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# ANTIGEN-PRESENTING CELLS IN TYPE 2 LUNG INFLAMMATORY RESPONSES

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This dissertation is dedicated to my grandparents, Antonio Alves Teixeira and Virginia Gonçalves Teixeira, for their wisdom and inspiration.

Dedico esta tese aos meus avós, Antonio Alves Teixeira e Virginia Gonçalves Teixeira, que me deram a sua sabedoria e sempre me inspiraram.

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# Abbreviations

APC: antigen-presenting cell

BAC: bacterial artificial chromosome

BATF3: basic leucine zipper ATF-like transcription factor 3

BAL: broncho-alveolar lavage

BMDC: bone marrow-derived dendritic cell

cDC(1,2): conventional dendritic cell (type 1, 2)

CFU: colony-forming units

$C_T$: cycle threshold

DC: dendritic cell

Der p 1: *Dermatophagoides pteronyssinus* antigen P1

FACS: fluorescence-activated cell sorting

Foxp3: Forkhead box P3

GAG: glycosaminoglycan

GATA3: GATA-binding protein 3

HDM: house dust mite extract

HSV2: herpes simplex virus type 2

IC: immune complex

(r)IL: (recombinant) interleukin

i.t.: intratracheal, intratracheally

IRF(4, 8): interferon regulatory factor (4, 8)

KLF4: Kruppel-like factor 4

LdLN: lung-draining lymph node

MHC(-I, -II): major histocompatibility complex (class I, II)

mLN: mesenteric lymph node

moDC: monocyte-derived dendritic cell

OVA: ovalbumin

pDC: plasmacytoid dendritic cell

PTX: pertussis toxin

qPCR: quantitative polymerase chain reaction

RORγt: retinoic acid-related orphan receptor gamma, t isoform

$S1P_1$: sphingosine-1-phosphate receptor 1

$T_{CM}$: T central memory cell

TCR: T cell receptor

$T_{EM}$: T effector memory cell

Th(1, 2, 9, etc.): T helper (type 1, 2, 9, etc.)

Treg: regulatory T cell

$T_{RM}$: tissue-resident memory T cell

WT: wild type

# Abstract

Antigen presenting cells (APCs) are crucial mediators between innate and adaptive immunity, translating information regarding bodily insults into durable and appropriate immune responses with the goal of restoring tissue homeostasis. The ability to mount numerous types of responses tailored to address a variety of diverse insults reveals heterogeneity among tissue APCs. The studies in this thesis address the signals that tissue APCs receive, how they integrate those signals, and how they propagate type 2 allergic lung inflammation, which can be a driving force in asthma. To this end, we first identify a process by which IL-33, a cytokine implicated in type 2 inflammation, stimulates the production of chemokines which attract allergen-bearing monocytes to the lung parenchyma during allergen challenge. Monocyte migration to the lung parenchyma during allergen exposure plays an important role in coordinating subsequent tissue eosinophilia, but not in promoting T cell responses to allergen. Instead, allergic T cell responses are driven by lung dendritic cells (DCs). We detail mechanisms by which interferon regulatory factor 4 (IRF4) promotes the migration of lung DCs to the lymph nodes and the expression of downstream factors that promote the education of T helper type 2 (Th2) cells. These IRF4-dependent processes are critical for promoting type 2 responses during allergic sensitization and during the effector response, but are not continuously essential for resident memory T cells ($T_{RM}$ cells) to mount memory responses. Finally, we investigate additional roles for IRF4-expressing DCs during intestinal infection with a colitis-inducing pathogen, suggesting that IRF4-expressing DCs also mediate Th22 responses in the gut. Together, these studies define novel mechanisms by which specific tissue APCs promote tailored responses to insults at mucosal barriers.

# Chapter I: Introduction

## Asthma and the allergic endotype

Asthma is a chronic obstructive disease of the airways affecting 339 million people worldwide (GAN 2018). Asthma is commonly diagnosed based upon its cardinal symptoms of wheezing, breathlessness, chest tightness, cough, and sputum production, which are thought to stem from bronchial hyperreactivity and persistent inflammation (GAN 2018). Disease management has gone through two distinct eras of approach: bronchodilators were introduced in the first half of the 20$^{th}$ century to address airway hyperreactivity, and corticosteroids emerged in the second half of the 20$^{th}$ century as inhibitors of inflammation (Pavord et al. 2018). While the efficacy of these treatments has demonstrated that these features are important contributors to asthma pathogenesis, these approaches are not appropriate for every patient. The traditional treatment modalities are not curative and do not sufficiently address the underlying causes of disease. Thus, despite these shared symptoms, it has recently become accepted that the term *asthma* refers to multiple diseases with distinct manifestations and etiologies (Pavord et al.

2018). An understanding of these disease subtypes, or "endotypes," is necessary to bring the field into the era of precision medicine.

A distinguishing characteristic among asthma endotypes is the extent and type of allergic inflammatory component. In fact, the worst outcomes arise from a combination of sensitization to multiple allergens and chronic wheeze (Simpson et al. 2010). The majority of children and approximately half of adults with asthma experience disease of allergic etiology (Lambrecht and Hammad 2015), and the immune component may take on various manifestations. While allergic asthma has classically been considered a type 2 inflammatory condition with prominent T helper type 2 (Th2) cells, eosinophilia, and antigen-specific IgE, the field also recognizes a predominantly type 17 inflammatory condition characterized by T helper type 17 (Th17) cells and neutrophilia, in addition to a spectrum of manifestations spanning combinations of these varieties (Martin et al. 2014). These comprise the main two groups of asthma endotypes, with the former commonly referred to as type 2 high asthma and the latter as type 2 low asthma.

