showed a modest (10-20%) increase in the number of DsRed positive cells (I, Fig. E3).

In basophils in the 10-12-day cultures, there was a basal level of approximately 19% of DsRed positive cells (Fig. 3B). As basophils express the IL-18 receptor(401), they were able to respond to IL-18 by expressing DsRed (37% positive cells). IL-33 was a slightly poorer DsRed inducer while IL-1 did not induce DsRsd above basal level.

It is important to note, that bone marrow derived cells require continuous presence of IL-3 in the culture as growth or differentiation factor. The basal level of DsRed in these experiments (I, Fig. 3C) is likely a consequence of this as are the high DsRed induction levels of IL-1 family cytokines. It is also possible, that an endogenous source of IL-33 was present in the culture. To test this, we added a neutralizing IL-33 receptor antibody to the culture (n=2). Indeed, the antibody decreased the expression of DsRed in unstimulated mast cells by approximately 50%, showing that an IL-33 source does exist in the culture (I, Fig. 3D).

Additionally, we tested the ability of mature, splenic basophils to respond to IL-1 family cytokines. Interestingly, unlike cultured basophils, splenic basophils were able to upregulate DsRed in response to IL-1 combined with IL-3 (I, Fig. E4).

5.1.4 MyD88 and STAT5 are needed for IL-33-induced IL-13 expression in BMMCs

It has been shown previously, that *MyD88*$^{-/-}$ mouse BMMCs were defective in their response to IL-33(396) and that the mice also had an impaired T cell response to IL-1 and IL-18(388). In addition to this, basophils did not respond to IL-33 with the production of IL-13 in these mice(245). To study the requirement for MyD88 signaling during IL-33 stimulation, we cultured BMMCs from *MyD88*$^{-/-}$ and WT mice (n=2 for both) and measured intracellular IL-13 by flow cytometry. MyD88 KO mice did not express any IL-13 upon stimulation with IL-33, with or without additional IL-3 (I, Fig. 4A). In WT BMMCs, 17-19% of cell became positive for IL-13 after 5-hour stimulation with IL-33 or IL-33+IL-3. *MyD88*$^{-/-}$ basophils parallel this, as stimulation was unable to induce any IL-13 production in them, while in WT basophils 14-15% of cells stained positive for IL-13 after IL-33 stimulation (I, Fig. E6).

Although bone marrow derived mast cells and basophils are dependent on IL-3, in short-term culture it is possible to replace the IL-3 with IL-4 or SCF. We studied the effect of STAT5 phosphorylation in the production of IL-13 by replacing the IL-3 in the culture medium of DsRed TG mice (n=2) with IL-4 for 32 hours. Compared to cells grown in IL-3, the cells in IL-4 showed about 50% less pSTAT5

(I, Fig. 4B). When stimulating these cells with IL-3, IL-33 or both, cells grown in IL-4 showed less DsRed production than cells that were kept in IL-3 (45% vs 64%). Adding IL-3 to the culture abolished this difference (I, Fig. 4C).

We also repeated the experiment but replaced IL-3 with SCF for 48 hours in DsRed (n=3) and WT (n=2) BMMC cultures. After culturing in SCF, the cells upregulated DsRed strongly when stimulated with IL-3+IL-33, but IL-33 alone was a poor inducer of DsRed (I, Fig. E7).

Finally, we examined the effect of MyD88 deficiency on STAT5 phosporylation after stimulation with IL-3 and IL-33. However, there was no difference in mast cells (I, Fig. 4D) or basophils (I, Fig. E8) in the level of pSTAT5.

5.1.5 IL-3 upregulates ST2 in peritoneal mast cells but not in BMMCs

We wanted to test the possibility that the stimulation with IL-3 via STAT5 was affecting IL-13 production by upregulating ST2, as STAT5 activation is essential for ST2 upregulation in Th2 cells(399). Peritoneal mast cells expressed high levels of the receptor even without stimulation, and stimulation with IL-3 for 16 hours induced a 1.5-fold induction in ST2 expression. IL-33 alone had no effect and the combination of IL-3 and IL-33 gave similar results than stimulation with IL-3 alone (I, Fig. 5B and 5C). In cultured BMMCs, adding IL-33 increased ST2 expression 1.4 fold, but when IL-3 together with IL-33 had no further additive effect (I, Fig. 5C). When BMMCs were cultured in SCF for 48 hours, their degree of STAT5 phosphorylation was lowered but ST2 expression remained unchanged (I, Fig. 4B and 5D).

5.2 The expression of IL-7Rα regulates TSLP signaling in mouse dendritic cells (II)

TSLP and IL-7 signal through a receptor complex that consists of a subunit specific for each cytokine (TSLPR for TSLP and γc for IL-7) and a common subunit, IL-7Rα (II, Fig. 1A). Signaling through IL-7Rα is mediated by the phosphorylation of STAT5(131). The main producers of TSLP appear to be epithelial cells at barrier surfaces(113,402). Extensive research has linked TSLP to allergic inflammation: although *TSLPR*$^{-/-}$ mice develop normally(403), their ability to produce an allergic response in an ovalbumin-induced allergy model is impaired(126). TSLP overexpression induces allergic inflammation in the skin and lungs of mice(69,126,132). Dendritic cells have been shown to be targets of TSLP in the

initiation of type 2 responses(68,113,114). However, only migratory DCs from lymph nodes and skin seem to express low levels of IL-7Rα(404) so it seemed likely that there are factors that regulate the expression of IL-7Rα in DCs. In this study, we examined especially the role of IL-4 in the regulation of IL-7Rα. IL-4 is a critical cytokine in type 2 responses(45). We found that freshly isolated mouse splenic DCs are unable to respond to TSLP, but during overnight culture, especially in the presence of IL-4, they upregulate IL-7Rα robustly, without substantial change in the expression of TSLPR or γc. This upregulation results in the phosphorylation of STAT5 following stimulation with IL-7 or TSLP. When administered intraperitoneally to mice, IL-4 decreased IL-7Rα in $CD4^+$ T cells while upregulating it in DCs.

5.2.1 *In vitro* culture renders DCs responsive to TSLP and IL-7

DCs from B6 mouse spleens were enriched as described in materials and methods, and their ability to respond to TSLP and IL-7 freshly *ex vivo* was tested by stimulating them with the cytokines for 15 minutes. As a positive control, GM-CSF was used. After the stimulation the phosphorylation of STAT5 was measured by flow cytometry in $CD11c^{bright}MHCII^{bright}$ DCs (II, Fig. 1B). As expected, GM-CSF produced a clear induction of pSTAT5, while TSLP or IL-7 had no effect (II, Fig. 1C and 1D). Next, we cultured DCs overnight on regular cell culture plates to assess their spontaneous activation(405). We repeated the stimulation with TSLP, IL-7 or GM-CSF and measured pSTAT5. The effect of GM-CSF was comparable to that of the *ex vivo* cells, but additionally, both TSLP and IL-7 induced a marked phosphorylation of STAT5 (II, Fig. 1C and 1D). The increase in pSTAT5 was highly significant for TSLP ($p<0.05$) and IL-7 ($p<0.01$) when compared to the stimulation of freshly isolated cells. Determined by the expression of CD80 and CD86, overnight culturing activated the DCs ($p<0.01$ for CD80 and $p<0.001$ for CD86) (II, Fig. 1E and 1F).

We wanted to examine, if this responsiveness to cytokines was due to an upregulation in the cytokine receptors. We stained IL-7Rα, TSLPR and γc of *ex vivo* and overnight cultured DCs. Culturing resulted only in a modest increase in staining for TSLPR and γc, whereas IL-7Rα was strongly upregulated (from approximately 1% positive cells to 20% positive for the receptor staining). The difference was statistically significant, $p<0.001$ (II, Fig. 2A and 2B).

5.2.2 Upregulation of IL-7Rα is most prominent in $CD4^+CD8^-$ DCs

As splenic $CD11c^{bright}$ DCs can be divided into three subclasses based on their expression of CD4 and CD8 ($CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$ DCs)(406), we analyzed DCs freshly ex vivo and after overnight culturing for their expression of IL-7Rα and CD4 and CD8 (II, Fig. 3A). None of these subclasses expressed IL-7Rα immediately after isolation. After 16 h incubation, IL-7Rα was slightly upregulated in $CD4^-CD8^+$ DCs (<10% positive cells) and clearly more so in $CD4^-CD8^-$ (<20% positive cells). In $CD4^+CD8^-$ DCs, the upregulation was the strongest: approximately 25% of the cells were positive for IL-7Rα. (II, Fig. 3B and 3C). The difference was significant between $CD8^+$ and $CD8^-$ DCs ex vivo ($p<0.01$) and after overnight incubation ($p<0.001$).

5.2.3 IL-4 and LPS enhance IL-7Rα upregulation in DCs *in vitro*

Next, we studied how different soluble factors might affect the expression of IL-7Rα during the overnight culture. IL-7 and IL-4 were of special interest as they downregulate IL-7Rα transcription in T cells(407). We also chose LPS to our experiment as it activates DCs through the TLR4/MyD88/NF-κB-pathway(408). After overnight culturing with medium only, IL-4, IL-7, LPS or IL-4+LPS, splenic $lin^-CD11c^{bright}MHCII^{bright}$ DCs were stained for IL-7Rα (gating in II, Fig. 4A). The effect of stimulations was compared to cells that were cultured in medium alone. IL-7 appeared to decrease the expression of IL-7Rα strongly ($p=0.019$) whereas IL-4 had a dramatically enhancing effect ($p=0.0001$). Adding LPS to the medium increased the expression of IL-7Rα ($p=0.0076$), but when LPS was combined with IL-4, there was no further increase (II, Fig. 4B). The expression of CD80 was upregulated after overnight culturing and IL-7 and LPS enhanced the upregulation, but IL-4 had the opposite effect (II, Fig. 4C).

We also determined the phosphorylation of STAT5 after the overnight stimulations by inducing pSTAT5 with TSLP and IL-7 (II, Fig. 4D). In line with the upregulation of IL-7Rα, we observed an increase in TSLP induced pSTAT5 after the culturing with IL-4 when compared to DCs cultured in medium only ($p=0.0037$). Interestingly, the IL-7Rα downregulation we noticed with the cells that had received IL-7 did not impair TSLP responsiveness. The upregulation of IL-7Rα that was seen with LPS did not enhance the phosphorylation of STAT5 after induction with TSLP or IL-7. In conclusion, only IL-4 was able to significantly increase the amount of pSTAT5.

In mouse lymph node DCs (II, supplemental figure 3), the expression of IL-7Rα was significantly increased after overnight culturing ($p=0.001$). IL-4 seemed to have and additive effect on the upregulation, but this increase was not statistically significant. IL-7 had a decreasing effect ($p=0.0118$). CD80 was upregulated after overnight culturing with IL-7 but not culturing with IL-4 had no effect.

5.2.4 IL-4 does not upregulate IL-7Rα in human blood DCs

We isolated myeloid dendritic cells from the peripheral blood of healthy donors ($n=5$) to examine, if human blood DCs would upregulate IL-7Rα in response to IL-4. Previous studies have shown that human airway mucosal DCs express TSLPR(409) and human peripheral blood myeloid DCs upregulate IL-7Rα and TSLPR during overnight culture(410). HLA-DR^{bright}CD11c^{bright} DCs (gating strategy in II, Fig. 5A) were surface stained and analyzed directly following isolation of after overnight culturing in medium only or with TSLP or IL-4. The cells were only modestly activated in medium only, as determined by the upregulation of CD80, but stimulation with TSLP induced a dramatic upregulation. IL-4 also caused upregulation, but clearly less (II, Fig. 5B and 5C). IL-7Rα was low on freshly isolated cells, but overnight culturing in medium only induced a strong, biphasic upregulation ($p=0.0012$). Adding IL-4 to the culture did not enhance the upregulation, unlike in mouse splenic DCs. Interestingly, TSLP combined with IL-4 prevented the upregulation entirely ($p=0.0024$). TSLP alone did not have such a complete inhibitory effect, but the biphasic upregulation pattern was not observed with it (II, Fig. 5B and 5C).

5.2.5 *In vivo* administration of IL-4 induces IL-7Rα in mouse splenic DCs

After observing the effect IL-4 had on IL-7Rα on splenic DCs *in vitro*, we became interested whether the same effect would happen *in vivo*. We subjected WT B6 mice ($n=5$ per group) to intraperitoneal injections of IL-4 complexed with anti-IL-4 or anti-IL-4 alone. The biological half-life of IL-4 is short and complexing it with anti-IL-4 is known to prolong the time it is available in the mouse body(391). As a positive control we then measured the expression of IL-7Rα in T cells as IL-4 has been shown to downregulate IL-7Rα in these cells(407). Additionally, we also stained IL-4Rα in T cells (T cell gating strategy in II, supplemental Fig. 4). Expectedly, the administration of the complex resulted in the downregulation of IL-7Rα in CD4$^+$

and CD8$^+$ T cells ($p<0.0001$ and $p=0.0013$, respectively). IL-4Rα, in contrast, was strongly upregulated ($p=0.0092$ for CD4$^+$ and $p<0.0001$ for CD8$^+$ T cells) (II, Fig. 6A and 6B). In splenic dendritic cells, complex induced a consistent and statistically significant, albeit small induction of IL-7Rα ($p=0.0005$). As in our in vitro experiments, CD80 was not affected by IL-4 (II, Fig. 6C). Anti-IL-4 had no effect on IL-7Rα. We confirmed that anti-IL-4 was a suitable control for this experiment by comparing the levels of IL-4Rα in the T cells of untreated and anti-IL-4 treated mice. The expression levels were similar in both mouse groups (II, Fig. 6D).

5.3 IgE responses to papain are unaffected in *Rras*$^{-/-}$ mice

The small GTPase R-Ras has multiple roles in in cellular processes such as adhesion, spreading and migration in which intergring ligand binding is crucial(347). Recently, R-Ras has been associated with the regulation of immune functions, namely in the formation of the immune synapse between dendritic cells and T cells. R-Ras KO mice have defects in DC maturation and cytoskeleton functions, resulting in reduced ability to prime T cells(344). Another study demonstrated that *Rras*$^{-/-}$ mice had a less severe disease course in an experimental autoimmune encephalomyelitis (EAE). In these mice, the number of tolerogenic DCs and regulatory T cells was increased(357). Finally, it was shown that R-Ras mediates inflammatory cytokine expression in an experimental skin cancer model(358). These findings prompted us to study further the role of R-Ras in the immune system, especially in type 2 responses as R-Ras deficiency had not yet been studied in this context. To this end, we immunized wild type and *Rras*$^{-/-}$ mice with papain. We found that there was no difference in the ability of WT and *Rras*$^{-/-}$ mice to mount an IgE response to papain. However, untreated *Rras*$^{-/-}$ mice had elevated MHCII expression and decreased CD80 expression in splenic DCs. Additionally, the number of conventional DCs in the lymph nodes of R-Ras KO mice was significantly lower than in WT mice.

5.3.1 Comparison of steady state DC phenotype in WT and R-Ras KO mice

We prepared total splenocytes from the spleens of R-Ras KO and WT mice (n=3 in both groups) and compared the numbers of B and T cells by flow cytometry. As reported earlier(344), R-Ras deficiency did not affect the numbers of these cells (III, Fig. 1A, gating strategy in supplemental Fig. 1A). We then isolated dendritic cells from the spleen and lymph nodes (LN) of KO and WT animals (n=3 in both groups)

as described in the Materials and methods, section 4.4.3. In the LN, the number of migratory DCs was similar between WT and KO, mice, but KO mice had less conventional DCs (p=0.01226) (III, Fig. 1B). Splenic DC numbers showed no difference between WT and KO mice (III, Fig 1C, gating in supplemental Fig. 1D), as shown before(344).

Next, we analyzed the DC surface markers that take part in DC/T cell interactions: CD86, CD80, MCHII and OX40L. In the case of DCs enriched from LN (n=3 for each mouse line), CD86 was decreased in conventional DCs (p=0.0241) of KO mice but not in migratory subsets. CD80 expression was reduced in both conventional (p=0.0078) and migratory DCs (p=0.0176) in KO mice. MHCII and OX40L expression in conventional DC's showed no differences between the two genotypes in these animals (III, Fig. 2A). In splenic DCs (n=3 for each mouse line), CD80 expression was slightly lower (p=0.0207) and MHCII expression was slightly higher in R-Ras KO mice (Fig 2B) in comparison to WT animals. Taken together, the main difference in DCs between WT and R-Ras KO mice is that the conventional DC's from R-Ras KO mice are lower in number and their expression of co-stimulatory molecule CD80 is lower.

5.3.2 Comparison of activated DC phenotype in WT and R-Ras KO mice

We next wanted to learn, how *in vitro* activation of the R-Ras KO DC's would affect the expression of CD86, CD80, MHCII and OX40L. We cultured splenic DC's (n=3 for each mouse line) on tissue culture plates for 16 hours, resulting in spontaneous (i.e. microbe and damage independent) activation of these cells(405). In addition, we supplemented the overnight cultures with TLR3 and TLR4 agonists (poly(I:C) and LPS) or Th2 cytokine IL-4. Interestingly, MHCII expression was higher in R-Ras KO DCs than in WT DCs under all stimulation conditions. After overnight culture in medium only, both CD80 and CD86 expression was slightly lower in R-Ras KO DC's as compared to WT DC's, but after stimulation with poly(I:C), LPS or IL-4 the differences disappeared (III, supplemental Fig. 2A, 2B, 2C and 2D)

5.3.3 Transcription factor and cytokine mRNA analysis in R-Ras KO mice

As R-Ras deficiency attenuates the disease course in EAE(357), we wanted to study if there was a skew towards a certain T helper cell differentiation type by analyzing key transcription factors related to T cell differentiation: Tbet, GATA3, Foxp3 and

RORγ by qPCR. Additionally, we measured the mRNA of IFNγ, IL-13 and IL-10. There was no difference between WT and KO mice (III, Fig. 2C). The mRNA level of IL-17A was undetectable in both mouse groups (n=4 for both mouse lines).

5.3.4 R-Ras deficiency does not influence papain-induced IgE expression

To investigate the possible effect of R-Ras deficiency on allergen-induced IgE expression, we subjected R-Ras KO mice and littermate WT animals (n=8 for each genotype) to papain immunization and measured the serum level of IgE as a read-out of type 2 response *in vivo*. The papain immunization protocol consists of two immunizations, on day 0 and day 14. We measured serum IgE levels on day 0 and again, when the mice were euthanized on day 16. There was no significant difference between the two genotypes in the amount of IgE. (III, Fig. 3A). To rule out the possibility that type 2 responses might be delayed in R-Ras KO mice due to the impaired immune synapse formation(344), we measured the IgE levels of WT and KO mice (n=17 and 18, respectively) two days after the second injection (on day 16). However, there was no difference in IgE levels between WT and KO animals (III, Fig. 3B).

5.3.5 ST2 expression is decreased in CD4$^+$ T cells in papain-immunized KO mice

After the immunization with papain, we examined the expression of IL-4Rα on splenic CD4$^+$ and CD8$^+$ T cells with flow cytometry, as IL-4 is a critical cytokine for type 2 responses. There was no difference in the receptor expression between WT and KO mice (n=5 in both groups) (III, Fig. 3E). We also stained IL-33 receptor ST2, because it has been shown to be upregulated during type 2 responses and it regulates the production of cytokines such as IL-5 and IL-13 in Th2 cells(399). In the CD4$^+$ T cells of R-Ras KO mice, the amount of cells positive for ST2 was decreased (III, Fig. 3F, gating strategy in supplemental figure 1B).

5.3.6 Comparison of DC phenotype in papain-immunized WT and KO mice

To study the phenotype of D's in the immunized animals, we determined the number of LN (n=4, each mouse line) and splenic DCs (n=5, each mouse line) on day 16 euthanized mice. Unlike in unmanipulated mice, which showed a clear difference in

the number of conventional DCs between WT and R-Ras KO animals (Fig 1B), we found no difference in the number of migratory or conventional DCs (III, Fig. 3C) in papain-treated animals. Similarly, the number of splenic DCs were alike in WT and R-Ras KO mice 16 days after the first immunization (III, Fig 3D).

Next we analyzed the activation markers of DCs enriched from LN and spleen on day 16. In the conventional and migratory DCs from the LN, no difference between the two genotypes was observed in the expression of CD86, CD80, MHCII or OX40L (III, Fig 4A). In splenic DCs, CD86 expression was slightly lower in R-Ras KO DCs (Fig 4B). MHCII and CD80 were similar in both mouse groups

5.3.7 RORγ mRNA expression is reduced in papain-immunized KO mice

To comprehend if papain immunization following Th cell differentiation was affected by R-Ras deficiency, we repeated the qPCR analysis of section 5.3.3 on day 16 of immunization on WT and KO animals (n=5 for both mouse lines). The expression levels of GATA3, Tbet and Foxp3 mRNA were identical between WT and R-Ras KO mice. The expression of mRNA for RORγ, the master regulator of Th17-differentuation, was slightly lower ($p=0.0492$) in the R-Ras KO mice. Cytokine mRNA analysis from the same samples indicated no difference between IFNγ, IL-13 or IL-10 (III, Fig 4C). Levels of IL-17A were undetectable in both mouse groups.

6 DISCUSSION

6.1 Cytokine signaling is in a key role in immune responses (I, II)

In this study, we have explored cytokine signaling from two perspectives: in study I, we showed by using a transgenic DsRed reporter mouse, that IL-13 production in mast cells requires a signal from two cytokines, IL-3 and IL-33. In Study II, we proposed that dendritic cells become responsive to TSLP by IL-4 stimulation. These findings fit in the principle of a two-tiered response, where cells that act as sensors in the beginning of an immune response, react to stimulus, such as allergen, by secreting "level 1" cytokines. These cytokines act on a second category of cells, causing them to begin the production of "level 2" cytokines that activate a final category of cells that perform actual effector functions, be it antibody production, pathogen killing and expulsion actions or tissue repair(15). For type 2 responses, barrier epithelial cells are a typical sensor and they react to insults by producing cytokines TSLP, IL-25, IL-33 and chemokines(5). The targets of these cytokines and chemokines are multiple, including innate lymphoid cells, T helper cells, dendritic cells, basophils, eosinophils and mast cells (229). These cells are the source of "level 2" cytokines, such as IL-4, IL-5, IL-9, IL-13, and AREG that act on cells which execute effector functions (229). Often the effector cells are the same cells that also act as sensors, but they are now equipped with new, distinct functions. For example, IL-13 mediates goblet cell metaplasia and mucus secretion by ECs(411). Many times the "level 1" cytokines work in pairs consisting of a STAT inducer (such as IL-6, IL-7 and TSLP) and a IL-1 cytokine (IL-1, IL-18 and IL-33)(412).

6.1.1 Mouse mast cells require IL-3 and IL-33 for efficient production of IL-13 (I)

In study I, we elucidated the mechanisms by which mast cells produce the effector cytokine IL-13. In the context of allergic inflammation, an important role for IL-13 has been established in airway hypersensitivity(411) and in atopic eczema(413)). It has been shown previously that the amount of available IL-13 is the limiting factor

in responses caused by the cytokine(102), so identifying the mechanisms that affect the production of IL-13 are of interest.

There is ample evidence that in T cells, a challenge with an IL-1 cytokine combined with a STAT inducer generates the production of other cytokines(412). Resting Th2 cells are able to produce IL-5 and IL-13, but not IL-4, in response to stimulation with IL-33 and IL-2, IL-7 or TSLP(399). Following this model, in our experiments mouse peritoneal mast cells produced IL-13 only when IL-3 was administered together with IL-33. It seemed that bone marrow derived mast cells responded differently, as only the addition of IL-33 was able to make them positive for DsRed that was a surrogate for IL-13 production. However, it would seem that this was rather a consequence of technical culturing requirements than actual biological fact, as BMMCs are grown in the continuous presence of IL-3 in the medium. When BMMCs were cultured in weaker STAT5 inducers IL-4 or SCF, the addition of IL-33 resulted in a weaker DsRed induction. In these cultures, restoring IL-3 to the medium raised the level of induced DsRed to that of cells that had received IL-3 continuously. Although our results show that both IL-3 and IL-33 are needed for efficient IL-13 production, without a STAT5 knockout mouse we cannot absolutely confirm that it is STAT5 that is essential for IL-13 production. Unfortunately, STAT5 is a critical factor for mast cell development and the STAT5 knockout mice are unable to produce peritoneal cavity mast cells(414). Similar to mast cells, the production of IL-13 in bone marrow derived basophils seems to rely on the combination of IL-3 and IL-33. Additionally, IL-18 could be used in substitution of IL-33. Previous work had identified IL-33 as a better inducer of IL-13 than IL-18(398), but we are unable to completely explain this difference compared to our results. Some technical differences between the two experimental settings could result in the observed difference. For example, the sorting of basophils could have activated them and increased their expression of ST2 which could increase the sensitivity to IL-33, or it could be related to the staining of different markers and gating of slightly different parts of basophil populations.

It would seem plausible that the signaling intermediates of IL-33 act directly on the *Il13* promoter as the functional inhibition of NF-κB in Th2 cells causes an immediate decrease in IL-33 induced IL-13 production(399).

It is interesting that neither mast cells nor bone marrow-derived basophils produced IL-13 when stimulated simultaneously with IL-1 and IL-3. Mast cells were also nonresponsive to IL-18, which can be explained by their lack of receptor expression. The lL-1 family cytokines, including IL-1α and β, are strongly associated with various inflammatory settings and it can be argued that they act as

DAMPs(415,416). It is likely that the different responsiveness of mast cells to IL-1, IL-18 and IL-33 reflects the versatile roles mast cells can take under varying stimulation conditions during infection and inflammation(reviewed in(417)). Indeed, studies have demonstrated that mouse mast cells can respond to IL-1 even by secreting type 2 cytokines, but this requires prior activation by ionomycin or cross-linkage of FcεRI(418). Mouse BMMCs also produce IL-6 after stimulation with IL-1β, but this too requires co-stimulation with IL-10 or SCF(419). This kind of co-stimulation was not included in our experiments. Studies in human mast cells are in support of this view of required additional stimulus(420,421). In our supplementary data we showed that mature splenic basophils actually were able to produce IL-13 in response to IL-1. Why this was not seen in cultured basophils is hard to explain, but is a reminder to the fact that cell differentiated in artificial cultures are never completely comparable to primary cells.

In mice, IL-3 is not absolutely necessary for mast cell generation but it is essential for the expansion of tissue mast cells and blood basophils and immunity against the nematode *Strongyloides venezuelensis*(422). During *Nippostrongylus brasiliensis* infection, T cell derived IL-3 enhanced the production and peripheral accumulation of basophils as demonstrated by the inability of IL-3 deficient or Rag2 KO mice to mount such a response. However, the steady state level of basophils is unaffected by the deficiency of IL-3(299). Additionally, IL-3 can enhance FcεRI-dependent production of IL-4 in basophils and FcεRI-dependent activation and mediator secretion in mast cells *in vitro*(298). The hypothesis that IL-3 controlled peripheral basofilia was supported by the observation that during *N. brasiliens* infection the recruitment of basophils to lymph nodes was dependent on IL-3(79). Although not usually regarded as a Th2 cytokine, IL-3 is a part of the orchestration of Th2 responses due to its important role in regulating mast cell and basophil functions and our results offer an interesting addition to how IL-3 affects these cells.

The role of mast cell derived IL-13 has not yet been fully investigated. In the case of mast cell derived IL-4, research has shown that it contributes to Th2 responses and Th1 responses(423,424), but is not essential. Unlike in Th2 cells(399), in mast cells the expression of the IL-33 receptor ST2 is inherently high and not affected by STAT5 phosphorylation or deficiency of MyD88. In peritoneal mast cells, but not in BMMCs, IL-3 increases the receptor expression to approximately 1.5-fold. Furthermore, IL-33 itself does not upregulate ST2 in peritoneal mast cells. This difference between mast cells and Th2 cells likely reflects the role mast cells have in the early phases of type 2 responses as they respond to "level 1" cytokines, such as IL-33, produced as danger signals from epithelial cells whereas Th2 cells might

become responsive to IL-33 later in the response cascade, after induction by innate stimuli. The IL-13 produced by mast cells is likely to represent a "level 2" cytokine response, but the exact impact of it remains to be studied.