## T helper responses in allergy and asthma

Early descriptions of T helper subsets were limited to distinguishing Th1 and Th2 cells, appreciating these subsets for their ability to control intracellular and extracellular pathogens, respectively (Kim et al. 1985, Mosmann et al. 1986). As our understanding has grown regarding the various types of challenges posed by the external environment, so too has our understanding of the diverse responses required to address these challenges. Thus, numerous varieties of helper CD4(+) T cells are now appreciated with Th1, Th2, Th9, Th17, Th22, Treg, $T_{FH,}$ and Tr1 among

them. Some of these subsets are well-delineated with T cells expressing lineage-defining transcription factors and particular cytokines, while others remain less-defined.

T helper responses in asthma can involve interplay between cells of the Th2, Th17, Th9, $T_{FH}$, and Treg lineages, with different lineages dominant in different asthma endotypes. For example, Th2 cells are the dominant T helper subset in eosinophilic asthma, in which they orchestrate responses driven by IL-4, IL-5, and IL-13. IL-4 drives B cell class switching for the production of IgE and IgG1, IL-5 is critical for the eosinophilic component of the disease, and IL-13 mediates goblet cell metaplasia, airway hyperreactivity, and endothelial activation (Lambrecht and Hammad 2015). Neutrophilic asthma displays a greater Th17 component (McKinley et al. 2008), whereas settings with a Th9 component display particularly strong roles for mast cells and goblet cells (Kearley et al. 2011). $T_{FH}$ cells mediate the humoral component of the allergic response as critical sources of IL-4 and IL-21, and may serve as a transitional phase for Th2 precursors during sensitization (Luthje et al. 2012, Ballesteros-Tato et al. 2016).

As is the case in other conditions manifesting with excessive inflammation, a deficiency in the ability of Tregs to mediate tolerance has been documented in asthma. In particular, depletion of natural, or thymic, Tregs during HDM sensitization exacerbates allergic inflammation by enabling exaggerated activation of lung DCs (Lewkowich et al. 2005). Further, mice deficient in induced Tregs develop spontaneous, asthma-like, type 2 inflammation in the lungs (Josefowicz et al. 2012). Thus, Tregs play a role in mediating tolerance to allergens in the lung and are among numerous other T helper fates that interact to contribute to or regulate asthma-like pathology.

## Antigen-presenting cells in the lungs

Mucosal barriers are the interface between a host and the external environment and are habitual sites of exposure to exogenous antigens. Antigen-presenting cells (APCs) reside in and surveil these tissues, providing a constant catalog of antigens in their surroundings to T cells that would otherwise be unable to detect these antigens directly. In the steady state, APCs are thought to be tolerogenic either by not inducing an immune response or by actively inducing regulatory cells (Matzinger 1994, 2002, 2007, Hawiger et al. 2001). However, insults such as allergens stimulate APCs to enact an inflammatory program. This activation may come from pattern- or danger- associated molecular patterns, or tissue-derived signals, which are all components that contribute to the cytokine milieu and are thought to contribute to education of naïve T cells (Seder and Paul 1994).

The lungs are home to at least two macrophage populations which are most commonly defined by their geographic location and are thus called alveolar and interstitial macrophages. The ontogeny of lung macrophages has been a topic of some controversy: it is now understood that the alveolar macrophage population is seeded during early life and possesses a considerable capacity for self-renewal. However, there is evidence that circulating progenitors may also contribute following exposure to inflammatory stimuli (Hashimoto et al. 2013, Guilliams et al. 2013). Blood monocyte progenitors give rise to a population referred to as exudative or inflammatory macrophages (Chung et al. 2015). Interstitial macrophages are present at birth and display a greater dependency upon recruitment from blood monocytes (Sabatel et al. 2017, Liegeois et al. 2018). As in other tissues and in circulation, lung monocytes are generally comprised of inflammatory or classical monocytes which express Ly6C and high levels of CCR2 and non-classical, patrolling, or alternative monocytes which do not express Ly6C but do express

CX3CR1. In addition to serving as progenitors to monocyte-derived macrophages and monocyte-derived DCs, lung monocytes have direct roles in inflammation with particular potency against bacterial infections (Serbina et al. 2008).

The broadest subclassifications of lung DCs are monocyte-derived DCs (moDCs), plasmacytoid DCs (pDCs), and conventional DCs (cDCs). The predominant role of moDCs during immune responses is the production of chemokines (Plantinga et al. 2013). pDCs are stimulated to produce type I interferon and proinflammatory cytokines in response to pathogens, and controversy has recently arisen whether they are DCs or members of the myeloid lineage at all (Dress et al. 2019). In contrast, cDCs efficiently migrate to tissue-draining lymph nodes and prime T cell responses upon activation. As inflammatory stimuli induce a broad range of T cell responses, a crucial line of inquiry has involved probing whether certain cDC subsets are specialized for the education of particular T helper responses or whether cDC subsets are multipotent in their capacity to educate multiple T helper cell fates in response to different stimuli.

Education of naïve T cells by DCs requires transduction of three major signals. The first signal relays information about the antigen. This signal is provided in the form of peptide presented on MHC-II and is received through the antigen-specific T cell receptor. The second signal is comprised of costimulatory signals, often in the form of B7 family members or TNF superfamily members, which are received through CD28 family members or TNFR superfamily members. The third signal provides information related to the nature of the insult, often in the form of type-polarizing cytokines which are most often thought to come from DCs. For example, the third signal is comprised of IL-12 production by DCs for Th1 responses, whereas there are roles for IL-6, TGFβ, IL-21, IL-1 and, IL-23 in polarizing Th17 cells (Walsh and Mills 2013). In

contrast, there has long been controversy regarding the identities and sources of Th2-polarizing cytokines (Schuijs, Hammad, and Lambrecht 2019). While IL-4 is known to be sufficient for Th2 specification of naïve T cells, particularly in vitro, neither DCs nor naïve T cells have been shown to produce IL-4. This has led to a search for the identity of the initial IL-4-producing cell. For a time it was thought that basophils were the necessary source of IL-4 for the education of Th2 cells (Sokol et al. 2009, Perrigoue et al. 2009, Yoshimoto et al. 2009), but it was soon discovered that DCs, not basophils, were necessary and sufficient (Hammad et al. 2010, Phythian-Adams et al. 2010), as the previous studies had depleted FcεR1(+) DCs in addition to basophils. Further, IL-4 is not always necessary for Th2 immunity in vivo (Pulendran, Tang, and Manicassamy 2010, Halim et al. 2014). Thus, additional insight is needed as to the mechanisms by which Th2 responses develop. Our lab and others have previously proposed that DC-derived Th2-polarizing cytokines include IL-33 and IL-10 (Iwasaki and Kelsall 1999, Bandukwala et al. 2007, Tjota et al. 2013, Williams et al. 2013, Tjota et al. 2014), which is a topic to be visited later in this thesis.