6.1.2 The expression of IL-7Rα regulates TSLP signaling in mouse dendritic cells (II)

In study II, we investigated how the TSLP and IL-7 receptor subunit IL-7Rα is regulated on dendritic cells that are implicated as direct targets of TSLP. It has been suggested that dendritic cells induce type 2 responses upon stimulation with TSLP, possibly through upregulation of OX40L(68,425). As reported earlier, IL-7Rα was expressed only in a small portion of splenic or LN DCs(404,410) which was mirrored in their inability to phosphorylate STAT5 when stimulated with IL-7 or TSLP. Also in accordance to a previous study(405), DCs were spontaneously activated during overnight culture, upregulating IL-7Rα. In our experiments, this upregulation enabled STAT5 phosphorylation through signaling via the receptor.

If dendritic cells truly are targets of TLSP, it is interesting that their expression of IL-7Rα is very low. This would mean that a mechanism for upregulating the expression of the receptor must exist. Mechanisms that induce TSLP itself are known to some extent. It is released mainly by ECs following trauma or after stimulation with microbial products and TLR agonists, inflammatory cytokines TNF, IL-4 and IL-13 and vitamin D3(113,114,127,426,427). The factors affecting IL-7Rα regulation on DCs are less clear. In our in vitro experiments, IL-4 and LPS enhanced IL-7Rα induction. It is possible that microbe-derived LPS or other TLR4 agonists, or TLR4 stimulating allergens(115,427) not only trigger TSLP production by ECs but also render DCs responsive to it by upregulating IL-7Rα. This might be important in asthma, in which acute exacerbations are often caused by infections(428). In a somewhat similar manner, early IL-4 from basophils(67) might act on both ECs and DCs, orchestrating locally both the production and responsiveness to TSLP.

We noticed that IL-7 seemed to inhibit IL-7Rα expression, but it should be considered that this might be a technical issue rather than true downregulation. It is possible that the cytokine IL-7 added to the culture medium blocks the binding of the antibody that was used in flow cytometric staining. If the receptor was downregulated we would have expected to see less pSTAT5 upon TSLP stimulation, but the notion that pSTAT5 still was induced supports the theory that the receptor still is available at the surface of the cell for signaling, only inaccessible for staining.

The other discrepancy in our results was the upregulation induced by IL-4 that did not seem to translate into STAT5 phosphorylation by IL-7 stimulation. This could be explained by the receptor subunit γc that is shared between IL-4 and IL-7. IL-4 in the medium could prevent IL-7 from binding into it, thus lessening the signaling of IL-7 through γc-IL-7Rα complex. Related to the issue of bioavailability, the expression of IL-7Rα on DCs could serve as a way to reduce the availability of IL-7 in biological fluids, a mechanism that has been speculated with T cell IL-7 receptor expression(407).

We found some intriguing differences between mouse splenic and human blood DCs. It has been shown that both IL-7Rα and TSLPR are upregulated in human blood conventional DCs during overnight culture(409,410) and unlike murine splenic DCs, human blood DCs are negative for TSLPR freshly *ex vivo*. This would suggest that there are differences in the regulation of TSLPR between the two anatomic locations, or alternatively, between the two organisms, stemming either from genetic differences or even from infectious background differences between humans and mice in standardized housing facilities. We found that although overnight culturing upregulates IL-7Rα in human DCs, the additive effect of IL-4 that was seen in murine DCs was not present in human cells. The expression of CD80 was also rather low on human freshly isolated DCs and overnight culturing only modestly upregulated it. Compared to murine splenic resident DCs and human tissue DCs, blood DCs subsets represent immature precursor forms that corresponds to those in tissues but with a less activated phenotype(429,430). It is worth noting that human DCs in different anatomical locations vary in their expression of TSLPR(409,410) and that the ability to respond to TSLP could be restricted to mature DCs in the vicinity of barrier epithelial surfaces. It could even be that DCs in the blood are under inhibitory regulation that prevents them from inappropriate Th2 polarization in response to TSLP while in circulation.

In the future, more extensive work on identifying different anatomic human DC populations capable of TSLP responses as well as expressing TSLPR and IL-7Rα are warranted. In mouse, studies with IL-4, IL-4Rα, IRS-2 and STAT6 deficient mice would elucidate the regulation of IL-7Rα by IL-4. Lastly, it should be considered that an alternative receptor for TSLP exists. A recent study indicated that TSLP acts on non-hematopoietic cells as well(122), even though IL-7Rα has been considered to be expressed on hematopoietic cells only(431). TSLPR is abundantly expressed on murine B cells (Junttila IS, Leonard WJ and Paul WE unpublished observation) so it would be interesting to study if B cells of mice deficient in IL-7Rα would still be able to respond to TSLP and would this response be dependent on STAT5.

Genome-wide transcription analysis and cellular phosphorylation analysis in WT and IL-7Rα deficient mice would clarify the issue.

6.2 IgE responses to papain are unaffected in *Rras*$^{-/-}$ mice (III)

In the last part of the study, we have investigated the effect of the deletion of *Rras* in mice on the capability to mount an IgE antibody response. Several reports have verified a role for the proteins of Ras superfamily in the immune system and DC biology(432) but the role of R-Ras is elusive. Functionally, R-Ras has been shown to regulate the immunological synapse; the physical crosstalk of DC and T-cell, and DCs lacking R-Ras induce poorer proliferation of T-cells than their WT counterparts in response to allogenic stimulation. This is likely due to a defect in actin cytoskeleton organization that is crucial for DC maturation and stable interactions with T cells(344). The formation of the synapse regulates the direction of ensuing immune response. *In vitro*, the strength of TCR-engagement in cytokine-free environment regulates T-cell differentiation. Weak TCR-signal leads to spontaneous IL-4 production while strong TCR signal causes IFNγ production(59). We argued that this could, in principle, suggest that R-Ras deficient mice would have an inborn skew towards type 2 responses. Interestingly, the clinical course of EAE, a mouse model of multiple sclerosis, is less severe in *Rras*$^{-/-}$ mice than in WT mice, but this has been attributed to a local decrease in the number of naturally occurring Tregs and tolerogenic DCs(357) in *Rras*$^{-/-}$ mice. Also, in the skin of *Rras*$^{-/-}$ mice, a lowered mRNA expression of pro-inflammatory cytokines IL-1α, IL-6 and IL-17A has been reported(358).

We chose to study the induction of type 2 responses in *Rras*$^{-/-}$ utilizing papain, a cysteine protease that induces type 2 responses via the cooperation of IL-4 producing basophils and DCs. Papain triggers reactive oxygen species (ROS) production in DCs and epithelia cells, and ROS promotes TSLP production via TLR4. ROS also stimulates basophil-attracting CCL7 production from DCs(74). Although we were unable to show any differences between WT and KO mice in the production of IgE after papain immunization, we observed some differences in the DC activation markers. Contrary to the results by Singh and colleagues(344), we found elevated MHCII levels in splenic DCs of R-Ras KO mice both in the unmanipulated state and after stimulation with LPS, Poly(I:C) and IL-4, but no difference was found in CD80 and CD86 expression after the stimulations. CD80 and/or CD86 co-stimulatory molecules were slightly less expressed in DCs of the

LN and spleen during steady state. When DC's were analyzed on day 16 from mice undergoing papain-induced immune response *in vivo*, we observed a similar trend; elevated MHCII expression and slightly reduced CD86 expression in splenic DC's, but no difference in LN DCs. Elevated MHCII expression coupled with decreased CD80/CD86 expression would support the view, that R-Ras deficient mice are not incapable of presenting antigens to T-cells, but the formation of the immunological synapse could be impaired since the crosslinking between T cell CD28 and DC CD80/CD86 is inefficient due to lower expression of CD80/CD86 in R-Ras KO mice. It is possible that R-Ras affects the clustering of some of the co-stimulatory molecules, hampering their function. The defect in CD28 signaling, would then lead to decreased intracellular protein kinase C and Akt activation, ultimately leading to defects in NF-κB-mediated transcription. However, in the context of type 2 response, it seems that this impairment of immunological synapse is not critical for the completion of the response.

We found no difference in the expression of transcription factors associated with Th1/Th2 polarization between WT and R-Ras deficient mice during steady state or papain-induced immune response *in vivo*. However, the notion that RORγ-transcription factor was expressed at lower levels in R-Ras KO mice after immunization, when compared to WT counterparts, is of interest. As IL-17 production is decreased in CNS of *Rras*$^{-/-}$ mice during EAE(357) or carcinogen tumor development(358) and RORγ is known to play a critical role in the development of IL-17 producing CD4+ T-cells(36), this finding was logical. There is also evidence that papain is able to generate Th17 responses by inducing allergen specific Th17 cells when administered epicutaneously(433).

While we noticed no difference in the expression of IL-4Rα between the two genotypes in CD4^{+} or CD8^{+} cells, we did find that the percent of cells positive for the IL-33 receptor, ST2, was moderately lower on R-Ras KO mice. ST2 is upregulated in Th2 cells during type 2 responses and in Th2 cells it can regulate cytokines such as IL-5 and IL-13 together with a STAT5 activator(399). Papain-induced IgE production on mouse lung(195) and genital tract(335) has been shown to be dependent on IL-33 signaling. However, in the skin, the role of IL-33 in allergic sensitization is less clear(334). This difference in the expression of ST2 did not seem to affect IgE production in *Rras*$^{-/-}$ mice in our experiments, but although Il-4-mediated events (IgE production and IL-4Rα expression) were unaffected in KO mice, R-Ras deletion might still affect IL-4 independent, but GATA-3 dependent (such as ST2 expression) processes.

As far as we know, this study has been the first one to investigate the role of R-Ras during allergic sensitization and type 2 responses. Although only minor differences were found between wild type and KO phenotype mice, more work is still needed to define the exact role for R-Ras in type 2 immunity. For example, helminth infection models both IL-4 dependent and independent (such as *Trichurus muris* and *Nippostrongylus brasiliensis*) could be utilized. Additionally, given the possible defect in IL-17 mediated responses in R-Ras KO mice and the notion that papain is able to induce Th17 cells(433), it would be of interest to study if Th17 responses are affected in R-Ras KO mice after papain sensitization.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

Orchestrating allergic type 2 responses requires complex interactions between the cells of the innate and adaptive immune system. In this present study, we have investigated how type 2 cytokines IL-4, IL-3, IL-33 and TSLP signal in myeloid cells. As outlined in the section 3, "Aims of the Study":

1. Our aim was to study cytokine-induced production of IL-13 by mouse mast cells and basophils. In our study, we showed that IL-3 synergizes with IL-33 in the production of IL-13 in mouse mast cells and basophils, thus adding a new piece to the puzzle of how allergic inflammation might be established.
2. Our second aim was to examine the role of IL-7Rα in the regulation of sensitivity to TSLP in dendritic cells. To this end, we suggested a mechanism that via the upregulation of IL-7Rα, IL-4 could yield mouse dendritic cells responsive for TSLP, which in turn mediates the Th2 polarizing and T cell priming functions of DCs in the beginning stages of allergic responses.
3. Lastly, we aimed to investigate the effect of R-Ras in the development of allergic inflammation. We studied if small GTPase R-Ras would be involved in IgE responses and DC activation during a papain-induced allergy model in mice, but found little evidence of this.

As the burden of allergic diseases increases in modern societies, new treatment options for allergic inflammation are urgently needed. The expanding knowledge of the molecular mechanisms underlying type 2 responses has given rise to new treatments, including inhibitors of cytokines, their receptors and downstream signaling molecules, such as Jak-kinases. However, many of these innovations have been somewhat disappointing so far and more studies must be performed.

The recent discovery of innate lymphoid cells has been paradigm changing for the way type 2 responses are seen, explaining why IL-4 seems dispensable *in vivo* and how type 2 cytokines are induced even in the absence of TCR stimulation. Elucidating the role of epithelial cell derived cytokines, ILCs and other innate cells in the orchestration of allergic inflammation still requires great amounts of research. In the future, the cooperation of IL-33 and STAT5-inducing cytokines will be further

investigated. Studies will be aimed at revealing how IL-7Rα is regulated in allergic cytokine environment, for example in the papain model. As the exact mechanisms of papain-induced type 2 responses are still unclear, future experiments focusing on the role of IL-25 and pattern recognition receptors, such as toll-like receptors, in the response are likely to prove fruitful.

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10 ORIGINAL COMMUNICATIONS

Efficient cytokine-induced IL-13 production by mast cells requires both IL-33 and IL-3

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Background: IL-13 is a critical effector cytokine for allergic inflammation. It is produced by several cell types, including mast cells, basophils, and T_H2 cells. In mast cells and basophils its induction can be stimulated by cross-linkage of immunoglobulin receptors or cytokines. The IL-1 family members IL-33 and IL-18 have been linked to induction of IL-13 production by mast cells and basophils. In CD4 T_H2 cells IL-33–mediated production of IL-13 requires simultaneous signal transducer and activator of transcription (STAT) 5 activation.

Objective: Here we have addressed whether cytokine-induced IL-13 production in mast cells and basophils follows the same logic as in T_H2 cells: requirement of 2 separate signals.

Methods: By generating a bacterial artificial chromosome (BAC) transgenic IL-13 reporter mouse, we measured IL-13 production in mast cells and basophils.

Results: In mast cells harvested from peritoneal cavities, 2 cytokine signals are required for IL-13 production: IL-33 and IL-3. In bone marrow mast cells IL-13 production requires IL-33, but the requirement for a STAT5 inducer is difficult to evaluate because these cells require the continuous presence of IL-3 (a STAT5 activator) for survival. Poorer STAT5 inducers in culture (IL-4 or stem cell factor) result in less IL-13 production on IL-33 challenge, but the addition of exogenous IL-3 enhances IL-13 production. This implies that bone marrow–derived mast cells, like peritoneal mast cells and T_H2 cells, require stimulation both by an IL-1 family member and a STAT5 inducer to secrete IL-13. Basophils follow the same rule; splenic basophils produce IL-13 in response to IL-18 or IL-33 plus IL-3.

Conclusion: Optimal IL-13 production from mast cells and basophils requires 2 cytokine signals. (J Allergy Clin Immunol 2013;132:704-12.)

Key words: *Mast cells, basophils, allergic inflammation, cytokines, signal transducer and activator of transcription 5, tyrosine phosphorylation, BAC transgenic mouse, DsRed fluorochrome*

Mast cells are tissue-based effector cells of innate immunity. They participate in tolerance induction and immune/allergic inflammatory responses by secreting soluble factors that regulate responses of cells in tissues.[1,2] Among these factors are the type I cytokine IL-13 and, to a lesser extent, IL-4. Both these cytokines initiate airway hypersensitivity.[3,4] However, in mice that are actively immunized and then challenged with the immunogen, IL-13 is the principal inducer of allergic inflammation; IL-4 appears to play its major role in the induction of T_H2 responses.[5]

In keeping with IL-13's role as a major mediator of allergic inflammation, mast cells produce more IL-13 than IL-4 in response to cross-linkage of FcεRI[6] or treatment with ionomycin.[7] Importantly, mast cells also secrete IL-13 when challenged with certain cytokines.[8] IL-13 and IL-4 are also expressed in basophils independently of FcεRI activation in response to cytokines.[9,10] Such cytokine-induced cytokine production could be of importance in propagating allergic inflammation after an inciting allergen has been eliminated.

Cytokine-induced cytokine production appears to be a general phenomenon seen in CD4 T_H cells, natural killer T cells, and innate lymphoid cells, as well as mast cells and basophils.[11,12] In T_H1/T_H2/T_H17 cells cytokine-induced cytokine production depends on stimulation by 2 agents, one of which activates a member of the IL-1 family of receptors, with the other inducing signal transducer and activator of transcription (STAT) activation.[11] In mast cells IL-33 appears to be the critical IL-1 family member for functional modulation. *In vivo* injection of IL-33 in the mouse ear induces an inflammatory skin lesion that is significantly reduced in mice that lack mast cells.[13] Furthermore, *in vivo* injection of IL-33 in *Rag-2*$^{-/-}$ mice induces airway hyperreactivity and goblet cell hyperplasia and increases the production of IL-4, IL-5, and IL-13 independently of lymphocytes.[10] *In vitro* type I cytokine production by mast cells has been reported to be mediated simply by the addition of the IL-1 family member IL-33.[8] Here we show that mast cells and basophils also follow the "2-signal model," with IL-13 production dependent on an IL-1 family member and an inducer of STAT5, mainly IL-3, in both mast cells and basophils. In keeping with the basophil's expression of both IL-18 receptor (IL-18R) α[14] and IL-33 receptor, both IL-18 and IL-33 are active in these cells; mast cells, expressing very low levels of IL-18Rα but high levels of IL-33 receptor, respond to IL-33 but not IL-18.

METHODS

DsRed transgenic mice

Transgenic mice expressing DsRed under IL-13 regulatory elements were prepared by using bacterial artificial chromosome (BAC) recombineering

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Abbreviations used
BAC: Bacterial artificial chromosome
BMMC: Bone marrow–derived mast cell
IL-18R: IL-18 receptor
SCF: Stem cell factor
STAT: Signal transducer and activator of transcription
WT: Wild-type

technology with *galK*-selection.[15] The BAC clone (BAC172) contains the *Il4*, *Il13*, and *Il5* genes and the T_H2 locus control region. The ATG of the *Il13* gene in the BAC172 clone was targeted with a *galK* construct containing homology arms at both the 5′ and 3′ ends of the *galK* gene that had *Bam*HI restriction sites. *galK* was subsequently targeted with a DsRed (Clontech, Mountain View, Calif) construct. The new BAC172 construct was fully sequenced between the 5′ and 3′ homology regions. Microinjection of the construct into B6 oocytes was followed by transfer into pseudopregnant foster mothers. Genomic DNA of 121 tentative IL-13/DsRed–transgenic pups was digested with *Bam*HI, separated on 0.8% agarose gel, transferred to a nylon membrane, and probed with a 916-bp PCR fragment spanning the 5′ and 3′ homology arms of the *Il13*/DsRed construct.

For correlation of DsRed expression with simultaneous IL-4 expression in T_H2 cells, mice born from the founders were bled, blood cells were plated under T_H2 conditions for 3 days and stimulated with phorbol 12-myristate 13-acetate/ionomycin for 4 hours, and cells were stained for CD4 and IL-4.

Mice

Wild-type (WT) C57BL/6 mice were from Jackson Laboratories (Bar Harbor, Me) or Taconic Farms (Hudson, NY). B6 *MyD88*$^{-/-}$ mice were from Dr R. Caspi (National Eye Institute, Bethesda, Md), with permission from Professor S. Akira (Osaka University, Osaka, Japan).[16] Mice were housed at the National Institute of Allergy and Infectious Diseases pathogen-free animal facility, and all experiments were done under a protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Cell culture

Bone marrow–derived cells were prepared by culturing red cell–depleted bone marrow cell suspensions in 20 ng/mL IL-3 for 10 to 40 days. The cells were used between days 10 and 12 to obtain basophils. In IL-33 receptor blocking experiments unlabeled T1-ST2 antibody (10 μg/mL; MD Bioscience, St Paul, Minn) was added to the culture. Peritoneal cavities of mice were flushed with PBS containing 2 mmol/L EDTA; plated in 6-well plates in RPMI containing 2% FBS, penicillin/streptomycin, and L-glutamine (2 mmol/L); and maintained at 37°C with 5% CO_2 to study peritoneal cells. T_H2 and T_H17 *in vitro* differentiation was performed and evaluated, as previously described.[11] Briefly, naive CD4 T cells were purified, cultured under specific T_H2 or T_H17 culture conditions for 4 days, and rested for 3 days, and then after restimulation with phorbol 12-myristate 13-acetate/ionomycin, the production of IL-17 or IL-4 was measured by using RNA analysis, cytoplasmic anticytokine staining, and ELISA.

Cell stimulation, flow cytometry, and quantitative PCR

Cells were stimulated with IL-1β, IL-33, IL-3, IL-5, GM-CSF, stem cell factor (SCF; PeproTech, Rocky Hill, NJ), thymic stromal lymphopoietin, IL-7 (R&D Systems, Minneapolis, Minn), or IL-18 (MBL International, Woburn, Mass), as indicated. All stainings for CD4, c-Kit, FcεRI (eBioscience, San Diego, Calif), IL-18Rα (BioLegend, San Diego, Calif), and IL-33 receptor/T1-ST2 (MD Biosciences) were performed in the presence of the FcγR II and III blocking antibody 2.4G2 and 0.1% mouse serum. Phospho-STAT5 staining was performed, as previously described,[17] IL-6 and IL-13 staining was performed with 0.5% Triton X in staining buffer. Anti–IL-6 and anti–IL-13 antibodies were purchased from eBioscience, and anti–phospho-STAT5 was purchased from BD (Franklin Lakes, NJ). For analysis of DsRed expression in bone marrow–derived mast cells (BMMCs) and bone marrow–derived basophils, the cultures were washed twice with PBS and plated with indicated cytokines for 16 hours. The unstimulated wells contained a basal level of IL-3 to maintain cell viability. For IL-4 and SCF cultures of bone marrow cells, cells were washed with PBS 3 times and grown thereafter either in IL-3–, IL-4–, or SCF-containing media, as indicated. In subsequent analysis a live cell gate was used.

Cell sorting was performed with FACSDiva software (BD). DsRed and IL-13 mRNA was measured after cell sorting by using isolating total RNA with an RNeasy kit (Qiagen, Hilden, Germany), and IL-13 protein was measured by using ELISA (R&D Systems). For measurement of RNA expression, total RNAs were reverse transcribed to cDNA by using SuperScript II First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, Calif). Quantitative PCR reactions were performed with a 7900HT sequence detection system (Applied Biosystems, Foster City, Calif). The probe sets for IL-3, IL-13 (FAM-MGB probe), and 18s ribosomal RNA (VIC-MGB-probe) were from Applied Biosystems. All mRNA levels were normalized to 18S ribosomal RNA.

RESULTS

Construction of an IL-13 reporter mouse

We used the 120-kbp BAC172/pBACBelo11 construct (kindly provided by Gap Lee and Richard Flavell) containing the *Il4*, *Il13*, and *Il5* genes, as well as the *Il4*/*Il13* locus control region. We inserted a DsRed-encoding construct immediately after the translation-initiating ATG for *Il13* using recombineering technology (Fig 1, *A*, and see the Methods section).[15] BAC-containing mice were screened by means of Southern blotting of their genomic DNA (Fig 1, *B*). CD4 T cells from distinct founder lines were cultured under T_H2 conditions, revealing a good correlation between the proportion of cells that were DsRed$^+$ and those that produced IL-4 on restimulation (see Fig E1 in this article's Online Repository at www.jacionline.org), implying a direct relationship between the number of copies of the transgene and expression of IL-4.

We chose one line, K-1, for further study. It had approximately 5 copies of the recombineered BAC. We analyzed both IL-13 protein production and RNA expression as a function of DsRed brightness of the cells. For protein analysis, we independently stimulated BMMCs from 3 different K-1 mice on day 40 of the culture for 4 hours with IL-33 and then sorted DsRed^{hi}, DsRed^{int}, and DsRed^{lo} BMMCs (c-Kit$^+$/FcεRI$^+$) and continued these separated cultures o/n. We then measured IL-13 concentration in the supernatants (Fig 1, *C*). For RNA expression, we sorted BMMCs from 2 K-1 mice after a 5-hour *in vitro* stimulation with IL-33 on day 21 of culture and measured the relation of IL-13 mRNA expression to DsRed mRNA expression, as determined by using quantitative PCR (Fig 1, *D*). On the basis of the good correlation of both IL-13 RNA and protein to DsRed brightness, we regard DsRed expression as a reliable surrogate for IL-13 expression.

IL-3 is required for IL-33–induced IL-13 production in peritoneal mast cells

It has been reported that addition of IL-33 alone strongly upregulated IL-13 expression in BMMCs.[8,10] IL-33 participates in inducing IL-13 production in CD4 T cells, but a STAT5 signal is also required in T cells.[11] BMMCs are traditionally elicited by culturing bone marrow progenitors in IL-3, a STAT5 activator, and thus the possibility existed that such basal IL-3/STAT5 synergized with IL-33 in inducing IL-13.

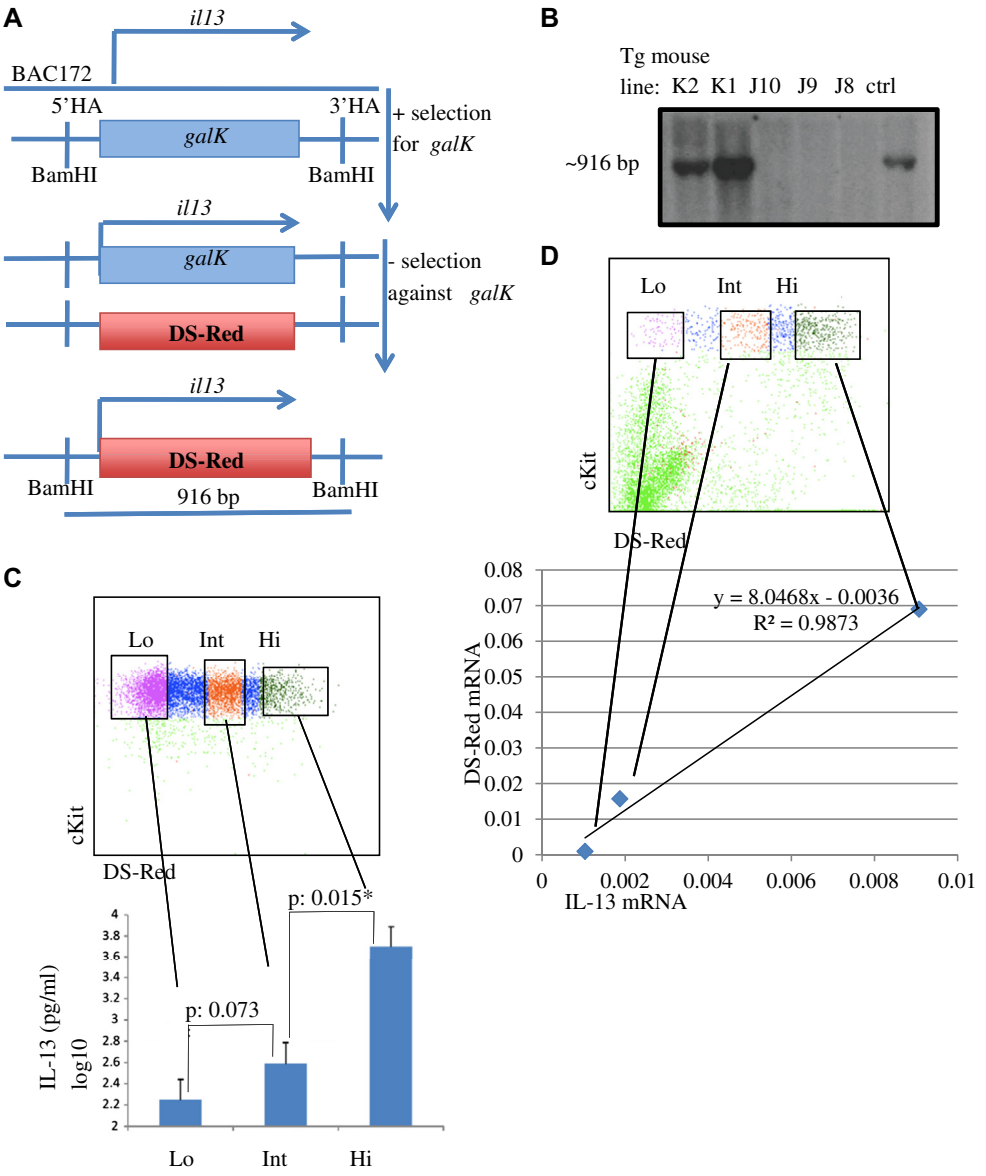

FIG 1. Generation and characterization of IL-13 DsRed reporter mice. **A,** Strategy and transgenic construct used to generate IL-13 DsRed mice. **B,** Southern blot analysis identifying DsRed$^+$ pups. **C,** Correlation of IL-13 protein production with DsRed intensity. **D,** Correlation of IL-13 and DsRed mRNA expression in purified BMMCs.

We tested the cytokine requirements for IL-13 production in mast cells directly harvested from the peritoneal cavity (see Fig E2 in this article's Online Repository at www.jacionline.org). Peritoneal cells were stimulated with nothing or with the IL-1 family members IL-1, IL-18, or IL-33 (10 ng/mL) with or without 10 ng/mL IL-3 for 16 hours (Fig 2, *A*). WT B6 mice showed a background frequency (1% to 3%) of apparently DsRed-expressing cells under all stimulation conditions. Unstimulated c-Kit$^+$/FcεRI$^+$ peritoneal cells from K-1 mice showed no DsRed$^+$ cells above background (n = 3; mean ± SEM, 1.3% ± 0.3%). IL-1 or IL-18 alone (n = 2) resulted in no induction of DsRed$^+$ cells above the WT background (2% each), and IL-33 (n = 3) alone caused essentially no induction of DsRed$^+$ cells (mean ± SEM, 3% ± 1.5%). IL-3 alone (n = 2) induced a very small proportion of cells to be DsRed$^+$ (approximately 5%). The combination of IL-3 plus IL-18 produced no more DsRed$^+$ cells than IL-3 (n = 2) alone, whereas IL-3 plus IL-1 caused approximately 10% of the c-Kit$^+$/FcεRI$^+$ cells to express DsRed (n = 2). However, the combination of IL-3 plus IL-33 (n = 3) led to a striking induction of DsRed$^+$ cells; on average, approximately 47% of the c-Kit$^+$/FcεRI$^+$ cells were DsRed$^+$ (mean ± SEM, 46.7% ± 4.8%).

We then asked whether IL-33 induced substantial numbers of DsRed$^+$ cells in the presence of other STAT5-inducing cytokines. We cultured peritoneal mast cells in 2 independent experiments with 20 ng/mL IL-33 and 20 ng/mL of several STAT5-inducing cytokines (IL-5, GM-CSF, thymic stromal lymphopoietin, and IL-7). Only IL-3 combined with IL-33 caused a major fraction of the peritoneal mast cells to express DsRed (Fig 2, *B*). In parallel, of the tested STAT5 inducers, only IL-3 induced STAT5 phosphorylation in peritoneal mast cells (n = 2; Fig 2, *C*).

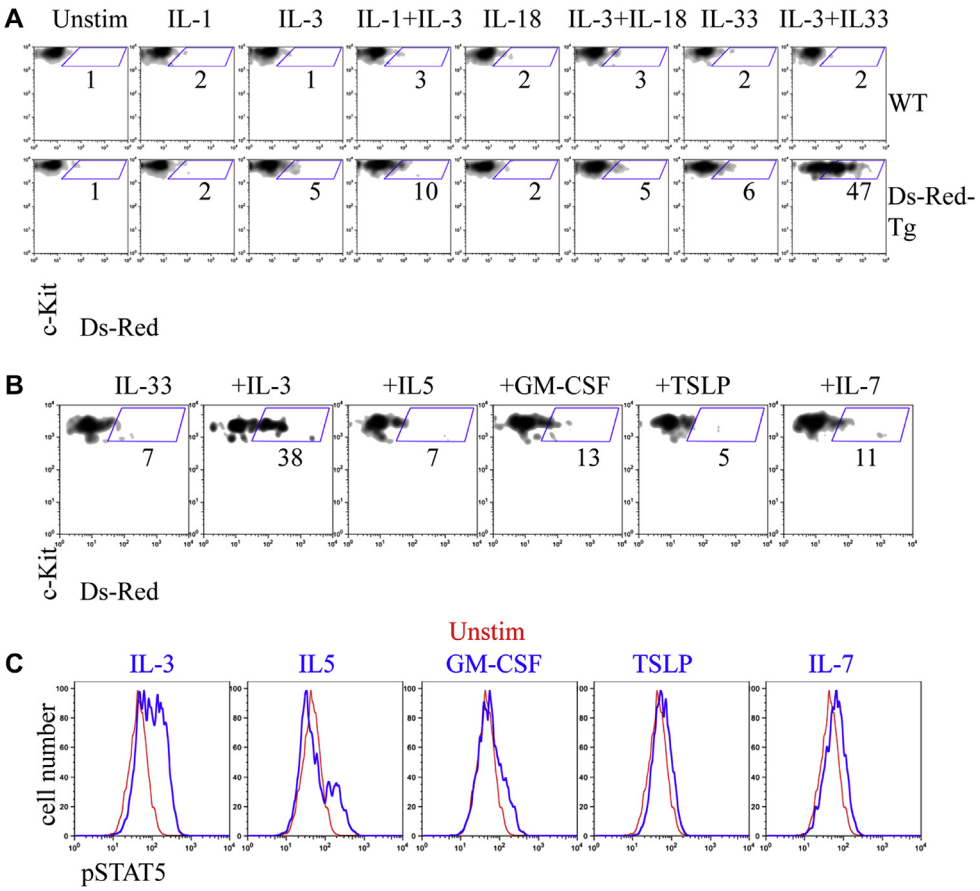

FIG 2. Peritoneal cavity mast cell IL-33–mediated IL-13 production requires an IL-3–STAT5 signal. **A,** DsRed expression in c-Kit$^+$/FcεRI$^+$ cells from peritoneal cavities of WT and DsRed mice on cytokine stimulation. **B,** DsRed expression in response to various STAT5 activators among c-Kit$^+$/FcεRI$^+$ cells. **C,** STAT5 activation in response to different cytokines in c-Kit$^+$/FcεRI$^+$ cells of the B6 peritoneal cavity. The *red line* indicates the basal level of STAT5 phosphorylation in unstimulated cells. *TSLP,* Thymic stromal lymphopoietin.

BMMCs and bone marrow–derived basophils produce IL-13 in response to addition of IL-33

We prepared bone marrow cultures from DsRed transgenic mice to determine BMMC and basophil cytokine requirements for IL-13 production. Approximately 11% (SEM, ±0.6%) of BMMCs (n = 3) from 10- to 12-day cultures in IL-3 were DsRed$^+$ without further activation (Fig 3, *A*, left panel); this probably represents a low level of IL-13 expression because staining with anti–IL-13, which is relatively insensitive, did not indicate "background" (Fig 4, *A*, upper panel). On 16-hour culture with IL-1 family members, we observed that IL-33 was capable of strongly inducing DsRed expression (approximately 69% [SEM, ±9%] of cells, n = 3), whereas neither IL-1 (n = 3) nor IL-18 (n = 3) caused any significant increase in the frequency of DsRed$^+$ cells (Fig 3, *A*, and see Fig E3 in this article's Online Repository at www.jacionline.org). The mean fluorescence intensity of DsRed in the positive gate was approximately 3-fold higher in cells stimulated with IL-33 as opposed to IL-3 alone, and thus not only did IL-33 increase the frequency of DsRed$^+$ cells approximately 7-fold, it also enhanced the degree of expression of DsRed approximately 3-fold on a single-cell basis. These results were obtained in 3 independent BMMC cultures grown in IL-3 for 40 days. Such cultures are essentially pure mast cells, ruling out the role of other cell types in DsRed induction. An important difference between the "short" and "long" culture durations was that prolonged culture decreased the background of DsRed$^+$ cells to approximately 5%. In addition, both IL-1 and IL-18 consistently showed modest (10% to 20%) induction of DsRed expression, whereas IL-33 induced striking DsRed production in these cells (see Fig E3).

For basophils (FcεRI$^+$/c-kit$^-$ cells) in the 10- to 12-day cultures, IL-18 was the best inducer of IL-13 production. Approximately 19% of basophils became DsRed$^+$ when cultured in IL-3 alone. Addition of IL-18 resulted in 37% DsRed$^+$ cells. IL-33 was a slightly poorer inducer of IL-13 than IL-18, whereas IL-1 did not induce any IL-13 expression above baseline (Fig 3, *B*). In splenic basophils from 2 DsRed mice (see Fig E4 in this article's Online Repository at www.jacionline.org), stimulation for 4 hours with IL-3 alone induced some DsRed expression (22% to 24%, with backgrounds of 4% to 8% of cells); addition of either IL-18 or IL-33, in combination with IL-3, increased DsRed production to 35% to 47% of cells; and IL-1 plus IL-3 also caused an increase in the frequency of DsRed-expressing cells. In contrast to basophils from IL-3 bone marrow cultures, IL-18, or IL-33 alone caused little or no induction of DsRed expression in splenic basophils.

IL-6 is also upregulated by IL-33 in BMMCs.[8] Not surprisingly, unstimulated WT BMMCs (but responding to IL-3 as a result of its presence in culture medium) or cells to which further IL-3 was added showed essentially no IL-6 expression. Addition

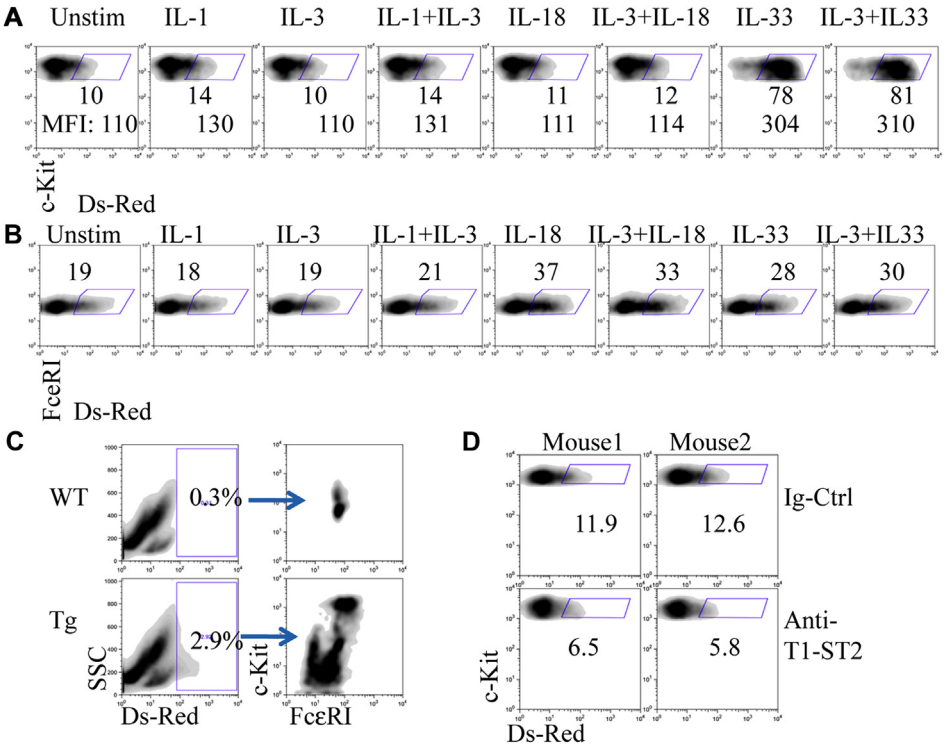

FIG 3. BMMCs and bone marrow–derived basophils respond to exogenous IL-18 and IL-33. **A,** DsRed expression in BMMCs from DsRed transgenic mice on cytokine stimulation. **B,** DsRed expression in bone marrow–derived basophils from DsRed transgenic mice on cytokine stimulation. **C,** DsRed background in bone marrow cultures is located in basophils and mast cells. **D,** T1-ST2 mAb decreases DsRed expression in BMMCs. *SSC,* Side scatter.

of IL-33 resulted in approximately 20% of BMMCs expressing IL-6 (see Fig E5 in this article's Online Repository at www.jacionline.org).

What is responsible for the background of DsRed$^+$ cells in the BMMCs and basophils obtained from the 10- to 12-day cultures? This background expression was observed in all experiments described here (Fig 3, *C*). It presumably resulted from stimulation by IL-3 (added as a growth/differentiation factor) and possibly from an endogenous source of IL-33. Interestingly, in another IL-13 indicator mouse (IL-13-GFP-KI), approximately 5% of BMMCs from heterozygous or homozygous reporter mice cultured in IL-3 were reported to be green fluorescent protein positive.[18]

To test whether a source of IL-33 existed in the cultures, we added an unlabeled neutralizing anti–IL-33 receptor (T1-ST2) antibody (clone DJ8)[19] from the outset of culture to 2 independent BMMC cultures from different DsRed mice. The presence of T1-ST2 decreased DsRed expression by the unstimulated mast cells (ie, BMMCs that received only IL-3) by approximately 50% (Fig 3, *D*). This implies that a source of IL-33 exists in the early bone marrow cultures.

Both MyD88 expression and STAT5 phosphorylation are needed for IL-33–induced IL-13 production by BMMCs

It was reported that BMMCs from *MyD88*$^{-/-}$ mice did not make IL-13 when stimulated with IL-33[8] and that IL-1 and IL-18 responses were impaired in these mice.[16] Similarly, bone marrow–derived basophils from these mice are unable to produce IL-13 in response to IL-33.[10] We prepared BMMCs from 2 WT and 2 *MyD88*$^{-/-}$ mice. Because these animals lacked the DsRed BAC transgene, we relied on intracellular staining for IL-13 rather than expression of DsRed as a marker of IL-13 production. Our experience is that staining for IL-13 is much less sensitive than DsRed expression, and in consequence, cells harvested from an IL-3–only culture show no detectable IL-13 by using intracellular staining. As anticipated, BMMCs from *MyD88*$^{-/-}$ donors did not express IL-13 when cultured with IL-33 plus the IL-3 that was in the bone marrow culture or IL-33 plus additional IL-3. By contrast, 17% to 19% of WT BMMCs expressed intracellular IL-13 within 5 hours of stimulation with 10 ng/mL IL-33 or IL-33 plus additional IL-3 (Fig 4, *A*). For basophils in the same cultures, IL-33 induced 14% to 15% IL-13–expressing cells, and as expected,[10] *MyD88*$^{-/-}$ basophils did not respond to IL-33 with any additional IL-13–expressing cells (see Fig E6 in this article's Online Repository at www.jacionline.org).

A major difficulty in determining the need for IL-3–mediated STAT5 phosphorylation in IL-33 induction of IL-13 production is that cultured BMMCs and basophils are dependent on IL-3 for survival, and accordingly, STAT5-deficient mice lack mast cells.[20] However, for short-term culture, IL-4 or SCF can replace IL-3 and will maintain the viability of the cells. To test the importance of STAT5 phosphorylation in IL-13 production, we performed 2 independent experiments in which we compared the expression of DsRed in K-1 BMMCs that had been cultured for the last 32 hours only with IL-4 or IL-3, as well as the degree of STAT5 Y594 phosphorylation in these cells. Substantially fewer (approximately 50%) of the cells grown in IL-4 stained

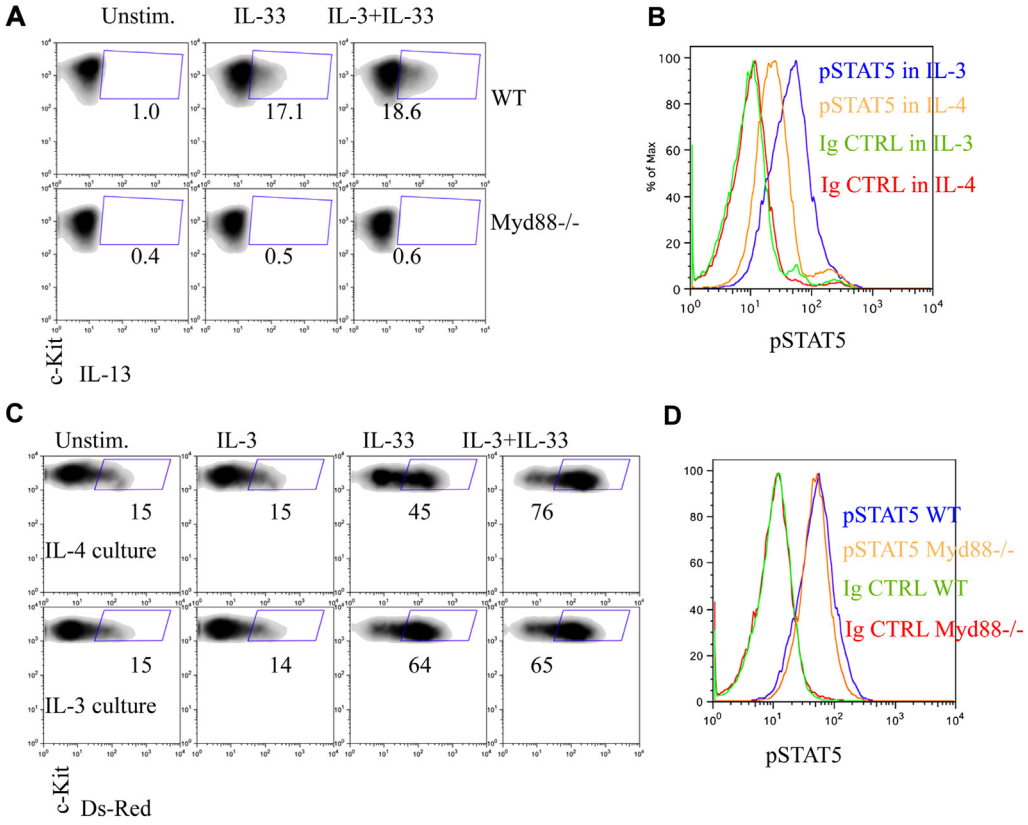

FIG 4. MyDd88 expression coupled with strong STAT5 phosphorylation is required for optimal IL-13 expression from BMMCs in response to IL-33. **A,** *MyD88*$^{-/-}$ BMMCs do not express IL-13 in response to IL-33. **B,** IL-3 replacement with IL-4 decreases STAT5 phosphorylation of BMMC STAT5. **C,** IL-3 replacement with IL-4 decreases IL-33–induced DsRed expression. **D,** STAT5 phosphorylation is not affected by deletion of MyD88.

with anti–phospho-STAT5 than did the cells grown in IL-3 for the same length of time. We then exposed these cells to no additional cytokines or to IL-3, IL-33, or both. Cells grown in IL-4 displayed substantially fewer DsRed^{+} BMMCs in response to IL-33 than did cells grown in IL-3 (45% vs 64%). This difference disappeared when IL-3 was added to the IL-4/IL-33 culture (Fig 4, *C*). Basophils tolerated replacement of the IL-3 with IL-4 much less well so that we were unable to analyze IL-13 production using the few basophils that remained viable after a 32-hour culture in IL-4. We also tested the capability of IL-33 to induce DsRed expression if IL-3 was replaced by SCF as a mast cell growth factor. We cultured BMMCs from 2 WT and 3 DsRed transgenic mice for 40 days in IL-3, washed out the IL-3, and cultured the cells in the presence of IL-3 or SCF for 48 hours, after which the cells were either left unstimulated or stimulated o/n with IL-33 alone or IL-3 plus IL-33. We found that SCF-cultured cells that were stimulated with IL-33 were poorly induced to produce DsRed. However, if these cells received IL-3 also, they induced DsRed strongly (see Fig E7 in this article's Online Repository at www.jacionline.org). In line with this, cells cultured in SCF for 48 hours had a substantially lower level of phospho-STAT5 than did cells that were cultured in IL-3 (see Fig E7).

We then studied the possibility that the failure of *MyD88*$^{-/-}$ cells to produce IL-13 in response to IL-3 plus IL-33 could be explained by diminished STAT5 phosphorylation in the *MyD88*$^{-/-}$ cells. As shown in Fig 4, *D*, there was no difference in STAT5 phosphorylation between WT and *MyD88*$^{-/-}$ BMMCs or basophils; this experiment was performed twice (see Fig E8 in this article's Online Repository at www.jacionline.org).

Role of IL-3 and IL-33 in IL-33 receptor expression

One possible mode of action of IL-3/STAT5 in IL-13 production could be through increasing the degree of expression of the IL-33 receptor. Indeed, in resting T_H2 cells, where IL-33 receptor expression is low, STAT5 activation is essential for upregulation of the IL-33 receptor.[11] We first confirmed the specificity of the T1-ST2 antibody (clone DJ8; Fig 3, *D*) by staining *in vitro* differentiated CD4 T_H17 or CD4 T_H2 cells with this antibody. As expected, we found high expression on T_H2 cells but none on T_H17 cells (Fig 5, *A*). We then tested whether peritoneal mast cells would increase T1-ST2 expression in response to IL-3 or IL-33. Peritoneal mast cells expressed high levels of T1-ST2 without stimulation (see Fig E9 in this article's Online Repository at www.jacionline.org). Stimulation of these cells for 16 hours with IL-3 resulted in approximately 1.5-fold induction of receptor expression (Fig 5, *B* and *C*), whereas IL-33 alone had no effect on T1-ST2 expression, and the combination of IL-3 and IL-33 was no better than IL-3 alone.

Exogenous IL-3 did not further enhance T1-ST2 expression on IL-3–cultured BMMCs. The addition of IL-33 increased receptor expression approximately 1.4-fold; no further enhancement was obtained by adding exogenous IL-3 to IL-33 (Fig 5, *B* and *C*). Thus the presence of IL-3 modestly enhanced T1-ST2 in

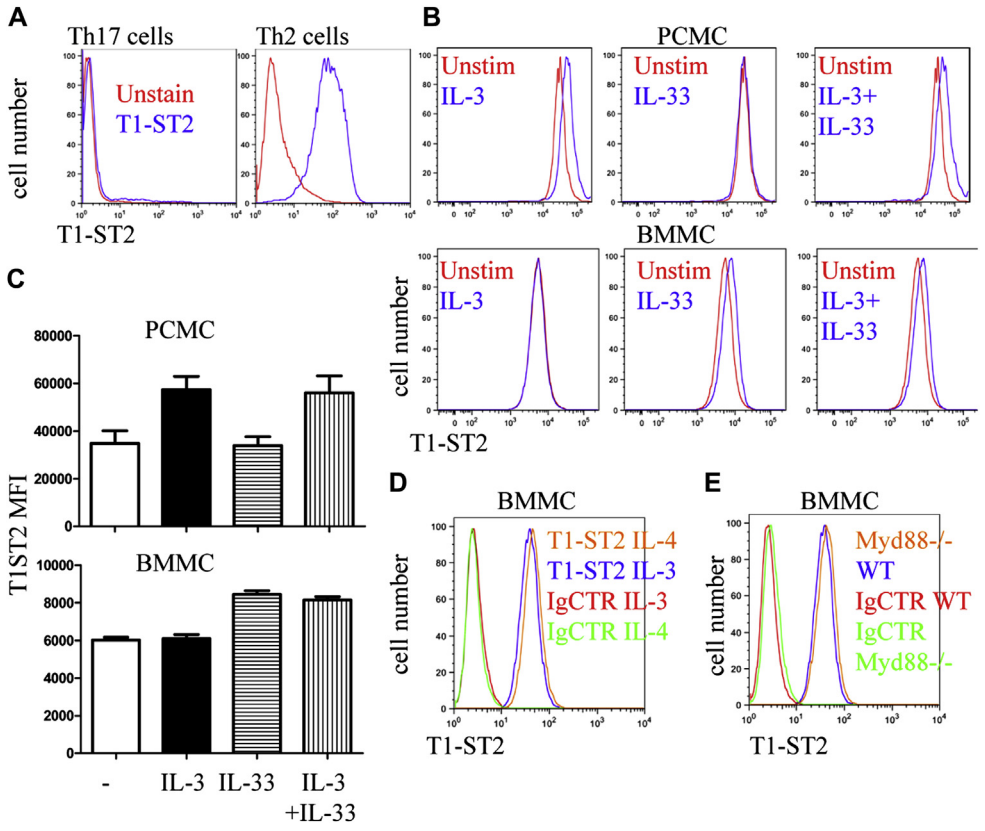

FIG 5. Expression of T1-ST2 in mast cells. **A,** T1-ST2 surface staining is specific because T_H2 cells express high levels of T1-ST2. **B,** Effect of cytokines on T1-ST2 expression in peritoneal cavity mast cells *(PCMCs)* and BMMCs. **C,** Quantitation of T1-ST2 expression in BMMCs and PCMCs. **D,** IL-3 replacement with IL-4 does not affect T1-ST2 expression in BMMCs. **E,** MyD88 does not regulate T1-ST2 expression.

peritoneal mast cells; whether it was important in BMMCs could not be ascertained, but additional IL-3 did not enhance receptor expression. Furthermore, BMMCs grown in IL-4 for the last 48 hours of the culture did not downregulate IL-33 receptor expression despite their diminution in STAT5 phosphorylation (Figs 4, *B*, and 5, *D*). We also tested the possibility that *MyD88*$^{-/-}$ BMMCs might express lower levels of T1-ST2 and thus be less responsive to IL-33 by comparing staining with anti−T1-ST2 in 2 WT and 2 *MyD88*$^{-/-}$ BMMC cultures (Fig 5, *E*). No difference was observed.

IL-18R expression in basophils and BMMCs

Because IL-18 is superior to IL-33 in stimulating IL-13 production by basophils while only IL-33 is active in BMMCs, we examined expression of receptors for these cytokines in bone marrow−derived basophils and BMMCs. In line with previous work,[10] we could not detect expression of IL-18Rα on BMMCs, whereas basophils reproducibly expressed IL-18Rα (Fig 6, *A*). We also observed that mature basophils from spleens expressed higher levels of IL-18Rα than did bone marrow−derived basophils (Fig 6, *A*, and see Fig E10 in this article's Online Repository at www.jacionline.org). In contrast, BMMCs showed very strong staining (mean fluorescence intensity, approximately 200) with anti−T1-ST2, whereas basophils stained far more weakly (mean fluorescence intensity, approximately 10; Fig 6, *A* and *B*). We quantitated these data by measuring the ratio of isotype control staining to the specific receptor antibody staining in 3 independent experiments (Fig 6, *C*).

DISCUSSION

Production of IL-13 is often of crucial importance for elimination of parasites in infection models, although this requirement varies for different parasites.[21] IL-13 plays a major role in many allergic inflammatory systems, particularly in airway hypersensitivity models.[4] Furthermore, because the functionality of IL-13 is related to the amount of the cytokine,[22] understanding the mechanisms controlling IL-13 production is of importance. To overcome technical limitations in measuring intracellular IL-13 production, we constructed a BAC transgenic indicator mouse in which the DsRed fluorochrome is controlled by IL-13 genetic regulatory elements.