## Lung macrophages in type 2 immunity

Alveolar macrophages are considered poorly able to induce responses from naïve T cells but adequate APCs for reactivating antigen-experienced T cells (Kugathasan et al. 2008). Nevertheless, both pro-allergic and anti-allergic effects have been demonstrated on the part of alveolar macrophages, indicating that they perform different roles depending upon how they are stimulated. Homeostatic, or quiescent, alveolar macrophages appear to support immune tolerance to allergic stimuli, as their depletion exacerbates lung inflammation (Thepen et al. 1992, Mathie et al. 2015). Demonstrated mechanisms of this activity have implicated IL-10, IL-12, or NO

production (Balhara and Gounni 2012). In another study, depletion of the quiescent AM population had no effect on the type 2 response to HDM (Machiels et al. 2017). However, activated alveolar macrophages may propagate the allergic response. For example, IL-33-activated alveolar macrophages differentiate from a quiescent to a pro-allergic phenotype via a mechanism dependent on IL-13 (Kurowska-Stolarska et al. 2009). Pro-allergic factors produced by AMs include reactive oxygen intermediates, TNF, IL-1β, IL-8, CCL2, CCL3, TNF, and products of the arachidonic acid pathway (Balhara and Gounni 2012). While alveolar macrophages have been well-studied and demonstrate considerable plasticity, the specific contribution of interstitial macrophages has only recently begun to be explored. Interstitial macrophages are considered primarily tolerogenic during airway allergy, mainly by responding to commensal microbial signals and by secreting IL-10 (Bedoret et al. 2009, Sabatel et al. 2017).

## Lung cDC1s in type 2 immunity

The lung cDCs expressing CD103, which are called cDC1s, are best-recognized as the most efficient cross-presenting DCs, and thus are potent stimulators of CD8(+) T cell responses. Due to their prominent role in responses to intracellular pathogens and cancer, cDC1s are associated with the Th1 response. However, there is surprisingly little evidence that migratory cDC1s prime Th1 responses in the lungs. While lung cDC1s are capable of promoting Treg responses to inhaled antigens (Khare et al. 2013), studies investigating the role of cDC1s in initiating type 2 immunity have been conflicting. In particular, one study found a prominent role for CD103(+) cDC1s in the development of lung Th2 responses. Using three different in vivo stimulation models, the authors demonstrated that while both CD11b(+) DCs and CD103(+) lung DCs phagocytose allergen, CD103(+) DCs stimulated Th2 cytokine-producing T cells after in

vitro coculture with naïve T cells (Nakano et al. 2012). In other studies, cDC1s were found to have no role in the Th2 response. For example, depletion of lung CD103(+) cDC1s using Langerin-DTR mice did not affect Th2 priming of CD4(+) T cells in the lung-draining lymph nodes shortly after sensitization with HDM or with *B. tropicalis*, a dust mite found in tropical and subtropical regions, and did not alter airway inflammation during effector response after challenge in either case (Plantinga et al. 2013, Zhou et al. 2014). In other studies, cDC1s were found to inhibit the Th2 response via constitutive production of IL-12 (Everts et al. 2016). Further, IL-12 production by cDC1s increases during the response to HDM and drives a small Th1 component of the response, which counteracts exacerbation of a Th2/Th17 response during chronic exposures (Conejero et al. 2017). Thus, the role of cDC1s in lung Th2 responses is not entirely clear, but this subset is not thought to be a principal mediator of allergic sensitization.

## Lung cDC2s in type 2 immunity

A robust literature supports the role of CD11b(+) cDC2s in the development of Th2 responses. The requirement for cDCs in general is evident from experiments in *Flt3l*$^{-/-}$ mice, which lack cDCs, and are greatly impaired in their ability to mount Th2 inflammation to HDM (Plantinga et al. 2013). As cDC1s do not significantly contribute to this response, cDC2s were suspected to be main contributors (Plantinga et al. 2013). Indeed, adoptive transfer of HDM-sensitized cDC2s demonstrates that cDC2s are sufficient for inducing the Th2 response (Mesnil et al. 2012, Plantinga et al. 2013). These studies demonstrate that cDC2s efficiently deliver antigen by migrating to the lung-draining lymph nodes where they can educate naïve, allergen-specific T cells. Further, the type 2 response to HDM is mitigated in mice lacking lung cDC2s due to deletion of IRF4 from the CD11c-expressing compartment, and can be restored by i.t.

transfer of lung CD11b(+) DCs (Zhou et al. 2014). In fact, mice that have normal numbers of IRF4-deficient lung cDC2s, due to deletion of IRF4 after its developmental requirement, also display mitigated Th2 responses, suggesting a role for IRF4 in Th2-skewing mature DCs (Williams et al. 2013). Further, the CD24-expressing subset of cDC2s was later shown to be necessary for type 2 responses to HDM in the lungs (Tussiwand et al. 2015). Therefore, CD24(+) cDC2s, which are dependent on IRF4 and KLF4, are considered most important for lung Th2 responses.