We observed that peritoneal mast cells did not become DsRed$^+$ (ie, to become IL-13 producers) in response to IL-33 alone. The need for stimulation by an IL-1 family member and a STAT activator to induce cytokine-induced cytokine production is in keeping with the requirements of primed CD4 T cells for such responses. Superficially, BMMCs appeared to respond differently. They produced IL-13 with only the addition of IL-33. However, we believe that this difference is purely technical. For BMMCs to survive, a STAT5-activating factor is essential.[20] That factor is typically IL-3. Indeed, without IL-3, these cultures rapidly lose viability. IL-4 or SCF, weaker STAT5 inducers, can

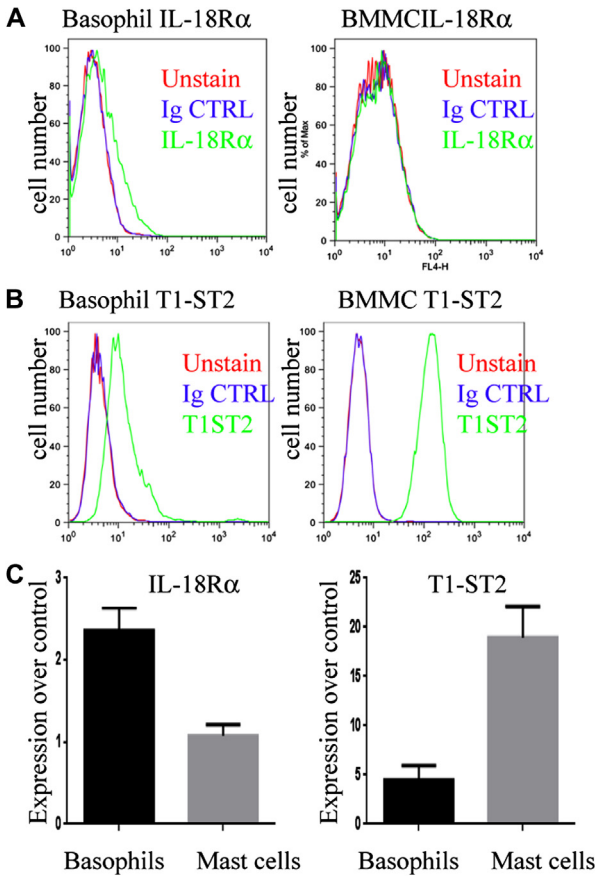

FIG 6. IL-18 and IL-33 receptor expression in BMMCs and bone marrow–derived basophils. **A,** IL-18Rα expression in bone marrow–derived basophils and BMMCs. **B,** T1-ST2 expression in bone marrow–derived basophils and BMMCs. **C,** Quantitation of IL-18Rα and T1-ST2 expression in bone marrow–derived basophils and BMMCs from 4 independent experiments.

maintain BMMC cultures. BMMCs from IL-4 or SCF cultures show weaker induction of DsRed in response to addition of IL-33 alone, which is in keeping with their poorer activation of STAT5. The addition of IL-3 to the stimulatory "cocktail" restores IL-13 production levels to those obtained when IL-33 was added to cells that had been stimulated with IL-3 from the beginning of the culture. Because BMMCs cannot be obtained from $STAT5^{-/-}$ knockout bone marrow,[20] we cannot unequivocally establish that STAT5 activation is essential for IL-13 production, but our results are certainly consistent with the conclusion that cytokine-induced IL-13 production by both tissue mast cells and BMMCs requires both IL-33 and IL-3.

For splenic basophils, IL-18 or IL-33 alone did not upregulate DsRed production, whereas their combination with IL-3 clearly did. Effectively, this means that IL-3–mediated STAT5 activation is important for IL-13 production by basophils as well. On the basis of the rapid expression of IL-13 and IL-6 in BMMCs (Fig 4, *A*, and see Fig E5), it seems likely that the effect of IL-33 is mediated through the direct action of its signaling intermediates on the *Il13* promoter; it is known that functional inhibition of nuclear factor κB results in decreased immediate IL-13 production by IL-33 in T_H2 cells.[11]

Our finding that IL-33 was not a better inducer of IL-13 than IL-18 in basophils in our experiments is somewhat different from published work. Kroeger et al[9] found that IL-33 induces IL-13 more strongly than does IL-18 in bone marrow–derived basophils. We cannot fully explain the discrepancy of our results. Although the expression of T1-ST2 is clearly less in basophils than in mast cells, tissue basophils and bone marrow–derived basophils both express T1-ST2 and respond to IL-33. One explanation might be that we did not use cell sorting to purify the cells, which might activate basophils and possibly upregulate their T1-ST2 expression. A further complication in the evaluation of IL-18's effect on basophils is the clearly higher IL-18Rα expression in splenic basophils compared with bone marrow–derived basophils.

There are interesting differences between the cytokine regulation of IL-13 production by resting T_H2 cells and mast cells. In BMMCs levels of the IL-33 receptors are inherently high and not altered by changes in STAT5 phosphorylation, nor does the absence of MyD88 impair the receptor expression. By contrast, in resting CD4 T_H2 cells, IL-33 receptor levels are quite low, and their upregulation requires both an IL-33–mediated signal and the presence of pSTAT5.[11] Although BMMC IL-33 receptor levels are not altered by diminishing or increasing the concentration of IL-3, peritoneal mast cells cultured in the presence of IL-3 show a 1.5-fold increase in IL-33 receptor expression compared with those cultured without IL-3, although T1-ST2 expression is very high on both populations. These results suggest that the main function of the IL-3–STAT5 axis in mast cells is in enhancing IL-13 transcription rather than increasing expression of IL-33 receptors. STAT5 is bound to STAT5 sites within the *Il13* gene in T_H2 cells, and therefore a role for direct regulation of transcription seems quite reasonable. Similarly, mast cells do not require an IL-33 stimulation event to maintain expression of the IL-33 receptor, as judged by the lack of difference in IL-33 receptor expression in WT and $MyD88^{-/-}$ BMMCs. Furthermore, IL-33 did not upregulate T1-ST2 in peritoneal mast cells, confirming that IL-33 signal does not regulate the expression of its receptor in these cells.

Our findings are consistent with a general requirement of 2 cytokine signals for FcεR-independent or T-cell receptor–independent IL-13 production by mast cells/basophils and T_H cells. One signal is delivered by an IL-1 family member and the other by a STAT5 activator.

We thank Julie Edwards for advice and expert cell sorter operation and Dr Lionel Feigenbaum (National Cancer Institute) for assistance in generating DsRed mice. We also thank Sanna Hämäläinen for help with IL-6 staining.

Key message

- **Mast cells and basophils follow the general rule observed in CD4 T cells for cytokine-induced IL-13 production: joint stimulation by an IL-1 family member and a STAT activator.**

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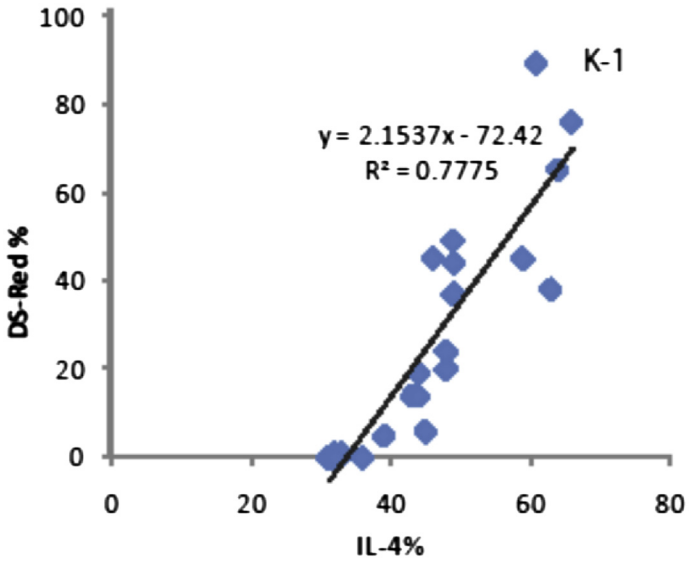

FIG E1. T_H2-differentiated CD4 T cells from different DsRed transgenic mice indicate good correlation between the proportion of $DsRed^+$ cells and the frequency of IL-4–producing cells. The founders of the mice used in experiments described in this report were K-1.

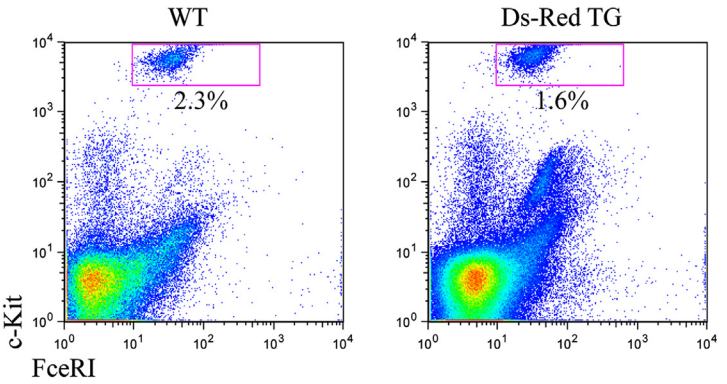

FIG E2. Identifying mast cells in mouse peritoneal exudate. Live cell gate indicates a small consistent c-Kit/FcεRI−positive population in peritoneal cavities of both B6 and DsRed transgenic *(Tg)* mice.

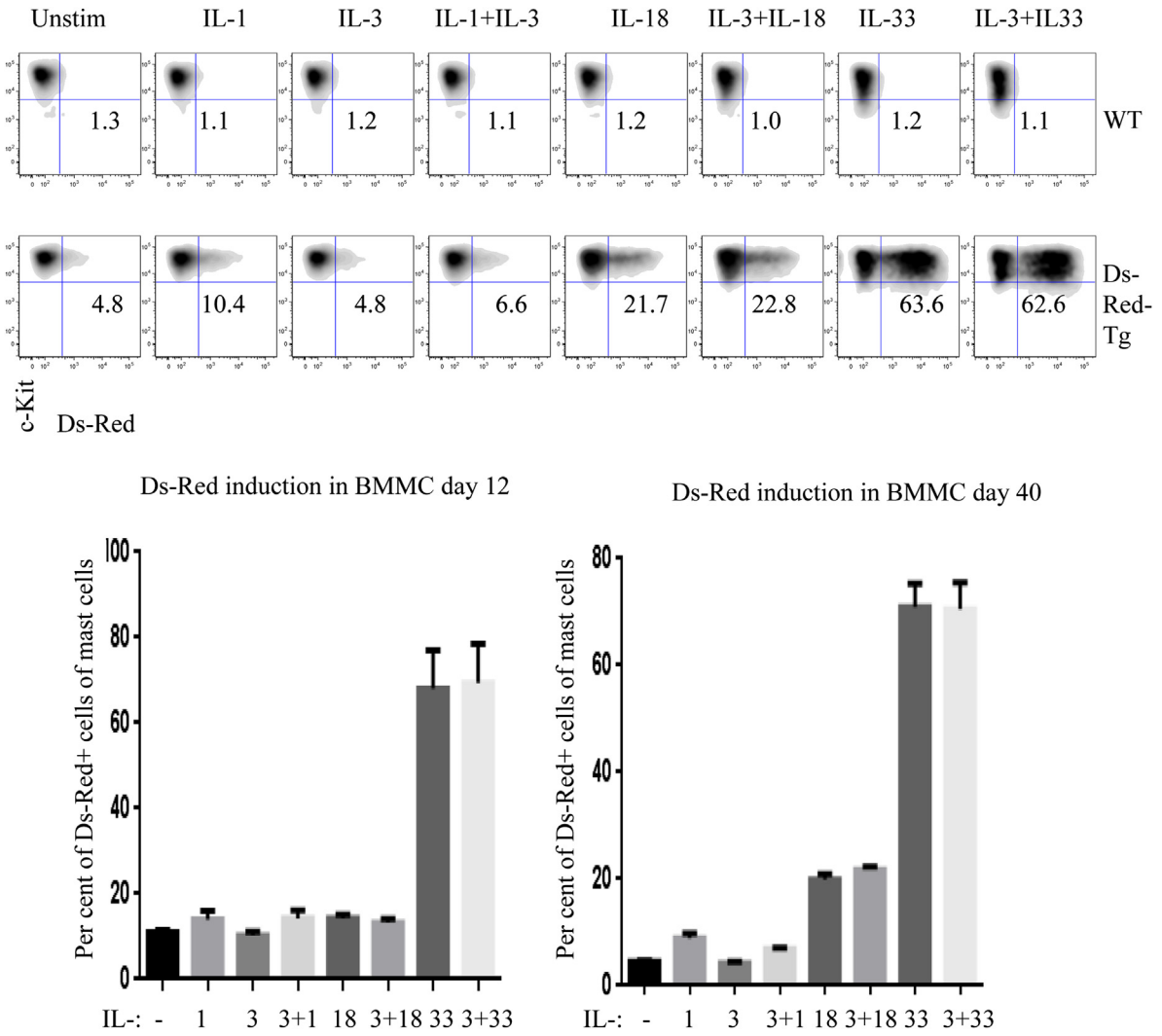

FIG E3. DsRed production in response to IL-1 family members and IL-3 in bone marrow–derived mast cells cultured for 40 days in IL-3. To quantitate the data, the induction of DsRed in 3 experiments on day 12 and day 40 BMMCs is shown with means and SEMs of $DsRed^{+}$ cells.

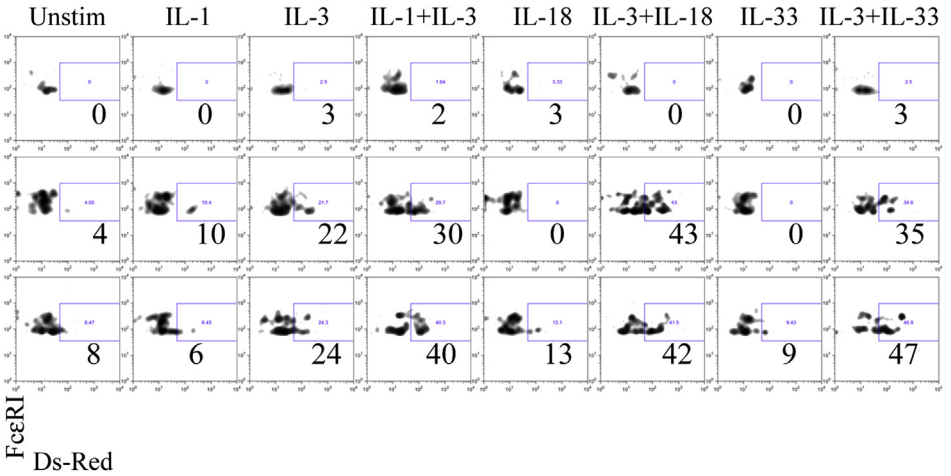

FIG E4. Splenocytes from 1 WT mouse *(upper panel)* and 2 DsRed transgenic mice (*middle* and *lower panels*) were stimulated as indicated for 4 hours. Subsequently, DsRed expression of $Fc\varepsilon RI^{+}/B220^{-}$ cells was measured *(x-axis)*. *Numbers* indicate the percentage of $DsRed^{+}$ $Fc\varepsilon RI^{+}/B220^{-}$ cells.

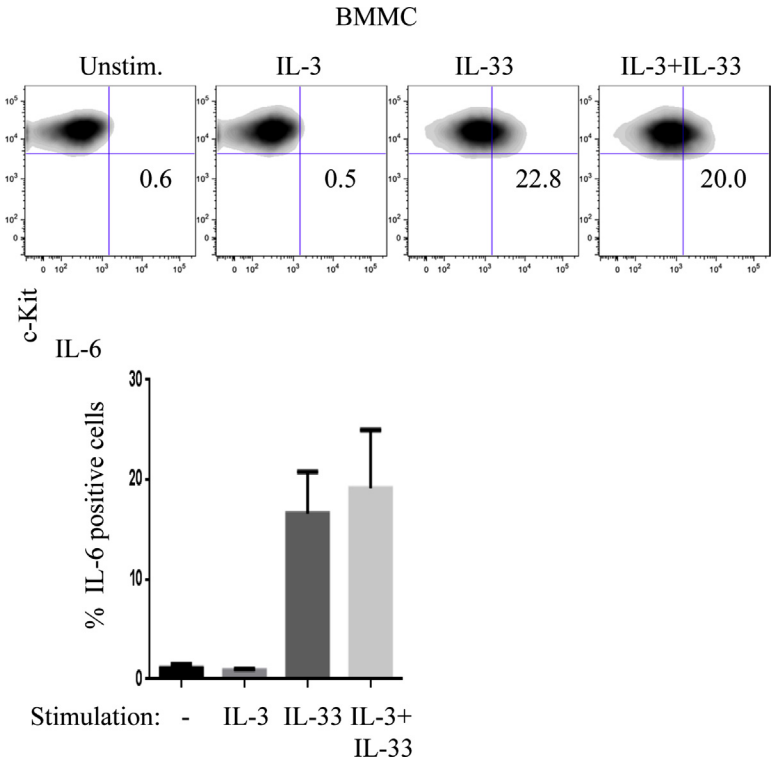

FIG E5. IL-6 production by WT BMMCs. Cells were stimulated as indicated for 4 hours in the presence of brefeldin A and stained for FcεRI, c-Kit, and IL-6. Cells shown here are FcεRI^{+}/c-Kit^{+}. The *lower panel* shows means and SEMs from 3 independent experiments.

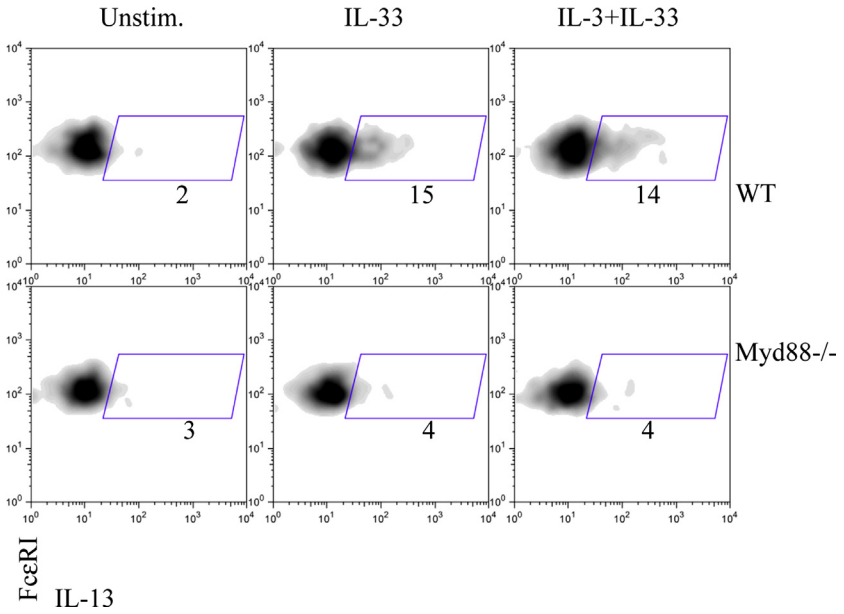

FIG E6. IL-13 production by WT and *Myd88*$^{-/-}$ bone marrow–derived basophils. Cells were stimulated as indicated for 5 hours and stained for FcεRI, c-Kit, and IL-13. Cells shown here are FcεRI^{+}/c-Kit^{-}.

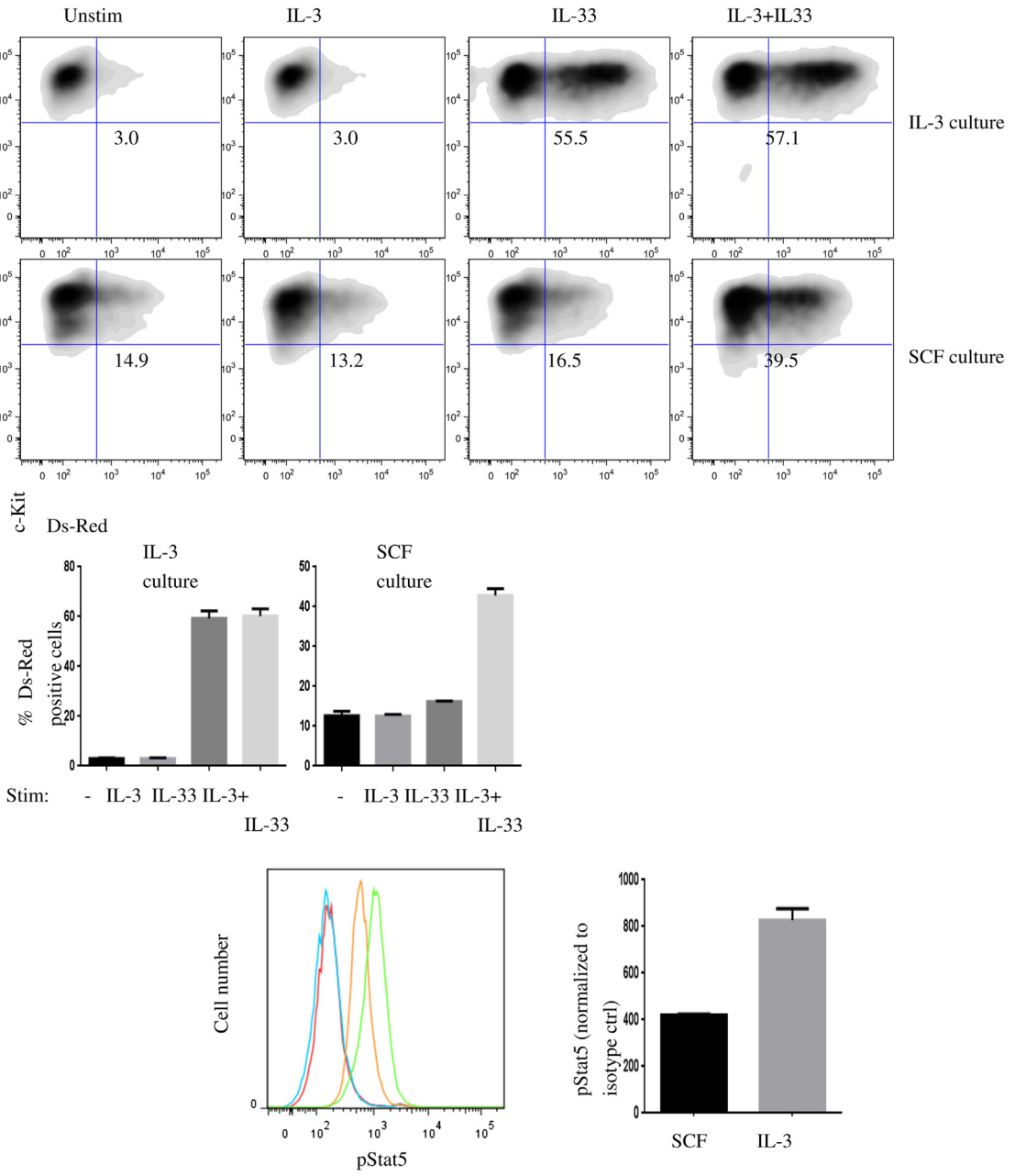

FIG E7. DsRed production in response to IL-3 and IL-33 by bone marrow–derived mast cells cultured for 40 days in IL-3, followed by a 2-day culture in IL-3 or SCF. Data from 3 experiments shows means and SEMs. STAT5 phosphorylation in BMMCs cultured in SCF or IL-3 is shown, as well as quantitation of the data from 3 experiments. Means and SEMs of specific STAT5 phosphorylation retracted by isotype controls are shown.

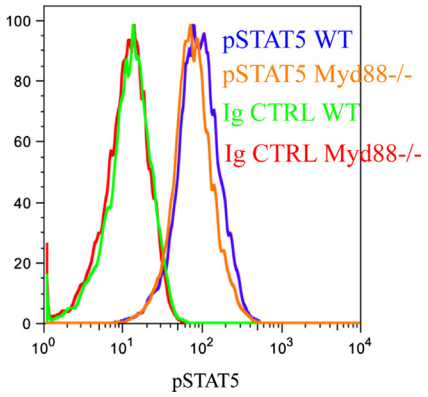

FIG E8. STAT5 phosphorylation in WT and *MyD88*$^{-/-}$ bone marrow–derived basophils. FcεRI^{+}/c-Kit^{-} cells from 10-day IL-3 culture were stained for phospho-STAT5 before the cells were stimulated with IL-33 or IL-3 plus IL-33 (as in Fig 4, *D*).

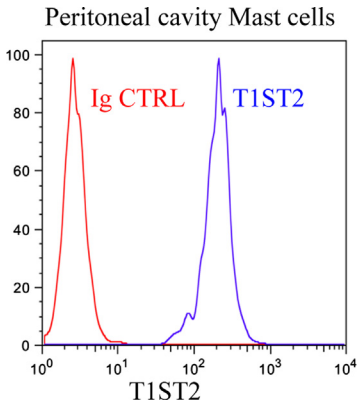

FIG E9. Expression of the receptor for IL-33 in peritoneal cavity mast cells. Cells from peritoneal cavity flushes were stained immediately after flushing for c-Kit and FcεRI. Simultaneously, cells were stained for T1-ST2 (*blue* histogram) or isotype control (*red* histogram). c-Kit/FcεRI double-positive cells that had neither T1-ST2 nor isotype control staining overlapped completely with isotype control staining.

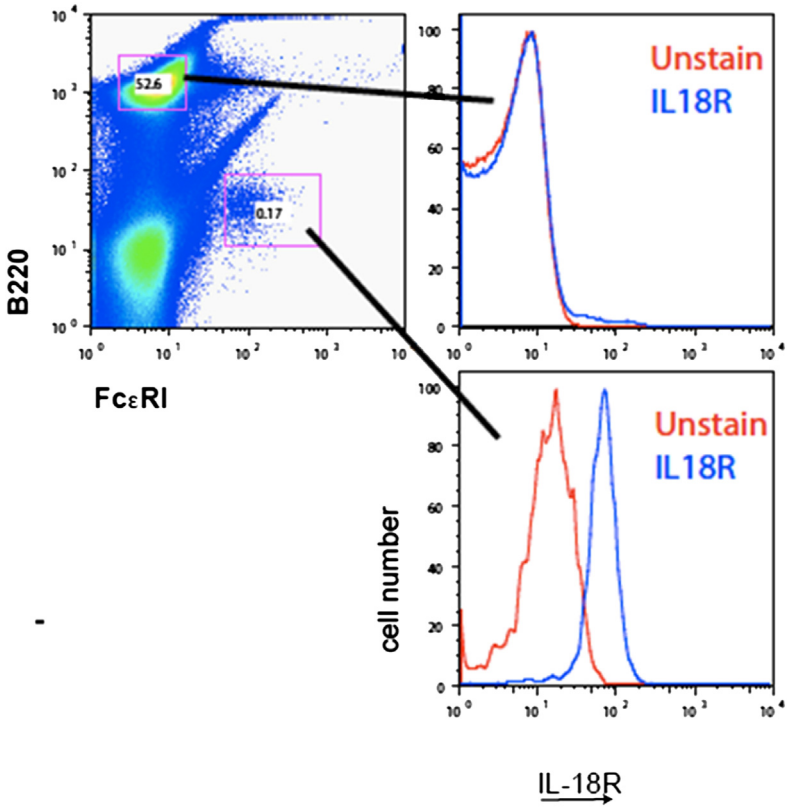

FIG E10. IL-18R expression by splenic basophils. Splenocytes from WT B6 mice were stained with B220/FcεRI and either IL-18Rα mAb or unstained control. The *upper right panel* shows IL-18Rα expression on B cells ($B220^+/Fc\varepsilon RI^-$). The *lower right panel* shows IL-18Rα expression on basophils ($Fc\varepsilon RI^+/B220^-$). Splenic basophil IL-18Rα expression is higher than bone marrow–derived basophil IL-18Rα expression (see Fig 6, *A*).

IL-7Rα Expression Regulates Murine Dendritic Cell Sensitivity to Thymic Stromal Lymphopoietin

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Thymic stromal lymphopoietin (TSLP) and IL-7 are related cytokines that mediate growth and differentiation events in the immune system. They signal through IL-7Rα–containing receptors. Target cells of TSLP in Th2 responses include CD4 T cells and dendritic cells (DCs). Although it has been reported that expression of TSLP receptor (TSLPR) on CD4 T cells is required for OVA-induced lung inflammation, DCs have also been shown to be target cells of TSLP. In this study, we show that murine ex vivo splenic DCs are unresponsive to TSLP, as they fail to phosphorylate STAT5, but in vitro overnight culture, especially in presence of IL-4, renders DCs responsive to both TSLP and IL-7. This induced responsiveness is accompanied by dramatic upregulation of IL-7Rα on DCs with little change in expression of TSLPR or of γ_c. In splenic DCs, the induction of IL-7Rα occurs mainly in $CD8^-$ DCs. In vivo, we found that IL-4 has a differential regulatory role on expression of IL-7Rα depending on the cell type; IL-4 decreases IL-7Rα expression on CD4 T cells whereas it upregulates the expression on DCs. Our results indicate that the induction of IL-7Rα expression on DCs is critical for TSLP responsiveness and that IL-4 can upregulate IL-7Rα on DCs. ***The Journal of Immunology*****, 2017, 198: 3909–3918.**

Signaling by thymic stromal lymphopoietin (TSLP) and IL-7 depends on ligand-mediated assemblage of a receptor complex consisting of a cytokine-specific binding chain and an auxiliary chain. The TSLP and IL-7 receptors share IL-7Rα, although they use this chain distinctly. IL-7 binds with high affinity to IL-7Rα, and this complex then binds common γ chain (γ_c), activating the Jak1/Jak3 kinases, followed by phosphorylation of critical tyrosine residues on IL-7Rα (1). Docking of STAT5 to the phosphorylated tyrosine residues of IL-7Rα results in Jak-mediated phosphorylation and subsequent nuclear translocation, as well as DNA binding of STAT5, resulting in regulation of STAT5-mediated transcription. In contrast, TSLP binds TSLP receptor (TSLPR), a close homolog of γ_c, followed by IL-7Rα recruitment to the complex. The K_D for TSLP binding to TSLPR is $\sim 7 \times 10^9$ (2), whereas the K_D for IL-7 binding to IL-7Rα is 1×10^{10} (3), indicating that initial cytokine binding to ligand-binding receptor chain occurs with higher affinity by IL-7 than by TSLP. As opposed to IL-7, the Jak1 and Jak2 kinases are activated by TSLP in human and mouse primary CD4 T cells with the consequent phosphorylation of STAT5 (1). In human myeloid dendritic cells (DCs), activation of STAT6 by TSLP has also been reported (4).

Functionally, IL-7 is a critical growth factor for B and T cell development and induces survival of memory T cells (5). Recently, the role of IL-7 in development of innate lymphoid cells has also been established (6). It is of interest that epithelial cells possess the capacity to produce IL-7, which might be important for survival of tissue lymphocytes locally (7). Memory cell competition for IL-7 has been suggested to be the mechanism of regulation of IL-7–mediated growth (8).

Although mast cells (9), basophils (10), and DCs (11) have been reported to produce TSLP, the main TSLP-producing cells appear to be the epithelial cells in humans and particularly the keratinocytes in mice (12–14). Thus, TSLP appears to be principally expressed at skin and mucosal barriers; in contrast, IL-7 production seems more a property of stromal cells in primary and secondary lymphoid organs (15). Effectively, this suggests a specialization of the functions of the two cytokines and particularly emphasizes the potential importance of TSLP in action at mucosal surfaces, be it in the induction of Ag-presenting competence in DCs or in the differentiation/expansion of memory/effector T cells in tissues.

To respond to TSLP, target cells need to express both IL-7Rα and TSLPR, whereas those responsive to IL-7 require expression of IL-7Rα and γ_c (16). Of conventional or plasmacytoid DCs from unmanipulated mice, only migratory and skin DCs express

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The online version of this article contains supplemental material.

Abbreviations used in this article: γ_c, common γ chain; DC, dendritic cell; MHC II, MHC class II; o/n, overnight; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor.

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IL-7Rα at a low level, although IL-7Rα expression appears to be important for DC development (17). Human myeloid DCs have been reported to alter their behavior in response to TSLP by directly upregulating CD40 and CD80 and by enhancing their capacity to induce T cell proliferation and chemotaxis, with the latter through expression of CCL17 and CCL22 (12, 18). Freshly isolated human myeloid DCs express low levels of both IL-7Rα and TSLPR, and overnight (o/n) culture of these cells strongly upregulates expression of both receptor chains (19). Recently, basophils were shown to be responsive to TSLP, and it has been reported that such basophils acquire a Th2 phenotype in that they produce more IL-4 in response to cytokine stimulation than do basophils cultured in IL-3 (20). IL-4 itself is a critical regulator of Th2 responses, and the cytokine binding receptor chain for IL-4 (IL-4Rα) is found virtually on all cells and, thus, basically all cells have the capability to respond to IL-4, though with different sensitivity (21).

Because IL-7Rα appears to be expressed at very low levels in murine DCs, we became interested in how they could be a direct target of TSLP. We reasoned that upregulation of IL-7Rα on DCs might be required. Indeed, we observed that neither TSLP nor IL-7 can cause freshly isolated splenic DCs to phosphorylate significant amounts of STAT5, but that o/n culture of splenic DCs, especially in the presence of IL-4, results in their responsiveness to both cytokines. We observed that TSLPR and γ_c were expressed on freshly isolated DCs and showed only modest enhancement as a result of o/n culture, but that IL-7Rα expression was dramatically upregulated by o/n culture, and more prominently so in the presence of IL-4. In spleen, IL-7Rα upregulation was less prominent in $CD8^+$ DCs, whereas $CD4^+$ DCs showed significant upregulation. This suggests that "tuning" of different DC subclasses to TSLP and IL-7 occurs via differential regulation of receptor chains for these cytokines and prepares these cells for differential responsiveness. We also discovered that IL-4 further upregulated IL-7Rα expression and cytokine responsiveness of DCs,suggesting yet another regulatory mechanism for IL-4 in orchestrating allergic responses. Furthermore, our results suggest that TSLP responses require both the induction of TSLP production and the induction of IL-7Rα on DCs, presumably with both events occurring in the same microanatomic location.

Materials and Methods

Mice and cell cultures

C57BL/6 mice (The Jackson Laboratory) were housed either in the National Institute of Allergy and Infectious Diseases pathogen-free animal facility or at the animal facility of the School of Medicine, University of Tampere. Experiments were performed under a protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee. $TSLPR^{-/-}$ mice were provided by Dr. W.J. Leonard (National Heart, Lung, and Blood Institute, National Institutes of Health). The spleens and lymph nodes from 6- to 10-wk-old mice were minced and incubated for 30 min at 37˚C in HBSS containing 75 μg/ml Liberase DL and 10 U/ml DNase (both Roche, Basel, Switzerland). The cells were filtered through a 40-μm cell strainer, and RBCs were lysed with 30 s ACK (Lonza, Basel, Switzerland) treatment. Where indicated, DCs were isolated using a MACS pan-DC isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were either cultured o/n in RPMI 1640 with 10% FBS, L-glutamine, and penicillin/streptomycin (Lonza) or used immediately. Where indicated, spleens were mechanically disrupted using a cell culture strainer to release DCs. In the in vivo experiment, mice were injected i.p. twice with either 6 μg of IL-4 (PeproTech, Rocky Hill, NJ) in PBS, complexed with 30 μg of anti–IL-4 Ab (clone BVD4-1D11; Thermo Fisher Scientific, Carlsbad, CA) as described (22), or 30 μg of anti–IL-4 alone in PBS. The injections were given 8 h apart. The mice were euthanized 12 h after the last injection. Human DCs were purified from peripheral blood of five healthy donors under ethical permission R21002 from the Ethics Committee of Pirkanmaa Hospital District using pan-human DC purification kit (Miltenyi Biotec). All reagents used were provided endotoxin free by the manufacturers and where applicable they were dissolved into sterile PBS containing BSA (low endotoxin; Sigma-Aldrich, St. Louis, MO). Strict aseptic techniques in biosafety level 2 sterile tissue culture hoods were used in all experiments.

Abs, cytokines, flow cytometry, and statistics

Mouse Abs and isotype controls were either from BD Biosciences (Franklin Lakes, NJ; pSTAT5, B220), eBioscience (Santa Clara, CA; CD11c, CD49/Dx5, MHC class II [MHC II], CD3, CD4, CD8, F4/80, IL-7Rα, γ_c, CD80, CD86, isotype controls for IL-7Rα and γ_c), or R&D Systems (Minneapolis, MN; TSLPR and polyclonal goat Ig as a control). Human Abs were from eBioscience (CD3, CD4, CD8, CD11c, CD14, CD19, CD56, HLA-DR), BD Biosciences (CD80), or Thermo Fisher Scientific (IL-7Rα and isotype control for IL-7Rα). Murine cytokines were from PeproTech (GM-CSF, IL-4, IL-7) or R&D Systems (TSLP). Human cytokines (IL-4, TSLP) were from PeproTech. Where indicated, 1 μg/ml LPS (InvivoGen, San Diego, CA), 20 ng/ml IL-4 with or without LPS, or 40 ng/ml IL-7 was added to the o/n culture. For pSTAT5 assays, cultured cells were starved for 2 h in RPMI 1640 (with L-glutamine, penicillin/streptomycin, and 1% FBS) at 37˚C and stimulated for 15 min with 100 ng/ml GM-CSF, IL-7, or TSLP. Intracellular pSTAT5 staining was done after surface staining by permeabilizing the cells with 90% ice-cold methanol (23). For surface staining, 0.1% BSA, 0.1% mouse serum, and CD64 and CD32 blocking 4G2 Abs were used. Cells were analyzed with FACSCanto II (BD Biosciences), and data were analyzed with the FlowJo (Tree Star, Ashland, OR) analysis program. The Prism program (GraphPad Software, La Jolla, CA) was used for statistics. Geometrical means ± SEM are indicated. The *p* values were calculated using a two-tailed, paired Student *t* test.

Results

Splenic DCs respond to TSLP after in vitro culture

The TSLP and IL-7 receptors share IL-7Rα (Fig. 1A). We became interested in how TSLP might regulate DC function, particularly because of a recent report showing that splenic or lymph node DCs of mice had no detectable IL-7Rα, with the exception of lymph node migratory DCs, and their dermal and epidermal counterparts showed low levels of IL-7Rα expression (17). Because several reports suggest a role for TSLP in the regulation of DC activities (24), the low level of expression of IL-7Rα in murine DCs seemed enigmatic. We chose to determine whether DCs were responsive to TSLP and IL-7 by measuring the appearance of an immediate signaling intermediate elicited by these cytokines, STAT5 tyrosine phosphorylated at Y594 (pSTAT5). We prepared single-cell suspensions from spleens of C57BL/6 mice by using DNase/Liberase treatment, followed by enrichment of DCs as described in *Materials and Methods*. The cells were incubated with the indicated cytokines for 15 min or left untreated, surface stained to allow their phenotyping, and permeabilized to allow the detection of pSTAT5. We analyzed the degree of pSTAT5 in $CD11c^{bright}$, MHC II^{bright} cells (Fig. 1B). As a positive control for pSTAT5 in DCs, we stimulated the cells with GM-CSF.

As expected, stimulation of splenic DCs with GM-CSF resulted in robust phosphorylation of STAT5 (Fig. 1C) in most of these cells. TSLP and IL-7 stimulated a modest or nonexisting response in the freshly isolated splenic DCs (Fig. 1C, 1D). We then asked whether "spontaneous" (i.e., DC damage–independent, microbial product–independent) DC activation caused by o/n culture altered TSLP or IL-7 responsiveness by these cells. We cultured splenocytes o/n on regular tissue culture plates (25) and stimulated them with GM-CSF, TSLP, or IL-7 for 15 min. GM-CSF induced STAT5 phosphorylation on most of the cultured DCs as it had on freshly isolated DCs. In contrast to freshly isolated splenic DCs, a substantial proportion of the o/n-cultured DCs showed robust phosphorylation of STAT5 in response to TSLP or to IL-7

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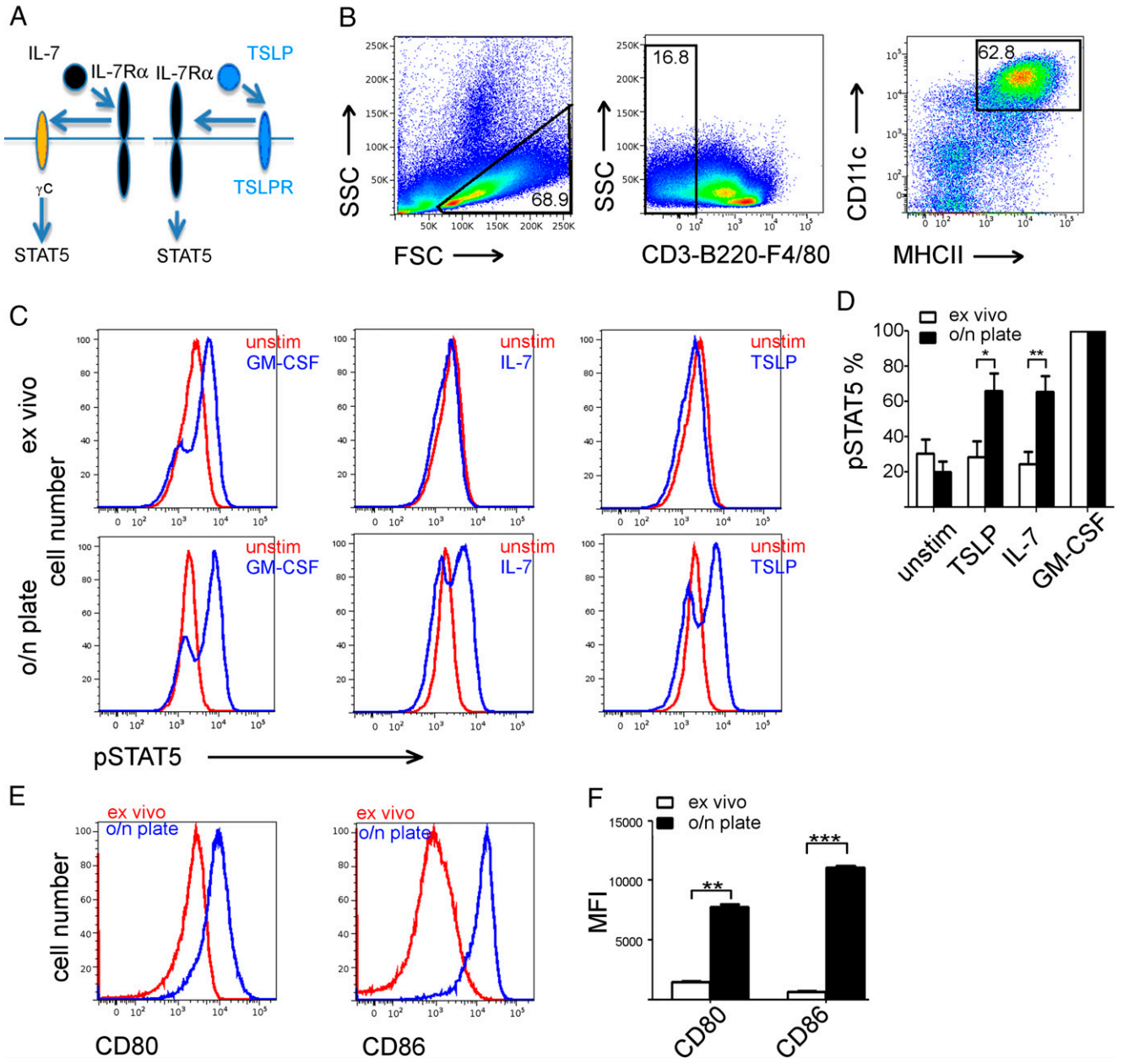

FIGURE 1. Splenic DC IL-7 and TSLP responsiveness requires o/n culture of the cells. (**A**) Schematic presentation of assemblage of functional IL-7 and TSLP receptor complexes. (**B**) Gating of enriched CD11c^{bright}/MHC II^{bright} splenic DCs. (**C**) STAT5 phosphorylation of DCs stimulated as indicated for 15 min either immediately after harvesting the spleens (ex vivo) or after 16 h of in vitro culture. Representative flow cytometer plots are shown. (**D**) Quantitation of pSTAT5 in DCs in response to cytokines. The number of pSTAT5$^+$ cells after GM-CSF stimulation was given the value of 100%. The bars represent the average percentage (±SEM indicated) of pSTAT5$^+$ cells among CD11c^{bright}/MHC II^{bright} cells after cytokine stimulation in six independent experiments. *$p < 0.05$, **$p < 0.01$, two-tailed, unpaired t tests. (**E**) The expression of CD80 and CD86 activation markers was measured from CD11c^{bright}/MHC II^{bright} cells either immediately after harvesting spleens or after 16 h of culture. Representative result is shown. (**F**) Quantitation of CD80 and CD86 expression. Statistical analysis of three independent experiments is shown. The bars represent the average of mean fluorescence intensity (MFI; ±SEM indicated). **$p < 0.01$, ***$p < 0.001$, two-tailed, paired t tests. FSC, forward scatter; SSC, side scatter.

(Fig. 1C, lower panel). Quantitatively, when percentage of GM-CSF–induced pSTAT5 was set to 100, stimulation of splenic DCs with IL-7 or TSLP after o/n culture showed a significant enhancement in the proportion of pSTAT5$^+$ DCs (Fig. 1D). The increase in the proportion of DCs that exhibited STAT5 phosphorylation in response to TSLP or IL-7 was highly significant ($p < 0.05$ and $p \leq 0.01$, respectively, by two-tailed unpaired t test Fig. 1D).

To establish that the DNase/Liberase treatment had not impaired the cytokine response, we compared IL-7 stimulation on ex vivo splenocytes prepared either mechanically or with DNase/Liberase treatment; IL-7 induced pSTAT5 to a similar degree on splenic CD4 T cells from both preparations (Supplemental Fig. 1, upper panel), indicating that IL-7Rα was not degraded by DNase/Liberase. As the DC yields were higher when using DNase/Liberase treatment, we chose to use this approach. Also, enrichment of DCs did not affect expression of IL-7Rα on freshly isolated DCs (Supplemental Fig. 1, lower panel). Overnight culture activated DCs; both CD80 and CD86 expression were significantly upregulated on DCs after 16 h of culturing the cells (Fig. 1E); the p values for upregulation were $p < 0.01$ for CD80 and $p < 0.001$ for CD86 (two-tailed paired t test, Fig. 1F).

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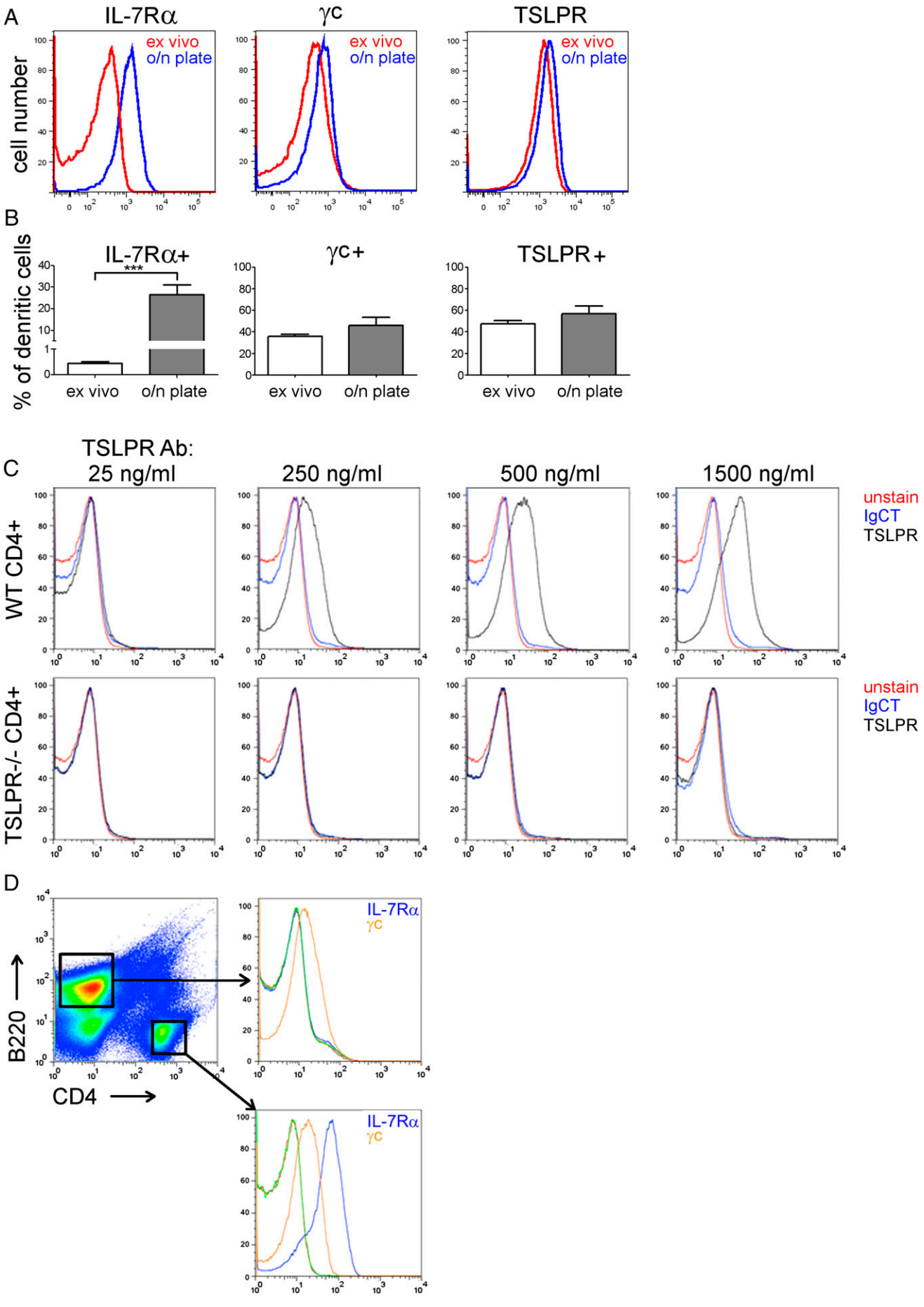

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Expression of receptors for IL-7 and TSLP on DCs

As IL-7Rα is a component of the receptors for both IL-7 and TSLP (Fig. 1A), the induction of responsiveness to these cytokines might reflect an upregulation of IL-7Rα expression on cultured DCs. As reported (17), we found that the great majority of ex vivo splenic DCs had no detectable IL-7Rα because isotype control and mAb against IL-7Rα stained the same amount of CD11c^{bright}/MHC II^{bright} cells (Supplemental Fig. 2). Overnight culture of splenocytes resulted in no change in the binding of the isotype control Ab to CD11c^{bright}/MHC II^{bright} cells (Supplemental Fig. 2), whereas anti–IL-7Rα staining showed a dramatic upregulation of this receptor chain (Fig. 2A). Abs to γ_c and to TSLPR stained essentially all freshly isolated DCs; o/n culturing resulted in only a modest enhancement in the intensity of staining for each receptor chain. Quantitatively, ~1% of the total ex vivo DCs expressed IL-7Rα whereas ~20% of the cells were positive for IL-7Rα after o/n culture (Fig. 2B). The upregulation of IL-7Rα on DCs was statistically highly significant ($p < 0.001$, two-tailed paired *t* test).

We confirmed the specificity of the polyclonal anti-TSLPR Ab by using various amounts of anti-TSLPR Ab or control Ig on cell type known to express TSLPR (splenic CD4 T cells) from wild-type and TSLPR-deficient mice (Fig. 2C). To test the specificity of IL-7Rα and γ_c Abs, we compared their staining profiles on B220$^+$ cells and CD4 T cells. As expected, anti–IL-7Rα showed strong staining on CD4 T cells, but no staining on B cells, that are known to lack surface IL-7Rα, whereas anti-γ_c staining was positive on both cell types (Fig. 2D).

Induction of IL-7Rα in different splenic DC subsets

The CD11c^{bright}/MHC II^{bright} splenic DCs are actually a group of subtypes characterized by differential expression of surface markers. Differential expression of CD4 and CD8 has been used to classify CD11c^{bright} DCs as CD4$^+$/CD8$^-$, CD4$^-$/CD8$^+$, or CD4$^-$/CD8$^-$ subclasses (26). The different subclasses of splenic DCs might have a different capacity to upregulate IL-7Rα. We stained splenic cells for IL-7Rα immediately ex vivo or after o/n culture as in Fig. 2, but we analyzed the expression of CD4 and CD8 simultaneously (26). We identified three major populations among the cells in the CD11c$^+$/MHC II$^+$ gate based on CD4 and CD8 expression (Fig. 3A). None expressed detectable IL-7Rα when tested immediately after harvest. Strikingly, the upregulation of IL-7Rα in o/n-cultured cells was most prominent on CD8$^-$ DC populations (Fig. 3B). Whereas o/n-cultured CD8$^+$ DCs showed levels of IL-7Rα that were modestly changed when compared with CD8$^+$ DCs ex vivo (<10% of the cells became IL-7Rα$^+$), ~25% of CD4$^+$ DCs became positive for IL-7Rα (Fig. 3C). CD4$^-$/CD8$^-$ DCs fell between CD4$^+$ and CD8$^+$ DCs; <20% of the cells became IL-7Rα$^+$ during o/n culture. Quantitatively, the difference of IL-7Rα expression on CD8$^+$ DCs was significant ex vivo and after o/n culture ($p < 0.01$ and $p < 0.001$, respectively, Fig. 3C).

IL-4 and LPS enhance IL-7Rα expression in cultured DCs

Next, we studied how various soluble factors might regulate the IL-7Rα expression in cultured DCs. In T cells, IL-7 itself downregulates IL-7Rα transcription and IL-4 has also been associated in downregulation of IL-7Rα (27). Additionally, LPS is a potent activator of DCs via the TLR4–MyD88–NF-κB pathway and could control the cytokine sensitivity by regulating the expression of cytokine receptors on DCs. For these experiments, the cells were left o/n in either 10% serum containing culture medium alone, or with IL-4 (20 ng/ml), IL-7 (40 ng/ml), LPS (1 μg/ml), or IL-4 plus LPS, and then measuring IL-7Rα in CD11c^{bright}, MHC II^{bright} cells, which were negative for CD3, B220, and F4/80 (Fig. 4A).

In splenic DCs, o/n culturing alone increased IL-7Rα expression significantly as compared with ex vivo–purified DCs ($p = 0.0003$). Strikingly, as compared with o/n alone–cultured DCs, adding IL-7 into the culture appeared to strongly downregulate IL-7Rα expression in DCs ($p = 0.019$), whereas adding IL-4 significantly increased IL-7Rα expression ($p = 0.0001$, Fig. 4B). LPS alone also induced IL-7Rα expression significantly, when compared with DCs cultured in medium alone ($p = 0.0076$), whereas adding IL-4 to LPS did not further increase the expression of IL-7Rα in DCs. The induction of IL-7Rα was not associated with further activation of DCs as judged by CD80 expression. CD80 was upregulated in o/n-cultured cells, but both IL-7 and LPS further induced CD80 expression, whereas IL-4 did not (Fig. 4C).

In line with changes observed in the expression of IL-7Rα, the induction of intracellular pSTAT5 by 15 min stimulation with TSLP was enhanced in splenic DCs that had been cultured in IL-4 when compared with DCs cultured in culture medium alone ($p = 0.0037$, Fig. 4D). Strikingly, no difference in TSLP responsiveness was observed between DCs that been cultured either in medium alone or in medium containing IL-7 ($p = 0.5008$), whereas IL-7 responses were slightly, but not statistically, significantly lower in IL-7–cultured cells when compared with medium alone ($p = 0.0998$). Albeit LPS upregulated IL-7Rα in splenic DCs (Fig. 4B), culturing these cells in LPS did not enhance their cytokine responsiveness (Fig. 4D). Taken together, TSLP-induced STAT5 phosphorylation was significantly enhanced only in DCs cultured in IL-4. The slight decrease in pSTAT5 responsiveness to IL-7 by DCs cultured in IL-7 was not due to decreased expression of γ_c by IL-7 stimulation (data not shown).