Mice lacking IRF4 in both $CD11c^{int}$ pre-cDC and $CD11c^{high}$ mature DC compartments fail to develop cDC2 populations in the lungs, lung-draining lymph nodes, small intestine, mesenteric lymph nodes, and spleen (Bajana et al. 2016, Schlitzer et al. 2013, Vander Lugt et al. 2014). The remaining cDC2s show reduced migration to tissue-draining lymph nodes during the initiation of type 2 immunity (Bajana et al. 2012). Because of systemic failure of cDC2 development in the absence of IRF4, it has been difficult to study the ongoing role for IRF4 in mature DCs during inflammatory responses in vivo. To circumvent this, many studies have instead focused on the functional capacity of cultured BMDCs in response to non-physiologic stimuli. These in vitro DC cultures primarily demonstrate that IRF4-deficient DCs display a diminished capacity for antigen processing and presentation (Vander Lugt et al. 2014). However, in vivo study of these processes in response to relevant allergic stimuli is necessary for our understanding of how IRF4 acts in mature DCs, and is a major focus of the studies in this thesis.

## Lung moDCs in type 2 immunity

While sharing features such as high MHC-II expression and antigen processing machinery with cDC2s, moDCs generally display poor migratory capabilities and thus are not thought to contribute to Th2 education in tissue-draining lymph nodes (Mesnil et al. 2012, Schlitzer et al. 2013, Plantinga et al. 2013). Further, lung moDCs are less adept at priming and inducing effector cytokine production from naïve T cells than are cDCs (Nakano et al. 2015, Moran et al. 2015). As such, $CCR2^{-/-}$ mice, which have reduced numbers of lung moDCs, mount robust Th2 responses to the dust mite *B. tropicalis* (Zhou et al. 2014). While moDCs assist in orchestrating inflammation by producing chemokines during the challenge phase of the allergic response (Plantinga et al. 2013, Medoff et al. 2009), they appear to contribute to induction of the Th2 response only in particular circumstances. For example, at very high doses of allergen, allergen-bearing moDCs may be found in the lung-draining lymph nodes after sensitization, but even in this setting they appear to initiate Th2 responses of lower magnitude than do cDC2s (Plantinga et al. 2013). Thus, current evidence suggests that moDCs are not of primary importance for initiating allergic T cell responses.

## Trafficking and recruitment of immune cells to tissues

Development and activation of immune cells is insufficient for a robust immune response. Immune cells must undergo a complex process of trafficking to appropriate tissues where the relevant immune insult has occurred. As leukocytes patrol, traveling through the systemic vasculature, they must recognize the endothelium of affected tissue. These sites are marked by chemokine gradients produced by sentinel cells resident in the affected tissue and by selectins upregulated on the activated endothelium (Vestweber 2015). Upon binding of selectins

and activation of integrins, leukocytes undergo attachment to the endothelium via capture, rolling, slow rolling, arrest, and crawling on the luminal side of the endothelium (Vestweber 2015). This is followed by transmigration through the endothelium, or diapedesis, at an exit site. Leukocytes then continue to migrate, following gradients of chemokines bound to glycosaminoglycans on cell surfaces and throughout the extracellular matrix until reaching the target site (Rahimi and Luster 2018). Immune insults initiate acute inflammation through PRR-dependent secretion of inflammatory mediators, like chemokines or endothelial activators, by macrophages, DCs, and mast cells (Griffith, Sokol, and Luster 2014) or through alarmin release by stressed or dying cells (Rider et al. 2017).

While fast-acting myeloid cells such as neutrophils and monocytes employ these mechanisms in acute responses, leukocyte migration is also essential in adaptive immune responses. Indeed, one component of T cell education by DCs involves the imprinting of memory as to where the DC encountered antigen, and therefore to where T cell homing should be encouraged. Intestinal DCs that educate T cells in the mesenteric lymph nodes produce CD4(+) T cells expressing the integrin α4β7 (Hamann et al. 1994, Iwata et al. 2004) and the chemokine receptor CCR9 (Kunkel et al. 2000, Iwata et al. 2004), which instructs T cell homing to the gut. Skin DCs educating T cells in skin-draining lymph nodes teach CD4(+) T cells to express the skin-homing glycan CLA (Berg et al. 1991) and chemokine receptors CCR4 (Campbell et al. 1999) and CCR10 (Sigmundsdottir et al. 2007). Our understanding of factors necessary for T cell homing to the lungs is less developed, but one study demonstrated that lung DCs imprint CCR4 expression upon T cells in the lung-draining lymph nodes, and that this CCR4 expression is important for lung homing (Mikhak, Strassner, and Luster 2013). These studies demonstrate that DCs provide organotropic signals to T cells during their education. In

order to do so, DCs integrate signals regarding the nature of antigens via PRRs and CLRs, as well as tissue-derived signals in this process (Matzinger and Kamala 2011).

## Resident memory T cells

In addition to "type" classification based on master transcription factor expression and cytokine profile, T helper subsets are also classified with regard to their degree of activation and the anatomic compartment they patrol. Venous blood sampling reveals circulating T cell populations which can be distinguished based on expression of the lymph node homing receptor CCR7. The CCR7-expressing cells include antigen-inexperienced naïve T cells and central memory T cells ($T_{CM}$ cells), which have been previously activated but retain the ability to surveil the lymph nodes and forego some extent of effector function in exchange for proliferative capacity (Sallusto et al. 1999). In mice, both cell types demonstrate high expression of CD62L, which recognizes lymph node high endothelial venules (Gallatin, Weissman, and Butcher 1983). Naïve T cells are low in CD44 (DeGrendele et al. 1997), which mediates binding to extracellular matrix components in tissues (Goodison, Urquidi, and Tarin 1999), whereas $T_{CM}$ cells express high levels of CD44. CCR7-negative cells include the T effector memory ($T_{EM}$) population, which contains memory cells that patrol tissues and also cells which can rapidly take on effector functions upon restimulation (Sallusto et al. 1999). Like $T_{CM}$ cells, these cells express CD44, but do not express CD62L.