In DCs purified from lymph nodes (Supplemental Fig. 3), o/n culture significantly increased expression of IL-7Rα ($p = 0.0010$), whereas IL-4 appeared to enhance IL-7Rα, but this difference was not statistically significant ($p = 0.0871$), whereas IL-7 clearly downregulated IL-7Rα ($p = 0.0118$) in these cells (Supplemental Fig. 3). For CD80 expression in lymph node DCs, IL-7 induced CD80 expression whereas IL-4 had no effect on CD80 expression (Supplemental Fig. 3).

Human DCs upregulate IL-7Rα during o/n culture

In human peripheral blood myeloid DCs, not only IL-7Rα but also TSLPR are upregulated during o/n culture (19), whereas in human airway mucosal myeloid DCs TSLPR is constitutively activated (28). This could suggest differential regulation of the TSLP receptor system between two anatomic locations. To learn whether

FIGURE 2. The increased responsiveness of splenic DCs to TSLP and IL-7 is due to robust increase in IL-7Rα expression upon o/n culture. (**A**) IL-7Rα, γ_c, and TSLPR expression of splenic DCs (CD11c^{bright}/MHC II^{bright} cells) was measured either directly after harvesting the spleens (ex vivo) or after o/n culture. A representative example is shown. (**B**) Statistical analysis of five to eight independent experiments. Bars represent the average percentage of receptor chain–positive cells (±SEM indicated). ***$p < 0.001$, two-tailed, paired *t* tests. (**C**) TSLPR Ab specificity was tested using splenic CD4 T cells from wild-type or TSLPR$^{-/-}$ mice. The experiment was done twice (one wild-type and one TSLPR$^{-/-}$ mouse per experiment). (**D**) The specificity of IL-7Rα and γ_c Abs was tested on splenic CD4 and B220 cells, as indicated with Ab-specific isotype controls (green and red lines). The staining was done twice with two different wild-type B6 mice.

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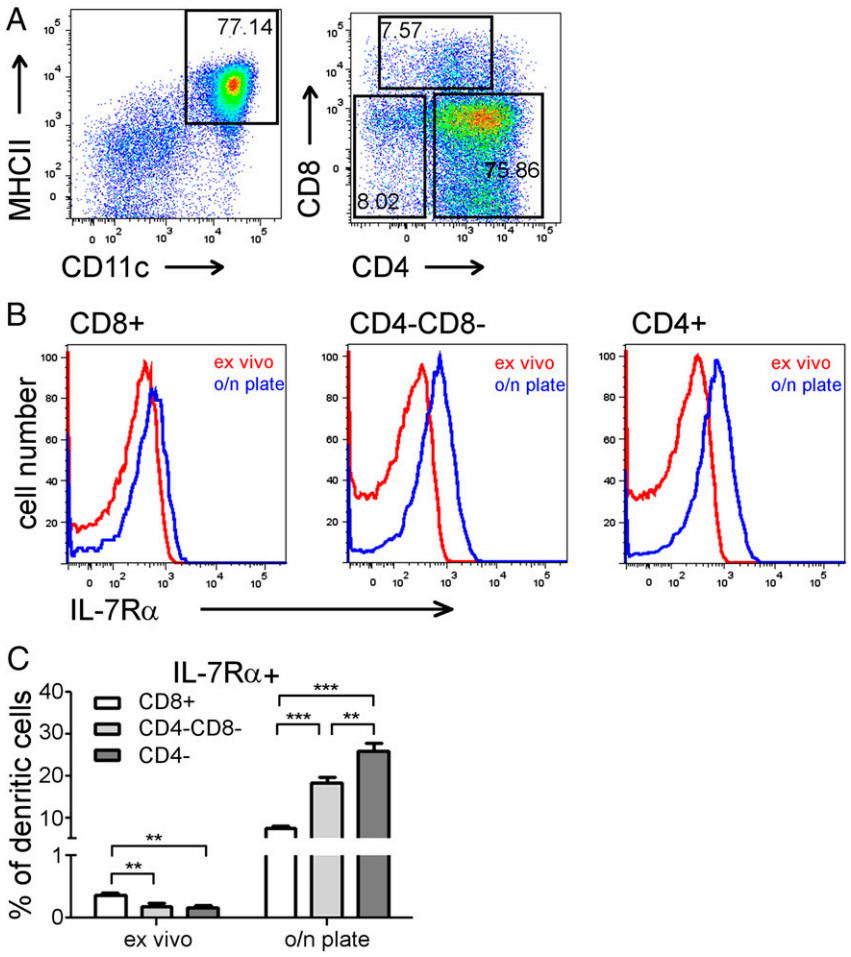

FIGURE 3. IL-7Rα upregulation occurs mainly in CD8$^-$ splenic DCs. (**A**) Gating the o/n-cultured splenic DCs on the basis of CD4 and CD8 expression. Numbers indicate percentage of cells of total CD11c/MHC II–positive cells. (**B**) IL-7Rα expression was studied in different splenic DC subpopulations ex vivo or after o/n culture. A representative result from one experiment is shown. (**C**) Statistical analysis of IL-7Rα expression on splenic DC subpopulations from three independent experiments. The bars indicate the percentage of IL-7Rα$^+$ cells in the indicated subpopulation (the means ± SEM are indicated). $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed, paired *t* tests.

the observed IL-7Rα upregulation we discovered in murine DCs would occur in human DCs and would be linked particularly to stimulation with IL-4, we studied human peripheral blood DCs from healthy donors ($n = 5$) that were enriched as described in *Materials and Methods*. We analyzed HLA-DR^{bright}/CD11c^{bright} cells either directly ex vivo or after o/n culture on tissue culture plates (Fig. 5A). For o/n culture, we either left the cells unstimulated or stimulated the cells with human IL-4 or human TSLP. Quite differently from murine DCs, CD80 expression in human DCs was only modestly upregulated during o/n culture, but IL-4 and particularly TSLP strongly upregulated CD80 expression (Fig. 5B). For IL-7Rα expression, low expression ex vivo was strongly upregulated during o/n culture ($p = 0.0012$) and the upregulation was biphasic, indicating that IL-7Rα upregulation was not a feature of all HLA-DR^{bright}/CD11c^{bright} cells. Alternatively, only a portion of the cells was able to overcome possible inhibitory factors in the o/n cultures that were keeping IL-7Rα expression low. IL-4 stimulation itself did not induce IL-7Rα expression in human DCs (for o/n culture versus o/n culture with IL-4, $p = 0.0933$) whereas combining IL-4 stimulation to TSLP strikingly downregulated IL-7Rα expression ($p = 0.0024$) in human DCs. Thus, for human total HLA-DR^{bright}/CD11c^{bright} DCs, IL-4 did not upregulate IL-7Rα. It will be of interest to characterize the DC subpopulations that clearly upregulate IL-7Rα and determine whether IL-4 specifically regulates IL-7/TSLP sensitivity in these subpopulations.

IL-4 increases IL-7Rα expression on mouse splenic DCs in vivo

The notion that IL-4 appeared to further upregulate IL-7Rα on o/n-cultured DCs in mice prompted us to ask whether IL-4 might have the same effect on DCs in vivo. Because the half-life of IL-4 in biologic fluids is rather short, we used the method described by Morris et al. (22), where complexing IL-4 to a soluble anti–IL-4 Ab prolongs the cytokine $t_{1/2}$. Wild-type B6 mice ($n = 5$ per treatment) were either injected with anti–IL-4 or anti–IL-4 complexed to IL-4 (complex). To verify that the complex administration resulted in IL-4–mediated responses, we measured IL-7Rα and IL-4Rα on T cells. As expected (27), the expression of IL-7Rα was decreased in CD4 T cells in response to the complex ($p < 0.0001$), whereas the complex significantly upregulated IL-4Rα ($p = 0.0092$) in these cells (Fig. 6A, gating strategy for T cells is shown in Supplemental Fig. 4). For CD8 T cells, we found that the results were similar; IL-4 downregulated IL-7Rα ($p = 0.0013$) and upregulated IL-4Rα ($p < 0.0001$) expression (Fig. 6B). Because IL-4 upregulated IL-7Rα on DCs in vitro (Fig. 4B), we next asked whether the same occurs in vivo. As we measured IL-7Rα expression from mice treated with anti–IL-4, no effect on IL-7Rα expression was observed. However, for mice treated with complex we noticed consistent and statistically significant induction in IL-7Rα on DCs (Fig. 6C, left panel, $p = 0.0005$). In line with observations from in vitro cultures, CD80 was not induced by IL-4 in vivo (Fig. 6C, right panel).

To confirm that anti–IL-4 was an appropriate control for these experiments, we compared IL-4Rα expression in CD4 and CD8 T cells. We found that IL-4Rα expression in either cell type was similar in untreated or anti–IL-4-treated mice, whereas complex-treated mice showed dramatic upregulation of this receptor chain (Fig. 6D).

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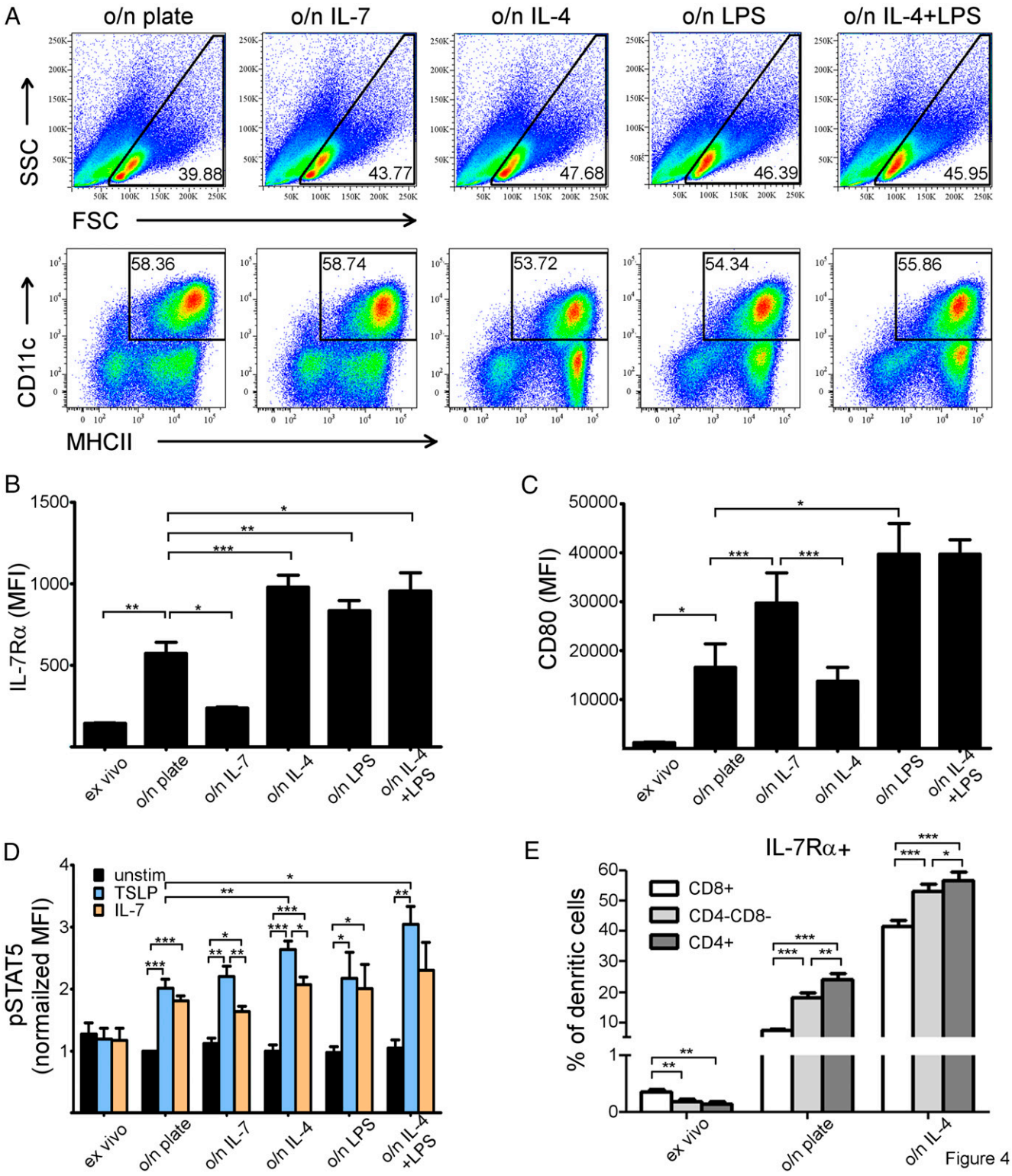

FIGURE 4. IL-4 induces IL-7Rα expression and TSLP responsiveness of splenic DCs. (**A**) Comparison of splenic DC populations after different o/n culturing conditions. (**B** and **C**) Statistical analysis of IL-7Rα (B) and CD80 (C) expression on splenic DCs. Bars indicate mean fluorescence intensity (MFI; with ±SEM). IL-7Rα and CD80 staining data are from three independent experiments (three animals used in each experiment). (**D**) STAT5 phosphorylation in splenic DCs (either ex vivo or o/n cultured with indicated stimulations) in response to either vehicle (PBS) or IL-7 or TSLP. The bars indicate normalized MFI of pSTAT5 (o/n-cultured unstimulated sample was given the value 1). Data are from three independent experiments (three animals used in each experiment). (**E**) IL-7Rα upregulation in different DC subpopulations in response to IL-4 was measured. MFI (±SEM) is indicated. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-tailed, paired *t* test was used. FSC, forward scatter; SSC, side scatter.

Discussion

The requirement of IL-7Rα for both IL-7- and TSLP-induced signaling coupled with the distinct anatomical distribution of the two cytokines prompted us to study how they act on DCs because these cells have been implicated as direct targets of TSLP. In accordance with previous studies, we found that only a small

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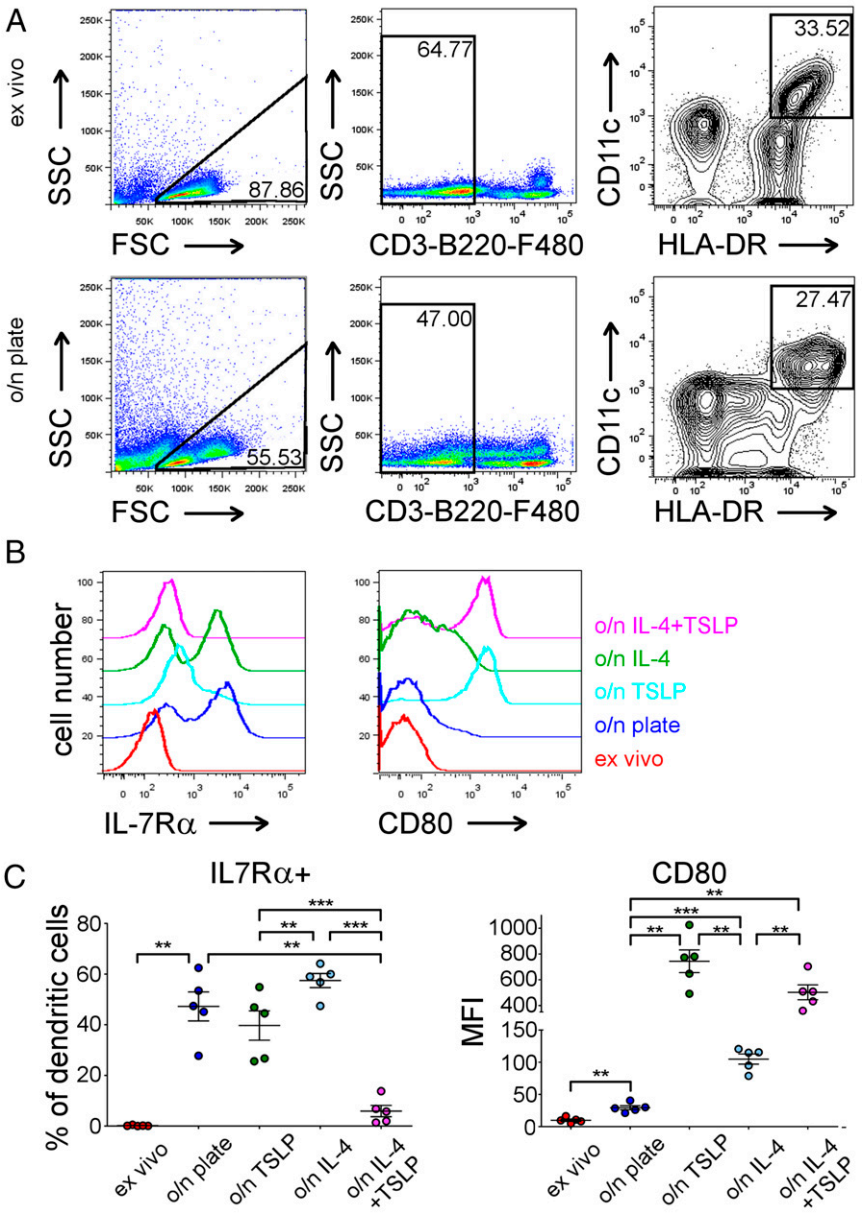

FIGURE 5. IL-7Rα expression is upregulated in human peripheral blood DCs during o/n culture. (**A**) Gating strategy of enriched human peripheral DCs ex vivo (upper panel) or after o/n culture (lower panel). (**B**) A representative experiment of expression of IL-7Rα (left panel) or CD80 in human DCs from one healthy donor after indicated culture conditions. (**C**) Statistical analysis of IL-7Rα and CD80 expression in peripheral blood DCs from five healthy donors after indicated culture conditions/stimulations. The means ± SEM are indicated. $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed, paired t tests. FSC, forward scatter; SSC, side scatter.

minority of freshly isolated splenic or lymph node DCs expressed IL-7Rα (17, 19) and that the great majority of these cells failed to phosphorylate STAT5 in response to either TSLP or IL-7. Overnight culture of these cells, which is known to activate DCs (25), resulted in upregulation of IL-7Rα and rendered a substantial proportion of the cells responsive to both IL-7 and TSLP. Evidence has been presented indicating that TSLP can cause skin or mucosal DCs to acquire Th2-inducing potential. The mechanism by which this occurs is still uncertain, but one possibility, at least in the human DCs, is the TSLP-mediated upregulation of OX40L expression (29).

The failure of freshly isolated splenic DCs to express IL-7Rα and to show responsiveness to TSLP implies that if TSLP truly plays a major physiologic role in Th2 differentiation through its effects on skin and/or mucosal DCs, then a dual induction process is required—the induction of TSLP synthesis/secretion by skin/mucosal epithelial cells and the induction of TSLP responsiveness by the local DCs. Induction of TSLP expression has been studied to some degree. Among the stimulants known to cause induction of TSLP are vitamin D_3, TNF-α, IL-4, and IL-13 (12, 13). Less is known about the mechanism through which IL-7Rα is induced on tissue DCs. Our study of splenic DCs implies that IL-4 and the LPS pathways regulate its in vitro induction. The notion that IL-7 appeared to inhibit IL-7Rα expression on DCs is interesting but it may also be a technical artifact. It is feasible that IL-7 in the culture media blocks the binding of the Ab recognizing IL-7Rα. This conclusion is supported by the fact that o/n IL-7 stimulation of DCs has no inhibitory effect on TSLP-induced STAT5 phosphorylation (Fig. 4C), indicating that the building blocks of the functional receptor for TSLP are available on the cell surface. The notion that addition of IL-4 into the o/n DC culture induced IL-7Rα expression but only modestly enhanced the responsiveness of the splenic DCs to IL-7 might be explained by the fact that IL-4 in the culture medium could keep the γ_c chain constantly occupied to the type I IL-4 receptor, thus limiting the increase in IL-7 signaling. Related to receptor bioavailability, increased expression of IL-7Rα on DCs could have another important consequence, namely reducing the bioavailability of IL-7 in biologic fluids, which plays a major role in survival of T cells (27).

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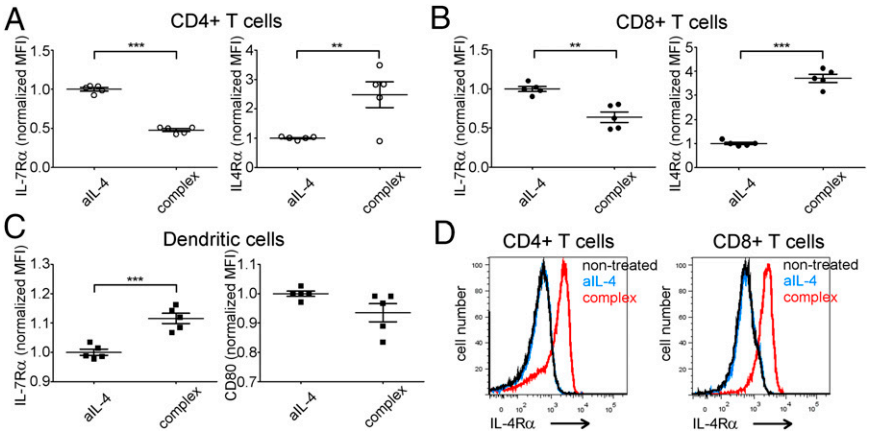

FIGURE 6. IL-4 affects surface expression of IL-7Rα in T cells and DCs differently in vivo. Anti–IL-4 Ab alone (aIL-4) or mixed with IL-4 (complex) were administrated i.p. to B6 mice (five per group) twice 8 h apart. Sixteen hours later, mice were euthanized and splenocytes were harvested and analyzed based on $CD3^+/CD4^+/CD8^+$ (**A**, **B**, and **D**) or $CD11c^+/MHC\ II^+$ (**C**) expression. (A) IL-7Rα (left panel) and IL-4Rα (right panel) expression in CD4 T cells. In all experiments dot represents expression level in one mouse (±SEM indicated). (B) IL-7Rα (left panel) and IL-4Rα (right panel) expression in CD8 T cells. (C) Expression of IL-7Rα (left panel) and CD80 (right panel) in splenic DCs. (D) Comparison of IL-4Rα expression in CD4 (left panel) and CD8 (right panel) T cells of untreated, aIL-4– and complex-treated B6 mice. $^{**}p < 0.01$, $^{***}p < 0.001$ two-tailed, paired *t* test.

Our studies imply that a coordinated induction of TSLP and IL-7Rα is essential for TSLP-mediated activation/differentiation of DCs. Note that recent work indicated that physiologically TSLP executes some of its functions via nonhematopoietic cells (30). This calls for further studies of TSLP signaling and the possibility that an alternative receptor may still exist because IL-7Rα is considered to be expressed solely by hematopoietic cells (31). Furthermore, comparison of murine B cells from WT or $TSLPR^{-/-}$ mice indicated that WT B cells express substantial amount of TSLPR, whereas they are negative for IL-7Rα. Would wild-type B cells from IL-7Rα–deficient mice respond to TSLP, and would this occur via Stat5 or independently of Stat5? Comparing genome-wide transcription analysis combined with cellular phosphorylation analysis in wild-type and IL-7Rα–deficient mice would clarify whether TSLP truly could signal without IL-7Rα.

The notion that IL-4, which itself induces TSLP production (14), also regulates a critical component of TSLP receptor complex on DCs in our experiments is interesting. A possibility remains that in the early induction phase of allergic inflammation, basophil-derived IL-4 (32) acts locally in barrier tissues on both keratinocytes to produce TSLP and simultaneously on DCs to upregulate the receptor for TSLP and, thus, IL-4 could orchestrate locally both production and responsiveness to TSLP on specific cell types. Further studies on functional characteristics of the IL-4/TSLP cytokine axis on IL-4–deficient mice will reveal how TSLP signaling and receptor expression (particularly IL-7Rα) are regulated by IL-4. Experiments following IL-7Rα expression on DCs utilizing mice genetically modified to lack the expression of IL-4, IL-4Rα, insulin receptor substrate 2, and Stat6 should answer the question of how IL-4 signaling regulates the expression of IL-7Rα on DCs.

Our observation that $CD8^+/CD4^-$ splenic DCs showed less induction of IL-7Rα as compared with $CD8^-/CD4^+$ or $CD8^-/CD4^-$ DCs implies that professional IL-12–producing DCs respond poorly to TSLP and IL-7 or that TSLP stimulation may divert the cells from acquiring or maintaining IL-12 producing capacity, at least in this experimental setting. As $CD8^+/CD4\_$ DCs are specific producers of IL-12, the failure of these cells to acquire IL-7Rα expression would be rational.

The benefit of the local regulation of IL-7Rα could be explained by locally regulated TSLP production depending on the signals that alveolar, bronchial, and skin epithelial cells receive. Whereas migratory DCs in lymph nodes and their skin counterparts (17) do express IL-7Rα at low levels, possibly allowing some cytokine signaling, the induction of IL-7Rα (and ensuing cytokine responsiveness) we observed in this study is quite drastic. The notion that in human freshly isolated DCs both TSLPR and IL-7Rα are expressed at low levels (19) indicates that human and mouse DCs may differ in the steady-state expression of TSLPR. Alternatively, TSLPR expression in human DCs may be regulated by infectious and genetic background differences that are absent in inbred mouse lines housed in standardized animal facilities. We found that in human peripheral blood DCs from small group of individuals ($n = 5$), the expression of IL-7Rα was induced during o/n culture as expected (19), but the additive IL-4–mediated effect seen in mice was not observed. One explanation could be that human DCs are continuously encountering Ags and are thus more activated than inbred mouse DCs from sterile housing. This idea is supported by the finding that CD80 expression was only modestly upregulated in human peripheral blood DCs as compared with splenic murine DCs. Thus, the phenotype of resting human DCs is quite different from corresponding mouse cells. Another point to consider is the fact that myeloid human DCs from different anatomical locations differ in their expression of TSLPR (19, 28), which could be explained by the fact that TSLP is expressed closer to epithelial and mucosal barriers and thus "sensing" of the cytokine is restricted to the cells closer these structures. Also, note that both IL-7Rα and TSLPR expression on DCs could be under active inhibitory regulation, particularly while in circulation. In murine CD4 T cells, IL-7Rα is downregulated by various cytokines (27), which could be the case for human DCs too. If this were the case, peripheral blood DCs would inherently express low levels of one or both of these receptor chains and only upon appropriate activation signal induce receptor chain expression, resulting in increased responsiveness to cytokine (TSLP/IL-7). Such a system would protect DCs against inappropriate activation by type 2–polarizing TSLP in the peripheral blood. Future work characterizing anatomy and activation status of various human DC populations in the context of TSLP (and IL-7) responses as well as expression of TSLPR and IL-7Rα will be a fruitful endeavor.

Another point to be considered is how much of the action of TSLP in Th2 responses is on CD4 T cells. Although there is the possibility that the STAT5 activation known to be critical to in vitro

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Th2 differentiation is mediated by TSLP in vivo, TSLP seems an unlikely cytokine to mediate its function in secondary lymphoid organs because its principal sites of production appear to be at mucosal and skin surfaces. If STAT5 activation is as essential for in vivo Th2 differentiation as it is for in vitro differentiation, IL-2 or IL-7 would appear to be far more likely to mediate this function during the initial phases of T cell priming. However, once activated CD4 T cells migrate to the tissues, TSLP-induced STAT5 phosphorylation could be important in completing or sustaining Th2 differentiation and/or survival.

Overall, our results provide a mechanistic explanation how DC responsiveness to TSLP in mouse can be modified simply by induction of IL-7Rα, the receptor chain required for both IL-7 and TSLP responses.

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Disclosures

The authors have no financial conflicts of interest.