Studies from recent years have described a population of tissue-resident memory T cells ($T_{RM}$ cells), which reside in nonlymphoid tissues including the lung. These cells arise during the development of the adaptive response and are retained in the tissue due to decreased sensitivity

to egress signals and increased expression of retention molecules expressed by lung tissue (Oja et al. 2018). $T_{RM}$ cells have some phenotypic overlap with $T_{EM}$ cells, as they are CD44(high) and CD62L(low). However, they do not access the vascular compartment and thus are not labeled during experimental intravascular injection with fluorescently-labeled antibodies against surface antigens they express (Anderson et al. 2014). Thus, $T_{RM}$ cells are notable for their proximity to and density at the physiological site of antigen entry in addition to their maintenance of an expression profile enriched for effector molecules and inflammatory cytokines, contributing to rapid effector function upon reintroduction of their stimulus (Szabo, Miron, and Farber 2019). It is now well-accepted that $T_{RM}$ cells sense perturbations to tissue homeostasis and respond by proliferating, producing proinflammatory cytokines, promoting DC maturation, and recruiting circulating cells to assist in the response (Masopust and Soerens 2019). $T_{RM}$ cells have been well-studied in multiple tissues particularly in the context of type 1 immunity, but less is known pertaining to the development of these cells in type 2 lung inflammation, such as in allergic asthma. However, the essential roles emerging for $T_{RM}$ cells in other contexts implicates $T_{RM}$ cells that form in response to allergen exposure as relevant effector cells in allergic asthma.

In settings of type 2 inflammation, mucosal CD4(+) $T_{RM}$ cells have been studied in the skin, the gut, and in the lungs. Naïve T cells educated during allergic sensitization give rise to a mixture of $T_{CM}$ and $T_{RM}$ cells, which bear clonal resemblance to their naïve precursors but display altered homing and effector functions in allergic contact dermatitis (Gaide et al. 2015). This study found that in an allergic setting, the $T_{RM}$ population grows more abundant with repeated exposures and potentiates immediate, robust responses, in contrast to $T_{CM}$ cells which mediate delayed and attenuated inflammation (Gaide et al. 2015). Infection with a strictly intestinal nematode, *H. polygyrus*, generates ST2(+) $T_{RM}$ cells which mediate memory responses

to reinfection by proliferating and producing Th2 effector cytokines (Steinfelder et al. 2017). Lung Th2 $T_{RM}$ cells develop during infection with *N. brasiliensis* and during responses to HDM. These cells are sufficient to mediate protection against *N. brasiliensis* reinfection independently of tertiary lymphoid structures (Thawer et al. 2014). IL-4Rα signaling is not required for the generation or maintenance of $T_{RM}$ cells in this setting, but is required for IL-13 production and for mediating protection during reinfection (Thawer et al. 2014). Effector T cells responding to HDM sensitization require IL-2 signaling for their appropriate expression of CCR4 and homing to the lungs, evidenced by defective accumulation of $CD25^{-/-}$ $T_{RM}$ cells one month after challenge (Hondowicz et al. 2016). The long-term maintenance of the majority of lung Th2 $T_{RM}$ cells and nearly all airway Th2 $T_{RM}$ cells depends on IL-7 signaling (Yeon et al. 2017). Studies suggest that CD86-expressing cDC2s increase in frequency during $T_{RM}$ cell-mediated recall responses (Turner et al. 2018). However, we lack an in-depth understanding regarding the identity and role for particular APC subsets in educating naïve T cells to become Th2 resident memory cells and in reactivating $T_{RM}$ cells during subsequent responses.

## Summary

In this thesis, I address the interplay between mucosal APCs and the effector molecules such as cytokines and chemokines that incite T cell-dependent inflammatory responses in vivo. First, I specify a mechanism by which IL-33 promotes the recruitment of monocytes to the lung through upregulating chemokines during an acute allergic exposure. I proceed to delineate the mechanisms by which IRF4-expressing lung DCs educate naïve T cells during allergic sensitization and thereby mediate effector and memory allergic responses. These studies are followed by an investigation of the role for IRF4-expressing DCs during infectious colitis,

highlighting that mucosal antigen presenting cells are capable of assessing inflammatory stimuli and executing tailored responses to address the insult at hand.

# Chapter II: Materials and Methods

**Mice.** *Chapter 3.* C57Bl/6 mice (WT) were purchased from Harlan Laboratories. FcγRIII$^{-/-}$ mice and CCR2$^{-/-}$ mice were purchased from Jackson Laboratory. IL-33$^{-/-}$ mice were a gift from S. Nakae (University of Tokyo, Tokyo, Japan) (Oboki et al. 2010). Animals were housed in a specific pathogen-free facility maintained by the University of Chicago Animal Resources Center. The studies conformed to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. *Chapter 4 and 5.* C57Bl/6 mice (WT) were purchased from Harlan Laboratories (Indianapolis, IN). B6 CD45.1 (B6.SJL-*Ptprc*$^{a}$*Pepc*$^{b}$/BoyJ, stock #002014, (Yang et al. 2002, Schluns et al. 2002, Janowska-Wieczorek et al. 2001)) and conditional *Irf4* mutant (*Irf4*$^{fl/fl}$; B6.129S1-*Irf4*$^{tm1Rdf}$/J, stock #009380, (Klein et al. 2006)) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). *Irf4*$^{fl/fl}$ mice were bred to CD11c-Cre-GFP transgenic mice (CD11cCre; C57BL/6J-Tg(Itgax-cre,-EGFP)4097Ach/J, stock #007567, (Stranges et al. 2007)), which were developed and kindly provided by Dr. Alexander Chervonsky (University of

Chicago). OTII mice were bred and maintained at the University of Chicago. In all experiments, mice were matched for sex and age and blinded by ear tagging. Animals were bred and housed in a specific pathogen-free facility maintained by the University of Chicago Animal Resources Center. These studies conformed to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. The experiments were approved by the University of Chicago Institutional Animal Care and Use Committee.