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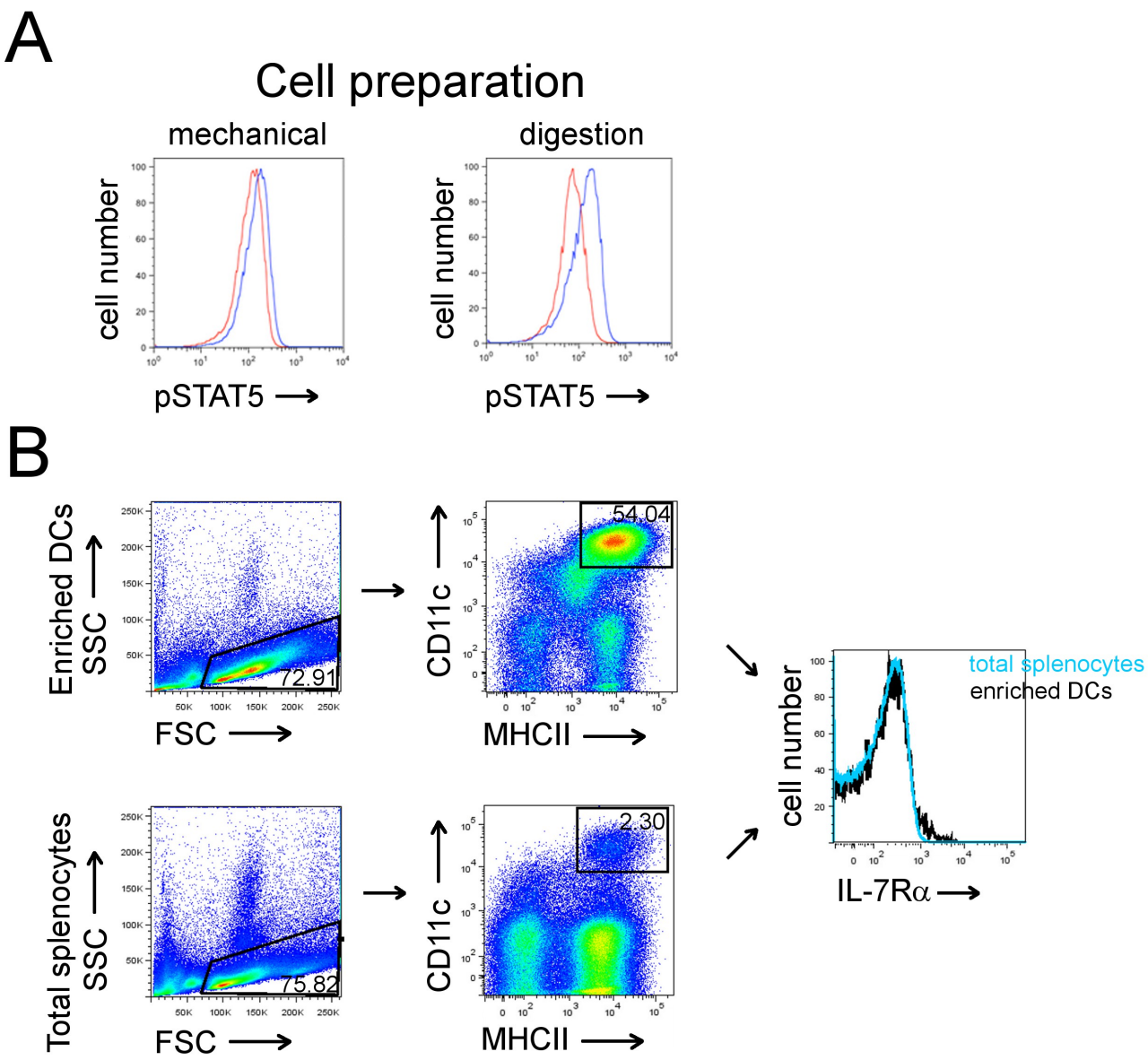
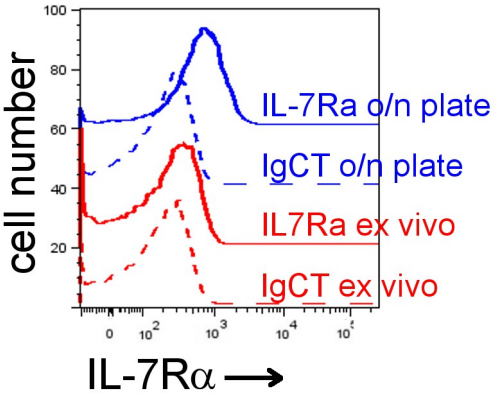


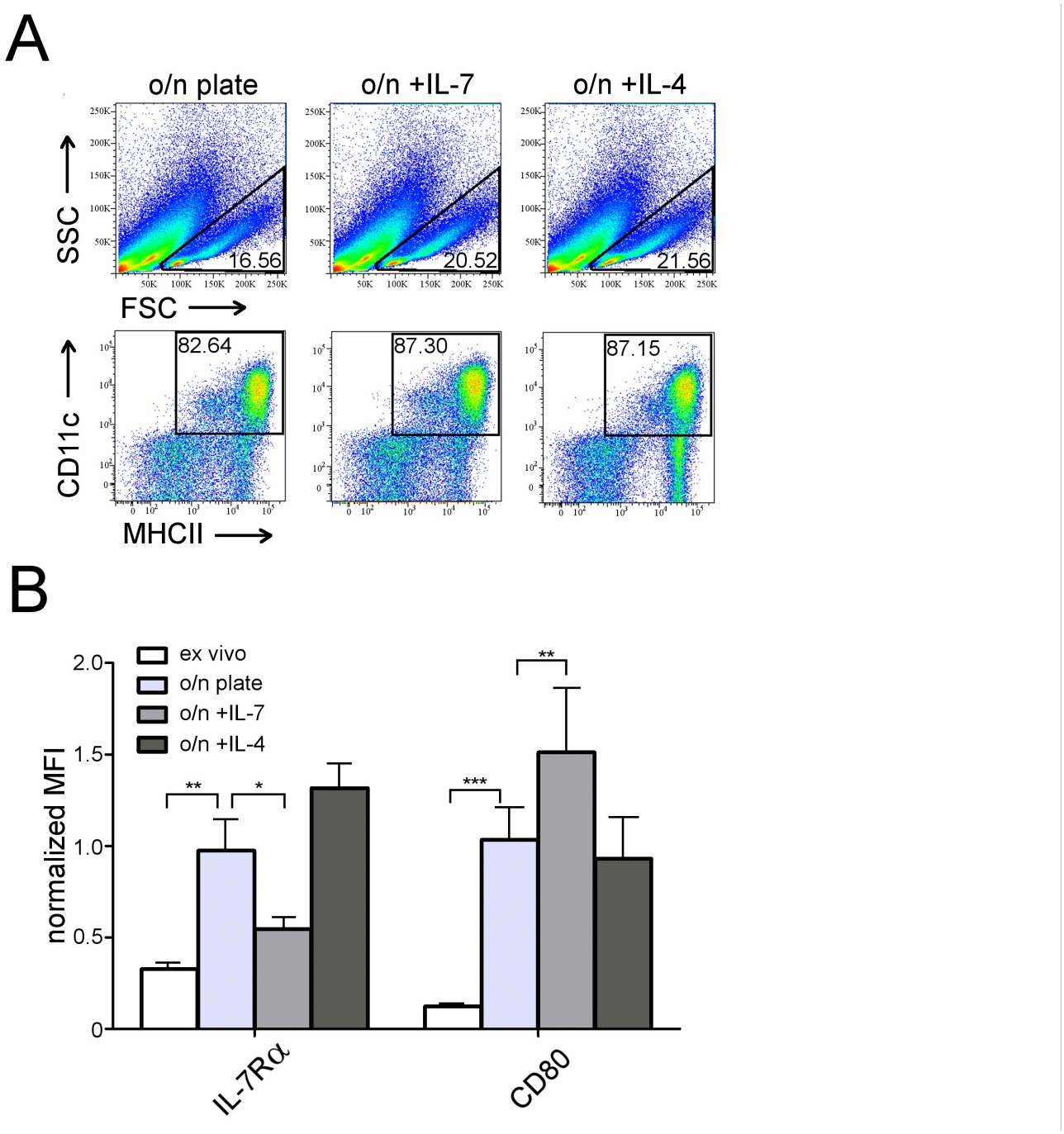
Supplementary figure 1. Dnase/Liberase treatment of spleen does not degrade IL-7Ra and DC enrichment does not affect IL-7Ra expression of ex vivo DC's.

Supplementary figure 1. Dnase/Liberase treatment of spleens does not degrade IL-7Rα and DC enrichment does not affect IL-7Rα expression of ex viv splenic DC`s. (A) To verify that IL-7Rα is not degraded on DC's by chemical dissociation of the spleen, single cell suspensions were prepared from spleens of WT B6 mice by either mechanical disruption (left panel) or Dnase/Liberase (right panel) treatment was used. After resting the splenocytes fo hour, the cells were either left un-stimulated or stimulated with IL-7 for 15 minutes. CD4 surface staining was followed by intracellular staining of pSTAT The experiment was repeated twice (1 mouse spleen for each experiment).
(B) To verify that DC enrichment did not affect IL-7Rα expression on these cells, IL-7Rα expression of total splenocytes and enriched DC`s was compared. The gating in these experiments was to CD11chi/MHCIIbright cells.



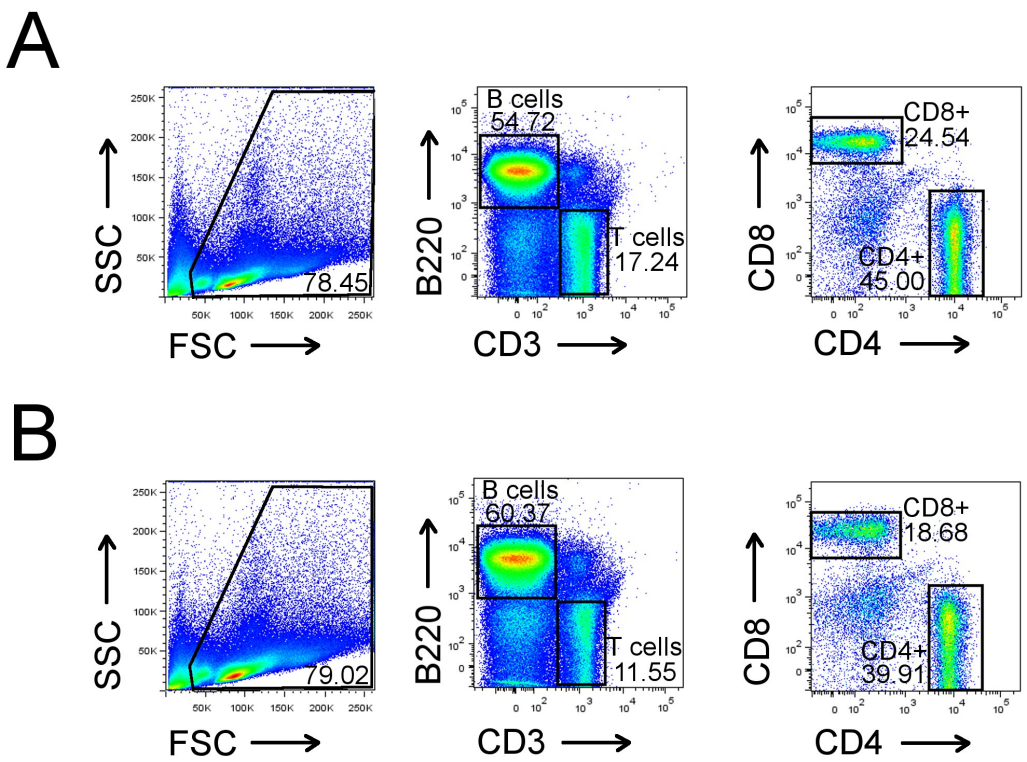
Supplementary figure 2. IL-7Ra staining is increases during o/n culture of splenic DC's while isotype control binding to these cells remains the same.

Supplementary figure 2. Increase of IL-7Rα antibody binding to DC`s is not due to generally increased binding of antibodies to DC`s. CD11cbright/ MHCIIbright enriched splenic DC`s were stained either with isotype control or monoclonal IL-7Rα antibody ex vivo or after overnight culture.



Supplementary figure 3. A. Gating strategy for lymph nodes DC's. B IL-7Ra and CD80 in lymph node DC's cultured under different stimulations.

Supplementary figure 3. Gating strategy and CD80 and IL-7Rα expression in lymph node DC's cultured in IL-7 and IL-4. A. Gating strategy to identify DC's in murine lymph nodes. Lymph nodes from three mice were collected and pooled. Single cell suspensions and DC enrichment were performed similarly to splenocytes. Cells were then stained for DC markers. B. IL-7Rα and CD80 surface markers from lymph node DC's were measured either e vivo or after overnight culture with indicated stimulations. The analysis of three independent experiments (9 mice overall) are indicated.



Supplementary figure 4. Gating strategy for splenic T-cells in mice undergoing anti-IL-4 (A) or anti-IL-4 - IL-4 complex (B) administration.

Supplementary figure 4. Gating strategy for splenic T cells from IL-4 treated mice. Spleens were harvested either from control mice (anti-IL-4-treated mice, A) or mice receiving IL-4 (IL-4 complexed to anti-IL-4, B). CD3 positive cells were further divided to CD4 or CD8 positive cells as indicated. Simil analysis was performed for all treated mice (5 controls and 5 IL-4 treated mice).

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ORIGINAL RESEARCH

R-Ras deficiency does not affect papain-induced IgE production in mice

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Abstract

Introduction: R-Ras GTPase has recently been implicated in the regulation of immune functions, particularly in dendritic cell (DC) maturation, immune synapse formation, and subsequent T cell responses.
Methods: Here, we investigated the role of R-Ras in allergen-induced immune response (type 2 immune response) in Rras deficient (R-Ras KO) and wild type (WT) mice.
Results: Initially, we found that the number of conventional DC's in the lymph nodes (LNs) was reduced in R-Ras KO mice. The expression of co-stimulatory CD80 and CD86 molecules on these cells was also reduced on DC's from the R-Ras KO mice. However, there was no difference in papain-induced immune response between the R-Ras WT and KO as measured by serum IgE levels after the immunization. Interestingly, neither the DC number nor co-stimulatory molecule expression was different between WT and R-Ras KO animals after the immunization.
Conclusions: Taken together, despite having reduced number of conventional DC's in the R-Ras KO mice and low expression of CD80 on DC's, the R-Ras KO mice are capable of mounting papain-induced IgE responses comparable to that of the WT mice. To our knowledge, this is the first report addressing potential differences in in vivo allergen responses regulated by the R-Ras GTPase.

Introduction

R-RAS is a small GTPase, a member of the extensive Ras superfamily, and shows about 55–60% amino acid identity with the classical RAS proteins (H-RAS, K-RAS, N-RAS) [1, 2]. Despite the close structural similarity to other members of the Ras family, the function of R-Ras is distinct from other Ras proteins. While all other members of the Ras family may cause malignant transformation, R-Ras has very little or no transforming activity. R-Ras plays a role in several cellular processes such as integrin signaling [3, 4], actin cytoskeleton organization [5], membrane ruffles, cell spreading [6, 7], cell adhesion [4, 8], and migration [9]. Concerning leukocytes, *Rras*$^{-/-}$ mice show impaired T cell trafficking [10] while murine macrophages expressing constitutively activated R-Ras show elevated phagocytotic activity [11].

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R-RAS can regulate inflammation [12–15]. Dendritic cells (DC's) from R-Ras deficient animals have reduced ability to prime T cell responses due to unstable immune synapse formation [14]. In experimental autoimmune encephalomyelitis model (EAE), *Rras*$^{-/-}$ mice showed attenuated disease course likely due to elevated numbers of peripheral tolerogenic and regulatory T cells (Tregs) [15]. Recently we showed that R-Ras is required for tumor development and progression in inflammation-dependent skin cancer model [13]. Although R-Ras was present only in the blood vessels and in skin, it was required both for the induction of pro-inflammatory cytokine production and for the extravasation of inflammatory cells to the skin [13].

Given the key role of DC's in adaptive immune responses and the role of R-Ras in regulating DC functions [14, 15] we wanted to know whether type2 immune response is regulated by R-Ras in vivo. We found that despite the fact that untreated R-Ras KO had decreased number of conventional DC's and the DC's show reduced expression of CD80/CD86 costimulatory molecules, these mice were capable of mounting a normal allergen-induced response after papain immunization.

Methods

Mice, immunizations, ELISA, cell culture, flow cytometry

R-Ras KO (= Rras$^{Gt(OST24882)Lex}$) mice (C57BL/6) have been described [16]. Animal experiment protocol was approved by Finnish National Animal Experiment Board (Permit: ESAVI/4738/04.10.07/2014). Administration of papain was performed as a modified version of the protocol described earlier by Sokol et al. [17]. Mice were injected subcutaneously either with PBS alone or PBS containing 0.5 mg papain (Merck Millipore, Darmstadt, Germany) on days 0 and 14. Blood samples were collected from the tail vein on day 0. Mice were euthanized on day 16 or day 21, and spleens and LNs were harvested. Blood was collected by cardiac puncture into serum blood tubes (BD, Franklin Lakes, NJ). IgE ELISA was from eBioscience (Santa Clara, CA).

Spleens and pooled LNs were incubated in 75 μg/ml Liberase DL and 10 U/ml DNase (Roche, Basel, Switzerland) for 30 min at 37°C. DC's were enriched with MACS Pan Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Alternatively, spleen cells were gradient centrifuged with Histopaque®-1077 (Sigma–Aldrich, St. Louis, MO).

Antibodies were (clones in parenthesis): CD3-APC (145-2C11) or -APC-eFluor780 (17A2), CD4-FITC or -APC-eFluor780 (both GK1.5), CD8-PerCP-Cy5.5 or -APC-H7 (both 53–6.7), B220-Pe-Cy7 or -APC-eFluor780 (both RA3-6B2), CD11c-PE-Cy7 (N419), F4/80-APC-eFluor780 (BM8), MHCII-FITC (M5/114.15.2), CD80-PerCP-eFluor-(16-10A1), CD86-APC (GL1), and OX40L-PE (RM134L) (all from eBioscience). Analysis was done by FACS Canto II (BD, Franklin Lakes, NJ) and FlowJo (Tree Star, Ashland, OR). Geometrical means and standard error of means are indicated.

Real-time PCR, statistical analysis

Spleens were harvested into RNAlater (Qiagen, Hilden, Germany). mRNA was extracted by Trizol Reagent (Life Technologies, Carlsbad, CA) and RNA was converted to cDNA by Thermo Maxima First Strand cDNA Synthesis Kit (Life Technologies). FAM-labelled Taqman probes (Applied Biosystems, Foster City, CA) for IL-10 (Mm01288386_m1), IL-13 (Mm00434204_m1), IL-17a (Mm00439618_m1), IFN-γ (Mm01168134_m1), FOXP3 (Mm00475162_m1), GATA3 (Mm00484683_m1), T-BET (Mm00450960_m1), and RORγ (Mm01261022_m1) were used with iQ^{TM} Supermix (Bio-Rad Laboratories Inc., Hercules, CA) for qPCR. VIC-labelled Eucaryotic 18S rRNA Endogenous Control (Applied Biosystems) was reference. Results were calculated by double delta method. Statistical analysis were done with Prism (GraphPad Software, Inc., La Jolla, CA) or R [18, 19] (for ELISA). *p*-values were calculated using two-tailed, unpaired student's *t*-tests.

Results and Discussion

R-Ras KO mice lacking functional R-Ras protein are phenotypically similar to WT counterparts [13, 16, 20]. However, DC maturation and immunological synapse formation with T cells is impaired in R-Ras KO mice and the capability of *Rras*$^{-/-}$ DC's to induce allogeneic or antigen-specific autologous T cell proliferation is reduced [14]. As described [14], we found that B- and T cell numbers in spleen of untreated R-Ras KO mice ($n = 3$) were normal (Fig. 1A, gating strategy in Supplementary Fig. S1A).

The less severe EAE in R-Ras KO mice is due to a local increase in the number of Tregs and tolerogenic DC's [15]. This led us to study the number and phenotype of DC's in WT and R-Ras KO mice. Analysis of magnetically enriched conventional and migratory DC's from the LN (gating strategy in Supplementary Fig. S1C) indicated that R-Ras KO mice had less conventional DC's than WT mice (Fig. 1B), ($p = 0.0126$), while the number of migratory DC's was similar between the two genotypes (Fig. 1B). As previously shown [14], the analysis of the enriched splenic DC's from spleen showed no difference between WT or R-Ras KO mice (Fig. 1C, gating strategy in Supplementary Fig. S1D). We chose to exclude plasmacytoid DC's from our analysis, as

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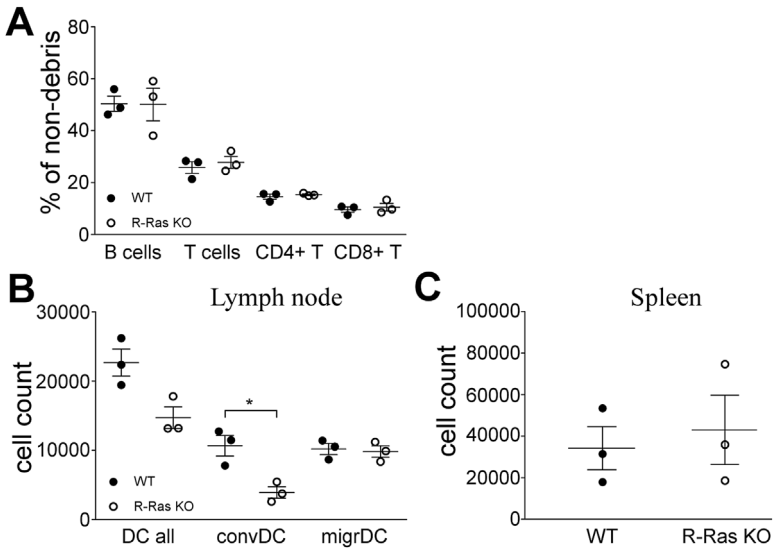

Figure 1. Immunophenotyping of un-manipulated R-Ras KO mice. (A) Lymphocyte populations of "off-the-shelf" wild type (WT, $n = 3$) or R-Ras deficient (R-Ras KO, $n = 3$) mice were compared by flow cytometry. (B and C) LNs (B) and spleens (C) were collected from untreated wild type (WT, $n = 3$) or R-Ras deficient (R-Ras KO, $n = 3$) mice. LNs from each mouse were pooled separately. DC's were enriched by magnetic separation (LN) or gradient centrifugation (spleens) and analyzed by flow cytometer. LN MHCII + CD11c+ (DC all) cell group includes two populations: conventional (convDC) and migratory dendritic cells (migrDC). (B) The amount of all MHCII + CD11c + dendritic cells (DC all) and their distribution between conventional (convDC) and migratory (migrDC) dendritic cells. (C) The amount of MHCII + CD11c + dendritic cells in the spleen. For statistical analysis unpaired two-tailed *t*-tests were used. $^{*}p < 0.05$.

they are currently considered less relevant to allergic responses: they are known producers of IFNγ upon TLR7/TLR9 activation during viral infections and autoimmune disorders [21].

The strength of T cell receptor(TCR)-engagement in cytokine-free environment regulates T cell differentiation. Weak TCR-signal leads to spontaneous, GATA3- and IL-2-dependent, IL-4 production while strong TCR signal causes, ERK-dependent, IFN-γ production [22]. The DC surface markers that take part in the DC/T-cell interaction include MHCII (antigen presentation to TCR), CD80/CD86 (co-stimulatory molecules associated with TCR) and OX40L (enhancement of Th2 differentiation at least in humans) [23]. MHCII expression was identical in both conventional and migratory LN DC's between WT and R-Ras KO mice (Fig. 2A). CD80 expression, in turn, was reduced in both conventional ($p = 0.0078$) and migratory ($p = 0.0176$) subsets from R-Ras KO mice (Fig. 2A), while CD86 expression was decreased in conventional DC subset of R-Ras KO mice ($p = 0.0241$), but not in migratory DC subset (Fig. 2A). OX40L expression was identical in conventional or migratory DC's between KO and WT mice (Fig. 2A). In spleen, MHCII expression was higher in the KO DC's ($p = 0.0129$) while CD80 expression was lower in the KO DC's ($p = 0.0207$) and, CD86 or OX40L expression were identical (Fig. 2B).

To better understand the role of R-Ras in tuning DC activation, we analyzed the response of splenic DC's from R-Ras deficient and WT mice to IL-4, LPS, and poly(I:C) during overnight plate culture, which "spontaneously" activates DC's [24]. Although LPS and poly(I:C) are not considered driving agents of Th2 polarization, we were interested to examine whether there was some general impairment of DC responses in $Rras^{-/-}$ mice and thus included these stimulants into the comparison.

Collectively, in splenic DC's MHCII expression is higher on DC's from R-Ras KO mice under all tested culture conditions. The differences observed in CD80 and CD86 expression between R-Ras KO and WT cells after overnight culture were abolished when the cells were further activated with poly(I:C), LPS or IL-4, while OX40L was induced by poly(I:C) and LPS, but R-Ras expression plays no role in the regulation of OX40L expression as no difference was identified between KO and WT cells (Supplementary Fig. S2A–D).

Taken together, the main difference in DC's between WT and R-Ras KO mice is that there is significantly less conventional DC's in R-Ras KO mice and these cells express less co-stimulatory molecules, particularly CD80. These results suggest, that antigen presentation via MHCII is not impaired in R-Ras DC's but the formation of immunological synapse due to decreased CD80/CD86 expression is impaired, which in turn might result in impaired CD28 signalling in T-cells.