**Mouse models of allergic airway disease.** *Chapter 3.* Grade V chicken egg OVA (A5503, Sigma Aldrich), rabbit anti–chicken egg OVA IgG (α-OVA) (C6534, Sigma Aldrich), recombinant mouse IL-33 (Biolegend), and pertussis toxin (516560, EMD Millipore Corporation) were used in murine experiments. Mice were administered α-OVA serum i.v. on day 0 followed by a fluorescently labeled OVA (OVA-FITC or OVA-AF647, Invitrogen) sensitization i.t. 24 hours later. At the time of sacrifice, the mice received an i.v. injection of anti-CD45 (30-F11, Biolegend). After five minutes, the lungs were perfused and harvested to allow for identification of cell localization in the vasculature (i.v. $CD45^+$) or lung interstitium (i.v. $CD45^-$). Lungs were processed into single cell suspensions as previously described for staining and analysis by flow cytometry (Tjota et al. 2014, Tjota et al. 2013). For some experiments, mice were further challenged on days 8, 9, and 10, sacrificed on day 11, and BAL was analyzed for cellular composition.

*Chapter 4.* House dust mite extract (Stallergenes Greer, part #XPB82D3A25, Lenoir, NC, USA) was resuspended in sterile PBS. In sensitization-only experiments, mice were administered 100 μg HDM via intratracheal instillation on day 0 and were sacrificed 12-18 hours later. Lungs and/or draining lymph nodes were made into single cell suspensions by mechanical disruption

followed by digestion in 600 U/mL collagenase IV (Sigma #C5138) and 20 µg/mL DNase I (Worthington DP grade) for one hour at 37C, and then additional mechanical disruption and red blood cell lysis. In experiments requiring fluorescent antigen tracking, HDM was labeled with Alexa Fluor 647 Protein Labeling Kit (Life Technologies #A20173, Eugene, OR, USA) per manufacturer's instructions. In experiments requiring allergen challenge, mice were administered 25 µg HDM via intratracheal instillation on days 7, 8, 9, and 10, and then sacrificed on day 13. In resting memory experiments, resting mice were sacrificed after 4-5 weeks. At the time of sacrifice, the mice received an intravenous injection of anti-CD45 (clone 30-F11, Biolegend). After five minutes, the lungs were perfused and harvested to allow for identification of CD45iv(+) cells located in the vasculature or CD45iv(-) cells in the lung parenchyma. In memory rechallenges experiments, mice were rechallenged during treatment with FTY720 (Enzo Life Sciences, #BML-SL-140). Mice were pretreated daily for two days with 25 µg FTY720, delivered by intraperitoneal injection. Mice continued FTY720 treatment while receiving 25 µg HDM via intratracheal instillation daily for four days. The mice continued FTY720 treatment until sacrifice three days later. Analysis of cells in the airways was conducted by bronchoalveolar lavage, in which sterile PBS was used to wash the airways four times via a tracheal cannula for a total recovery of approximately 3.0 mL. For histologic evaluation of lung inflammation, the left lobe was fixed in 10% neutral buffered formalin and then paraffin-embedded, cut into 5 µm sections, and stained with hematoxylin and eosin by the University of Chicago Human Tissue Resource Center.

When indicated, recombinant mouse IL-33 (Biolegend, San Diego, CA, USA #580506) or recombinant mouse IL-10 (Shenandoah Biotechnology, #200-84, Warwick, PA, USA) were used in murine experiments.

**Tetramer production.** *Chapter 4.* As previously described, I-$A^b$ containing the Der p 1 peptide 117-127 (CQIYPPNVNKI) were biotinylated and tetramerized with streptavidin-PE or streptavidin-APC (Prozyme) (Moon and Pepper 2018, Moon et al. 2007).

**T cell enrichment for adoptive transfer.** *Chapter 4.* Ly5.1 mice were sensitized and challenged as described above. On day 13, single cell suspension of lung cells was prepared as described above. Hematopoietic cells were isolated at the interface of 44% and 67% Percoll PLUS solutions (GE Healthcare #17-5445-01). CD4(+) T cells were then enriched by manufacturer's instructions using a MACS mouse CD4(+) T cell isolation kit (Miltenyi Biotec #130-104-454, Bergisch Gladbach, Germany) to yield a purity of 90-95% CD4(+) T cells, which were washed and resuspended in PBS for intravenous adoptive transfer of approximately 1 x $10^6$ cells per mouse. Recipient mice were promptly intratracheally instilled with 100 ng recombinant mouse IL-33.

**T cell enrichment, labeling, and coculture.** *Chapter 4.* Total LN and spleen cells were isolated from OTII mice and were passed through a nylon wool column. Cells were then labeled with CFSE and cocultured with sorted lung DCs at a DC : T cell ratio of 1:10 for 4 days in 96-well round-bottomed plates.

**Flow cytometric analysis.** *Chapter 3.* For flow cytometric analysis, $1\times10^6$ cells were resuspended in 100 µL of flow cytometric buffer (PBS containing 0.1% sodium azide and 1% BSA). Cells were blocked with 20 µL of anti-CD16/32 (2.4G2) supernatant and stained with fluorescently conjugated antibodies listed in Table 2-1. $Ly6C^-$ monocytes were gated as $SSC^{lo}CD11c^{lo/-}$MHC-$II^{lo/-}CD11b^+F4/80^+Ly6C^-$; $Ly6C^+$ monocytes were $SSC^{lo}CD11c^{lo/-}$MHC-$II^{lo/-}CD11b^+F4/80^+Ly6C^-$; $CD11b^+$ moDCs were $CD11c^+$MHC-$II^{hi}CD103^-CD11b^+Ly6C^+$;

$CD11b^+$ cDCs were $CD11c^+MHC\text{-}II^{hi}CD103^-CD11b^+Ly6C^-$; and $CD103^+$ cDCs were $CD11c^+MHC\text{-}II^{hi}CD103^+CD11b^-$. Flow cytometric analysis was performed on a BD LSRFortessa (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star, Inc.).

*Chapter 4.* For staining of dendritic cells, 1 x $10^6$ cells were suspended in 200 µL of staining buffer (PBS with 0.1% sodium azide and 2% BSA) and incubated for five minutes with 20 µL 2.4G2 supernatant. For staining of other cells, 5 x $10^5$ cells were suspended in 100 µL of staining buffer and incubated for five minutes with 10 µL 2.4G2 supernatant. Antibodies used include those in Table 2-1.

For intracellular cytokine staining, cells were incubated in culture medium with 10 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin for fixed with 2% formaldehyde overnight at 4C. They were then permeabilized with 0.5% saponin in PBS containing 0.1% sodium azide and 2% BSA and then incubated with the indicated antibodies. For intracellular transcription factor staining, samples were prepared using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (eBioscience #00-5523-00) following manufacturer's instructions. Flow cytometric analysis was conducted using an LSR Fortessa (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star, Inc.). Cell sorting was conducted using a BD FACSAriaIIIu (BD Biosciences). These instruments are maintained by the Flow Cytometry and Antibody Technology Core Facility at the University of Chicago.

Lung DCs were gated according to the following strategy (Figure 4-1). After gating out of SiglecF(+) eosinophils and alveolar macrophages, lung DCs were defined as CD11c(+) and MHC-II high. This also excluded lung interstitial macrophages, which are CD11c(-)

| Anti mouse | Clone | Manufacturer(s) |
|---|---|---|
| CD3 | 145-2C11 | BD Biosciences, Biolegend |
| | 17A2 | Biolegend |
| CD4 | GK1.5 | BD Biosciences |
| | RM4-5 | Biolegend |
| CD8 | 53-6.7 | Biolegend |
| CD11a | H155-78 | Biolegend |
| CD11b | M1/70 | BD Biosciences, Biolegend |
| CD11c | N418 | Biolegend |
| CD19 | 6D5 | Biolegend |
| CD24 | M1/69 | BD Biosciences, Biolegend |
| CD44 | IM7 | Biolegend |
| CD45 | 30-F11 | Biolegend |
| CD45.1 | A20 | Biolegend |
| CD45.2 | 104 | Biolegend |
| CD62L | MEL-14 | eBioscience, Biolegend |
| CD69 | H1.2F3 | eBioscience, BD Biosciences, Biolegend |
| CD80 | 16-10A1 | Biolegend |
| CD86 | GL-1 | Biolegend |
| CD88 | 20/70 | BD Biosciences, Biolegend |
| CD103 | 2E7 | eBioscience, Biolegend |
| CCR7 | 4B12 | eBioscience |
| F4/80 | BM8 | Biolegend |
| Foxp3 | FJK-16s | eBioscience |
| GATA3 | 16E10A23 | Biolegend |
| γ/δ TCR | GL3 | Biolegend |
| IL-4 | 11B11 | eBioscience, Biolegend |
| IL-5 | TRFK5 | Biolegend |
| IL-13 | eBio13A | eBioscience |
| IL-17 | TC11-18H10.1 | Biolegend |
| IL-22 | Poly5164 | Biolegend |
| I-A/I-E | M5/114.15.2 | Biolegend |
| Ly-6C | HK1.4 | Biolegend |
| OX40L | RM134L | eBioscience, Biolegend |
| RORγt | Q31-378 | BD Biosciences |
| SiglecF | E50-2440 | BD Biosciences |
| ST2 | DIH4 | Biolegend |
| Va2 TCR | B20.1 | eBioscience, BD Pharmingen |
| Vβ5.1, 5.2 TCR | MR9-4 | eBioscience, BD Pharmingen |
| PE conjugated streptavidin | | eBioscience |
| Brilliant Violet 711 streptavidin | | Biolegend |
| APC/Cy7 streptavidin | | Biolegend |

**Table 2-1.** Antibodies used for flow cytometry staining.

(Sabatel et al. 2017). The DCs in the lung either express CD103 or CD11b, except for a small population of pDCs. The CD103(+) DCs are conventional DCs known as cDC1. CD11b(+) DCs include populations of CD88(+) monocyte-derived DCs (Nakano et al. 2015), which have also previously been defined by Ly6C, CD64 (FcγRI), and MAR-1 (FcεR1α) (Plantinga et al. 2013). The CD11b(+) CD88(-) conventional DCs are all thought to be dependent on IRF4, but remain heterogeneous and can be further subdivided by CD24 expression. The CD24(+) subset is known to be KLF4-dependent and has been suggested to be the primary subset responsible for type 2 responses (Tussiwand et al. 2015). We conducted subsequent analyses for each of the following DC populations: moDCs, CD103(+) cDCs, CD24(+) cDC2s, and CD24(-) cDC2s.

**qPCR, qPCR array, and analysis.** *Chapter 3.* IL-33$^{-/-}$ mice were instilled i.t. with PBS (n = 4) or 2.5 μg of rIL-33 (n = 5) in a total of 50 μL PBS. The lungs were harvested six hours later, minced, and homogenized in RLT buffer using a QIAGEN TissueRuptor. RNA was purified from each mouse lung homogenate (QIAGEN RNeasy Plus Mini Kit) and cDNA was prepared for each sample (QIAGEN RT$^2$ First Strand Kit). RT$^2$ Profiler PCR Array Mouse Chemokines & Receptors kit (Qiagen) was used on pooled samples for qPCR array, which was performed on a CFX96 Real Time System (Bio-Rad). Array data were analyzed using the data analysis web portal at http://www.qiagen.com/geneglobe. $C_T$ values were normalized to the housekeeping genes *Gusb* and *Hsp90ab1* after which delta-delta $C_T$ calculations were performed and transcript fold change over PBS controls was determined using the 2^(-delta delta $C_T$) formula. Results for *Ccl2*, *Ccl7*, and *Ccl22* were confirmed by individual qPCRs performed on cDNA from individual mice. PCR primers were as follows: HPRT, forward 5'-TGATCAGTCAACGGGGGACA-3', reverse 5'-TTCGAGAGGTCCTTTTCACCA-3'; CCL2, forward 5'-GGCCTGCTGTTCACAGTTGC-3', reverse 5'-CCTGCTGCTGGTGATCCTCT-3';