Since EAE in R-Ras KO mice is less severe than in WT mice, we wanted to study if type 2 responses are affected in these mice. To analyze a possible "skew" in the steady-state

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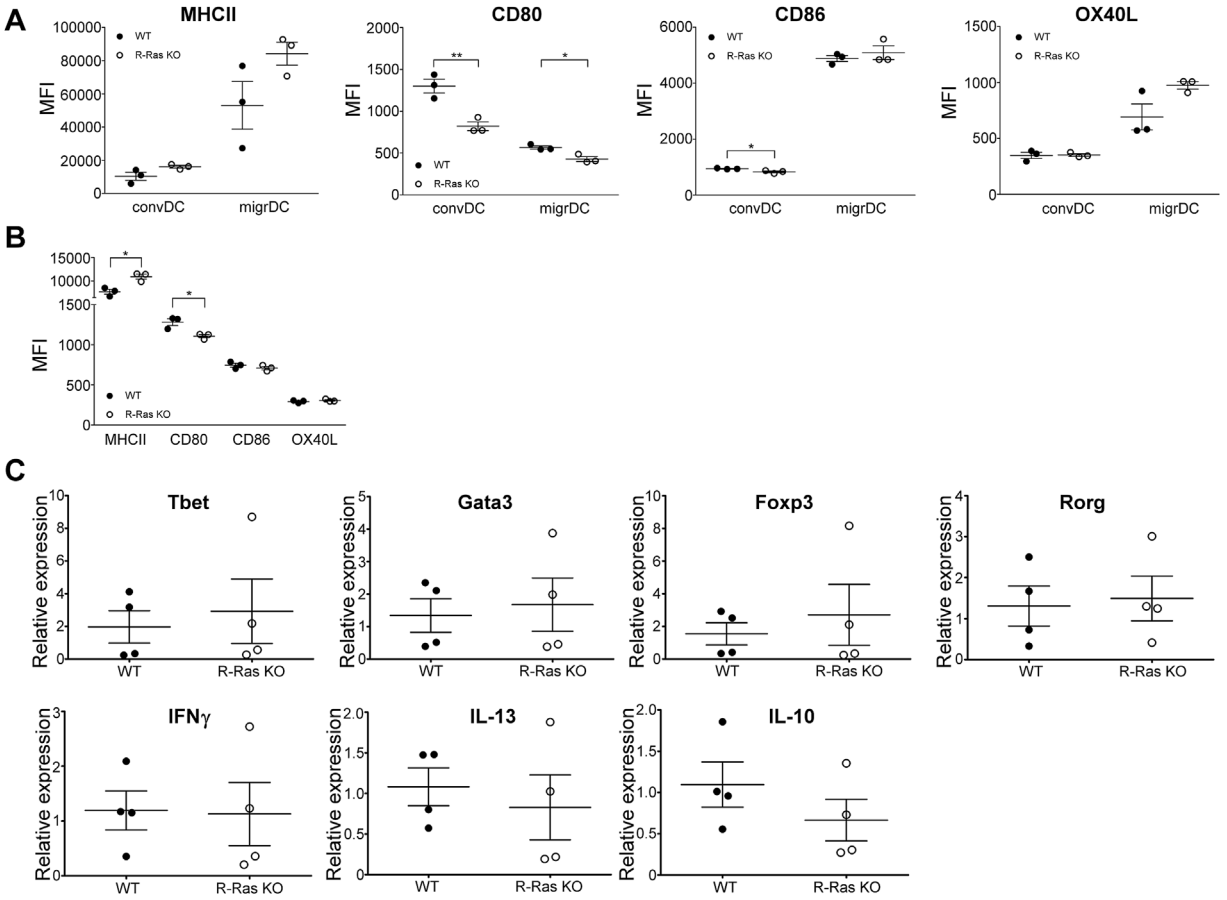

Figure 2. Comparison of the expression of dendritic cell surface proteins and expression of T-helper cell transcription factors and cytokines between WT and R-Ras KO mice. LNs (A) and spleens (B) were collected from untreated wild type (WT, $n = 3$) or R-Ras deficient (R-Ras KO, $n = 3$) mice and DC's were enriched as in Figure 1. (A) The expression of MHCII, CD80, OX40L, and CD86 surface proteins in conventional (convDC) and migratory dendritic cells (migrDC) from the LNs and (B) from the spleen. (C) Spleens of un-manipulated WT and R-Ras KO mice ($n = 4$ for each genotype) were harvested. mRNA was isolated from total splenocytes and transcribed into cDNA. The expression of Th-specific transcription factors and cytokines were investigated with qPCR. The differences within surface protein expression (A and B) or gene expression (C) values were statistically analyzed by two-tailed unpaired *t*-tests. $^{*}p < 0.05$, $^{**}p < 0.01$. Each dot represents an individual animal. Mean values with SEM are marked in the scatter dot plots.

CD4 T cells in R-Ras KO mice, we studied the mRNA expression of critical transcription factors related to helper T cell differentiation from the total splenocytes of WT and R-Ras KO mice, but found no difference in the mRNA expression of Tbet, GATA3, Foxp3, or Rorγ (Fig. 2C, upper panel). Also, the mRNA expression of IFNγ, IL-13, and IL-10 was identical between WT and R-Ras KO mice (Fig. 2C lower panel).

To study the allergen-induced response in R-Ras KO mice, we utilized papain-immunization as an in vivo model. Papain-induced responses are mediated via migratory DC's in skin [25] and we had already observed a decrease in CD80 expression in migratory DC's (Fig. 2A) in R-Ras KO mice. We subjected R-Ras KO mice and WT animals ($n = 8$ for each genotype) to papain injections twice: first immunization was performed on day 0 and re-immunization on day 14. We measured the serum level of IgE on day 0 and again when the mice were euthanized on day 21. We found no significant difference in the serum IgE levels between the two genotypes (Fig. 3A) 21 days after the first immunization. Next we wanted to rule out the possibility that the initiation phase of the type 2 response to papain might be delayed due to the impaired immune synapse formation [14] in R-Ras KO mice. For this, we measured the IgE levels of papain-immunized WT and KO ($n = 17$ and 18, respectively) mice only two days after the second immunization (i.e., 16 days after the first immunization). However, no difference in the serum level of IgE was found between R-Ras KO mice and WT (Fig. 3B). The concentration of IgE was approximately 10 μg/ml on day 16, while it was ∼50 μg/ml on day 21, indicating that the allergen response was still being induced after day 16 (Fig. 3A and B).

Next, we determined the number of DC's on day 16 after the papain immunization. Unlike in unmanipulated mice,

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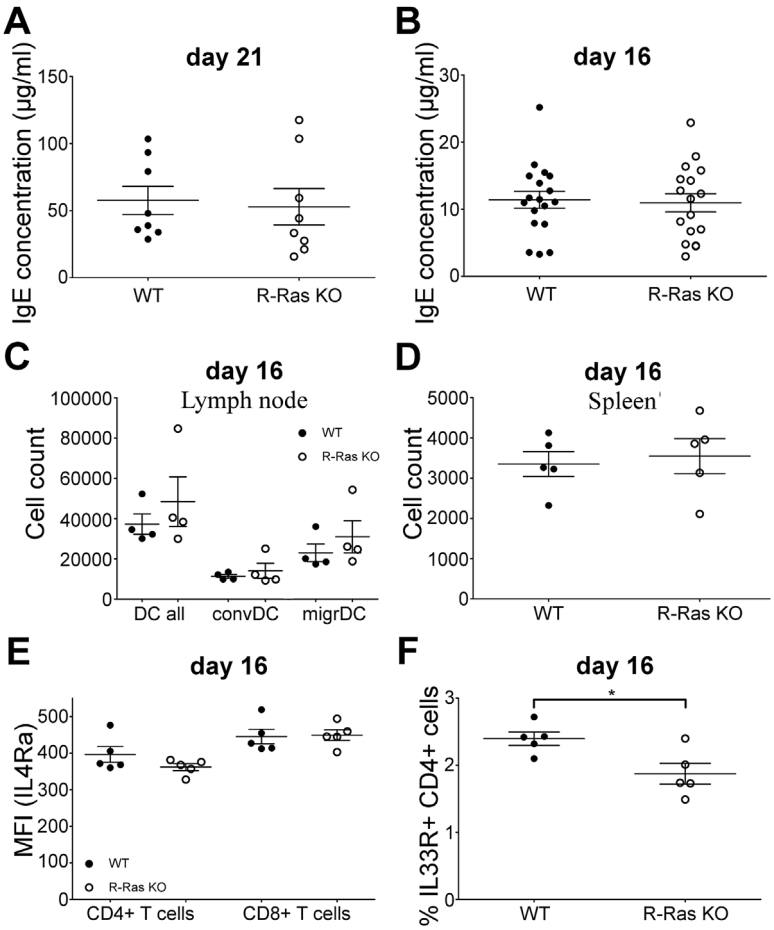

Figure 3. The effect of papain-immunization on WT and R-Ras-deficient mice. Wild type (WT) or R-Ras deficient (R-Ras KO) mice were treated with papain (500 µg/animal, subcutaneous injection on day 0 and day 14). Animals were euthanized either on day 21 or day 16. IgE concentration of blood serum was measured using ELISA on (A) day 21 or (B) day 16. Experiment in A was done twice with four mice of each genotype and in B the experiment was done four times with 4–5 mice of each genotype. (C and D) LNs ($n = 4$) and spleens ($n = 5$) were collected from papain-immunized (day 16) wild type (WT) or R-Ras deficient (R-Ras KO) mice and dendritic cells were enriched as in Figure 1. (C) The amount of all LN MHCII + CD11c + dendritic cells (DC all) and their distribution between conventional (convDC) and migratory (migrDc). (D) The amount of MHCII + CD11c + dendritic cells in the spleen. (E) Expression of IL-4Rα in CD4 and CD8 T cells of WT and R-Ras KO mice ($n = 5$ each genotype) on day 16 after papain immunization. (F) Percent of IL-33R positive cells of CD4 T cells from WT and R-Ras KO mice on day 16 after papain immunization. Mean values with SEM are marked in the scatter dot plots. Each dot represents an individual animal. $^*p < 0.05$ (unpaired *t*-tests).

which showed a difference in the number of conventional DC's between WT and R-Ras KO animals (Fig. 1B), we found no difference in their number (Fig. 3C) in papain-treated animals. Similarly, the number of splenic DC's was alike in WT and R-Ras KO mice (Fig. 3D). We also determined Th2-associated surface markers on CD4 T cells after papain immunization. The expression of the cytokine-binding receptor chain for IL-4 (IL-4Rα) is critical for CD4 T cell response to IL-4 and is up-regulated on CD4 cells during Th2 response. We found no difference in IL-4Rα expression between WT or R-Ras KO mice ($n = 5$) either in CD4 or CD8 cells (Fig. 3E). IL-33R is another cytokine receptor chain up-regulated in CD4 T-cells in Th2 responses. IL-33 can regulate ''effector cytokine,'' such as IL-5 and IL-13, expression in Th2 cells in concert with Stat5 activating cytokines, independently of antigens [26]. IL-33 has been shown to be indispensable for papain-induced IgE production in murine lung and genital tract [27, 28]. We found that the percent of IL-33R positive cells was reduced in R-Ras KO CD4 T cells (Fig. 3F, the gating strategy for IL-33R is shown is supplementary Fig. S1B). This might suggest that while IL-4 mediated events (IgE production, IL-4Rα expression) are intact in R-Ras KO mice, IL-4 independent (but GATA3-dependent) events, such as IL-33R expression can be affected by the deletion of R-Ras. The requirement of IL-4 for Th2 differentiation in vivo is discussed in detail in reference [29].

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Then we analyzed the expression of DC activation markers from papain-immunized mice. Unlike in the healthy mice, the conventional and migratory DC's from the LN showed no difference between the two genotypes in the expression of MHCII, CD80, CD86, or OX40L (Fig. 4A). In splenic DC's the expression of MHCII and CD80 were similar between WT and R-Ras KO animals, but CD86 expression was reduced after papain-immunization in R-Ras KO mice (Fig. 4B). Papain has been shown to induce Th2 polarization in multiple studies, determined by the IL-4 production of T cells [17, 28, 30] and T cell recall responses upon in vitro restimulation with papain [17, 28, 31, 32]. We measured the mRNA expression of transcription factors associated with T cell differentiation from splenocytes by qPCR after papain-immunization. The mRNA expression levels of Tbet, GATA3, and Foxp3 were identical between WT and R-Ras KO mice but for Rorγ, the master regulator of Th17-differentiation, the mRNA expression was significantly lower ($p = 0.0492$) in the R-Ras KO mice (Fig. 4C). Accordingly, hallmark cytokine mRNA analysis from these samples indicated no difference between IFN-γ, IL-13, or IL-10 expression (Fig. 4C). The notion that RORγ-transcription factor was expressed at reduced levels in R-Ras KO mice compared to WT is logical as IL-17 production is decreased in CNS of *Rras*$^{-/-}$ mice [15] undergoing EAE or carcinogen induced tumor development [13]. RORγ, on the other hand, plays a critical role in the development of IL-17 producing CD4+ T cells [33]. Also, a recent study showed that

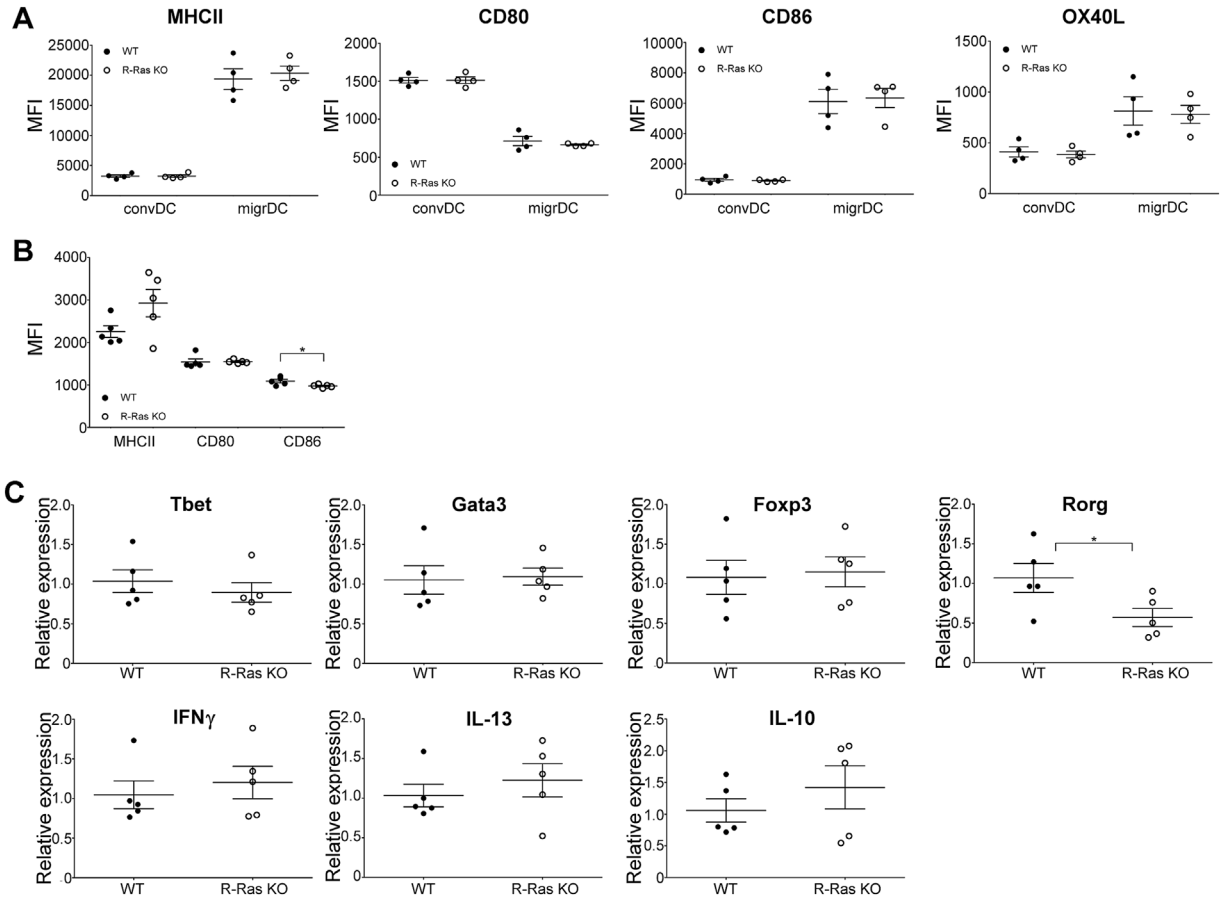

Figure 4. The expression of dendritic cell surface markers and mRNA expression of T-helper cell transcription factors and cytokines after papain-immunization in WT and R-Ras KO mice. (A) LNs were collected from papain-immunized (day 16) wild type (WT, $n = 4$) or R-Ras deficient (R-Ras KO, $n = 4$) mice and dendritic cells were enriched by magnetic separation. The expression of different surface proteins (MHCII, CD80, OX40L, and CD86) was investigated directly after euthanizing the mice. (B) Spleens were collected from papain-immunized (day 16) wild type (WT, $n = 5$) or R-Ras deficient (R-Ras KO, $n = 5$) mice and dendritic cells were enriched by gradient centrifugation. The expression of different surface proteins (MHCII, CD80, and CD86) was analyzed with flow cytometer. The differences within surface protein expression were statistically analyzed (unpaired two-tailed *t*-tests. $^{*}p < 0.05$). (C) Spleens were harvested from day 16 papain-immunized wild type (WT, $n = 5$) and RAS-deficient (R-Ras KO, $n = 5$) mice and prepared as in Figure 2. qPCR analysis for T cell transcription factors and cytokines was performed. Each dot represents an individual animal. Mean values with SEM are marked in the scatter dot plots. The differences within gene expression values were statistically analyzed by two-tailed unpaired *t*-tests. $^{*}p < 0.05$

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epicutaneous papain administration was able to induce the differentiation of allergen-specific Th17 cells [31], suggesting that papain induces not only Th2 but also Th17 responses.

To our knowledge, this is the first study that addresses the effect of R-Ras on allergen-induced immune response. Although we could not demonstrate any role for R-Ras in regulating the immune response toward papain-immunization, future work utilizing IL-4-dependent, and IL-4-independent Th2 polarizing models (such as *Trichurus muris* and *Nippostrongylus brasiliensis* infections) could specify a more defined role for R-Ras in immune response

Authors' Contributions

T.J., I.J., L.K., Z.O., S.P., M.V., and H.U-J. designed the research. L.K., Z.O., and M.V. performed the experiments. L.K., Z.O., M.V., S.P., H.U-J., T.J., and I.J analyzed the data. H.U.J. and M.V. contributed the genotyped mice littermates. L.K., Z.O., T.J., and I.J. wrote the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Gating strategies used in flow cytometry. (A) Spleens were harvested from euthanized mice. Single cell suspension was created as described in Materials and Methods and red blood cells lysed before staining the cells for surface markers and applying them onto the flow cytometer. Cell debris was excluded and remaining cells were studied for CD3 and B220 expression, and T cells were gated as $CD3^+$ population (T cell) and B cells as $B220^+$ group (B cell). T cells were further analyzed for CD4 and CD8 surface marker expression, and $CD4^+CD8^-$ and $CD8^+CD4^-$ subpopulation were identified as CD4+ T cells (CD4+) and CD8+ T cells (CD8+), respectively. (B) Strategy for IL-33R gating in CD4 cells. (C) For LN DC's, single cell suspensions of LN's were prepared as in Figure 1. $CD11c^+MHCII^+$ dendritic cells involve two subpopulations: conventional dendritic cells (convDC) show higher CD11c and lower MHCII expression while migratory dendritic cells express less CD11c but more MHCII. (D) For splenic DC's, spleens were prepared as described in Materials and Methods. Cell debris was excluded and cells were investigated for the expression of CD3 (T cell

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marker), B220 (B cell marker) and F480 (macrophage marker). $CD3^{-}B220^{-}F480^{-}$ cells (lin.neg.) were then studied further for the expression of CD11c and MHCII. The figure shows the gating of one representative sample for each case.

Figure S2. Expression of DC surface markers in WT and R-Ras KO splenic DC's in response to various stimulants. Spleens were prepared as described in materials and methods from WT ($N = 3$) an R-Ras KO ($n = 3$) mice. The cells were either stained directly (ex vivo) or after o/n culture with medium alone or with poly(I:C), LPS, or IL-4 as indicated. The expression of MHCII (A), CD80 (B), OX40L (C), and CD86 (D) was measured with flow cytometer.

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SUPPLEMENTAL INFORMATION FOR MANUSCRIPT: "R-RAS deficiency does not affect papain-induced IgE production in mice"

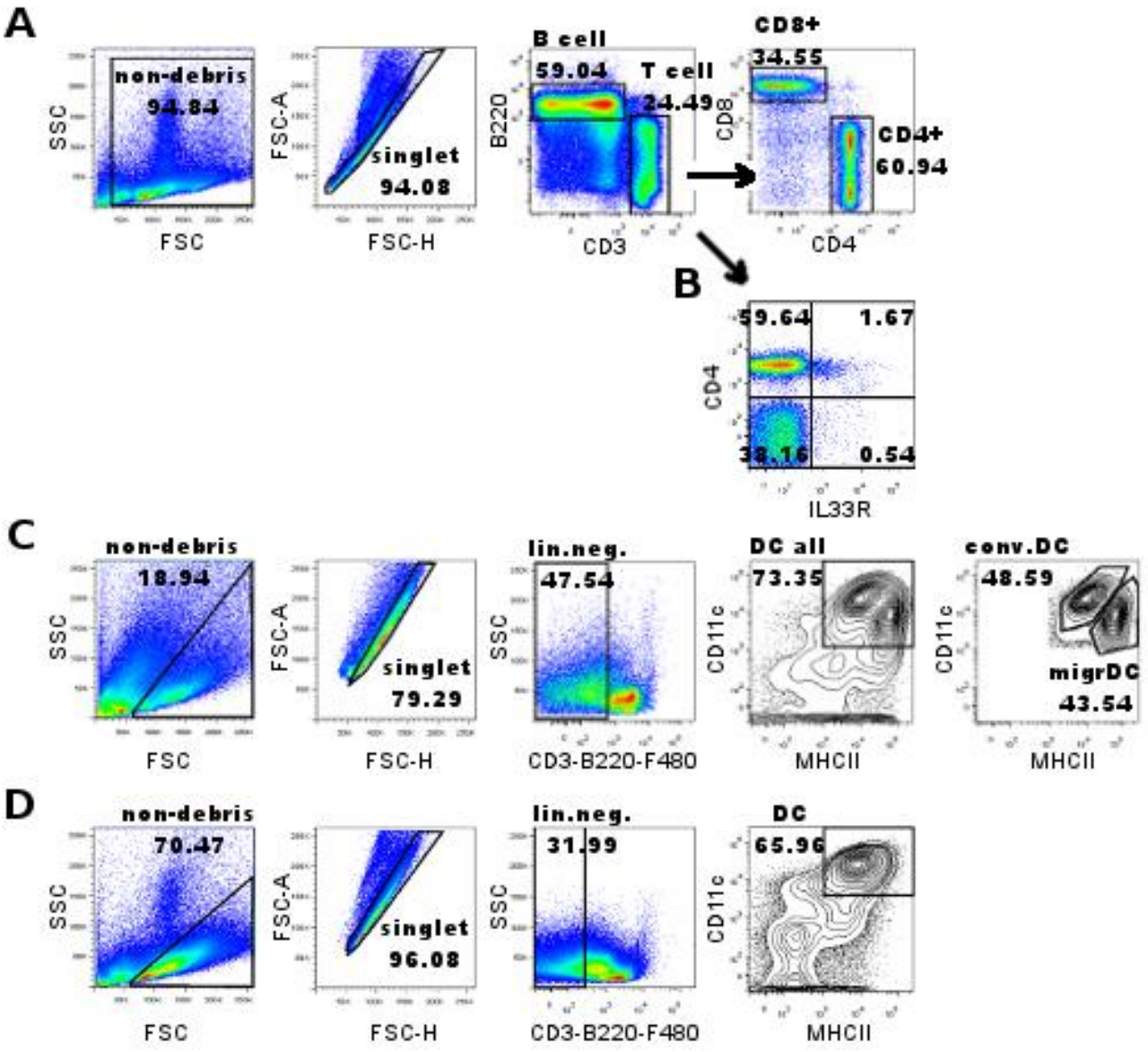
Laura Kummola, Zsuzsanna Ortutay, Maria Vähätupa, Stuart Prince, Hannele Uusitalo-Järvinen, Tero AH Järvinen, Ilkka S Junttila

SUPPLEMENTARY FIGURES

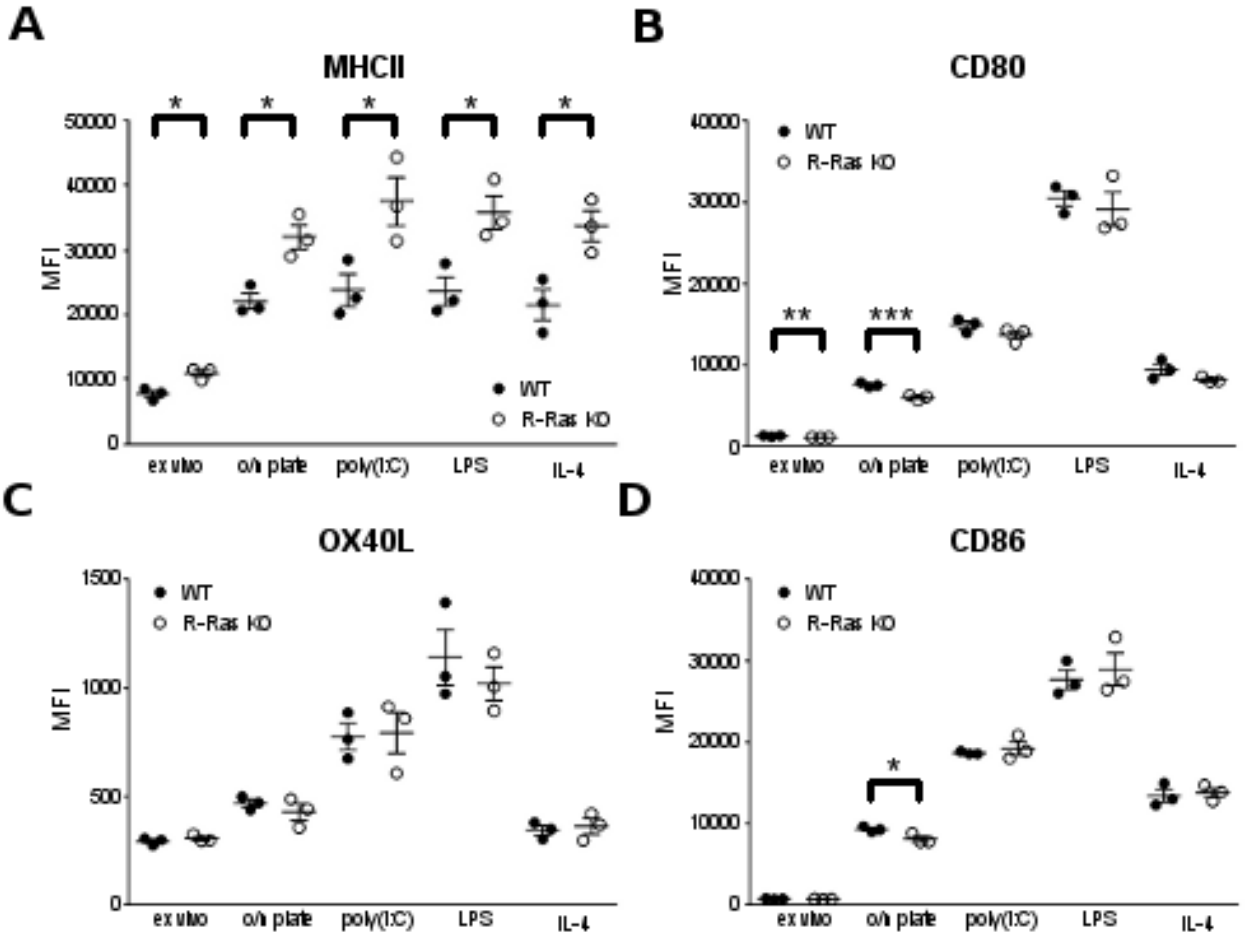
Supplementary figure 1. Gating strategies used in flow cytometry. (A) Splenic single cell suspension was created as described in Methods. Cell debris was excluded and remaining cells were studied for CD3 and B220 expression. T cells were gated as $CD3^+$ population (T cell) and B cells as $B220^+$ group (B cell). T cells were further analyzed for CD4 and CD8 expression, and $CD4^+CD8^-$ and $CD8^+CD4^-$ subpopulation were identified as CD4+ T cells (CD4+) and CD8+ T cells (CD8+). (B) Strategy for IL-33R gating in CD4 cells. (C) LN's were prepared as in Fig1. $CD11c^+MHCII^+$ dendritic cells involve two subpopulations: conventional dendritic cells (convDC) show higher CD11c and lower MHCII expression while migratory dendritic cells express less CD11c but more MHCII. (D) For splenic DC's were prepared as described in Methods. Cell debris was excluded and cells were investigated for the expression of CD3 (T cell marker), B220 (B cell marker) and F480 (macrophage marker). $CD3^-B220^-F480^-$ cells (lin.neg.) were then studied further for the expression of CD11c and MHCII. The figure shows the gating of one representative sample for each case.

Supplementary Figure 2. Expression of DC surface markers in WT and R-Ras KO splenic DC's in response to various stimulants. Spleens were prepared as described in materials and methods from WT (N=3) an R-Ras KO (n=3) mice. The cells were either stained directly (ex vivo) or after o/n

culture with medium alone or with poly(I:C), LPS or IL-4 as indicated. The expression of MHCII (A), CD80 (B), OX40L (C) and CD86 (D) was measured with flow cytometer.



Supplementary figure 1: Gating strategy



Supplementary figure2: Changes in surface markers of splenic dendritic cells after in vitro stimulation