CCL7, forward 5'-TGTGCCTGCTGCTCATAGCC-3', reverse 5'-ACATAGCAGCATGTGGATGCATTG-3'; CCL22, forward 5'-CGCAAGCCTGGCGTTGTTT-3', reverse 5'-CCTCCCTGGACCACACCAGA-3'. *Chapter 4.* RNA was isolated from sorted cells using a Quick-RNA Microprep Kit (Zymo Research #R1050) and cDNA was created for each sample using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher #4368814), according to manufacturer instructions. qPCR was conducted on a Bio-Rad CFX96 qPCR detection system where conditions included denaturation at 95°C for 2 min and then 30 cycles of 95° C for 30 sec, 53°C for 30 sec, and 72°C for 40 sec. $C_T$ values were normalized to the housekeeping genes *Hprt* or *Gapdh*. PCR primers were as indicated: *Gapdh*, forward 5'-TTCACCACCATGGAGAAGGC-3', reverse 5'-GGCATGGACTGTGGTCATGA-3'; *Hprt*, forward 5'-TGATCAGTCAACGGGGGACA-3', reverse 5'-TTCGAGAGGTCCTTTTCACCA-3'; *Il10*, forward 5'-GCCAAGCCTTATCGGAAATGATCC-3', reverse 5'-CACAGGGGAGAAATCGATGACAG-3'; *Il33*, forward 5'-CTGCGTCTGTTGACACATT-3', reverse 5'-CACCTGGTCTTGCTCTTGGT-3'.

**RNAseq.** Mice were sensitized to HDM and single cell suspension of the lungs was prepared nine hours later, as described above. Dendritic cells were stained and sorted, as described above. RNA was extracted in Trizol reagent and was isolated according to manufacturer's instructions using the Arcturus PicoPure RNA Isolation Kit (ThermoFisher #KIT0204). The Functional Genomics Facility at the University of Chicago generated full length cDNA and generated the sequencing library with Clontech Low Input Library Prep Kit followed sequencing using Illumina HiSeq 4000. Subsequent analysis was performed by the Center for Research Informatics at the University of Chicago: The quality of raw sequencing data was assessed using

FastQC. Illumina adapter/primer sequences were detected from sequencing reads. All RNA reads were first mapped to the mouse (mm10) reference genome using STAR release with default parameters (Dobin et al. 2013). Picard was used to collect mapping metrics. The resulting files from the previous alignment step in the RNA-seq analysis were taken individually as input to evaluate transcriptional expressing using subread::featureCounts (Liao, Smyth, and Shi 2014). Afterwards, several methods of differential expression analysis (DEA), including *DESeq2* (Love, Huber, and Anders 2014), *edgeR* (Robinson, McCarthy, and Smyth 2010), and/or *limma* (Ritchie et al. 2015) were employed to discover DE mRNA genes between pairwise groups based on expression estimation of individual mRNA genes using the criteria of fold change no less than 1.5 and FDR less than 0.1. To obtain groups with similar expression trend based on identified DE mRNA genes, several in-house scripts were implemented using R and Python languages. The identified DE genes were further used as input to functional analysis module for identification of enrichment of functional categories and regulatory networks.

**Statistical analysis.** *Chapter 3.* GraphPad Prism software was utilized to perform statistical analyses, and a P-value less than 0.05 was considered significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; # < $10^{-10}$; ns = not significant). An unpaired Student's two-tailed *t* test was utilized to analyze experiments with two groups. Error bars represent the SEM. *Chapter 4.* GraphPad Prism software was used for statistical analyses, and a P-value less than 0.05 was considered significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant). When data points came from a normal distribution, an unpaired Student's two-tailed *t* test was utilized to analyze experiments with two groups, and an ANOVA with Tukey's or Holm-Sidak's post-test was used for the comparison of more than two groups. Otherwise a Mann-Whitney *U* test was performed for the comparison of two groups, or a Kruskal-Wallis test with Dunn's multiple

comparisons test for comparison of more than two groups. Bars represent the mean, and error bars represent the SEM.

***Citrobacter rodentium* infection.** *Chapter 5. C. rodentium* (strain DBS100, ATCC Cat #51459) was grown overnight at 37°C while shaking. Mice were inoculated with 2 x $10^9$ colony-forming units (CFU), as measured by $OD_{600}$ in 0.2 mL PBS by oral gavage after 4 hours of fasting. Survival was compared between groups using the log-rank test and weight loss was compared using RM two-way ANOVA.

***Citrobacter rodentium* quantification.** *Chapter 5.* Every three days feces were collected, weighed, and homogenized in PBS. Serial dilutions grew on MacConkey agar and colonies were counted after overnight incubation at 37°C. Groups were compared statistically using the Kolmogorov-Smirnov test.

**Study approval.** The University of Chicago Animal Resources Center approved all animal procedures.

# Chapter III: IL-33 drives monocyte recruitment to lung interstitium through chemokine upregulation

**Note:**

The following section titled "IL-33 drives monocyte recruitment to lung interstitium through chemokine upregulation" is reproduced verbatim, with the exception of figure renumbering and addition of the final paragraph, from my co-first authored reference (Tjota et al. 2017). 

**Authors:**

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*These authors contributed equally to this work.

## Abstract

Tissue infiltration by circulating monocytes is a critical step in the initiation and augmentation of type 2 inflammatory responses in the lungs. Our studies demonstrate that IL-33$^{-/-}$ mice have a defect in monocyte extravasation from the vasculature to the lung interstitium during induction of type 2 inflammatory responses. This result suggests that monocyte migration to the lungs is IL-33 dependent, and we found that administration of exogenous recombinant IL-33 (rIL-33) is sufficient to restore monocyte localization to the lung interstitium. Further investigation of the effect of early administration of rIL-33 on the lungs identified upregulation of multiple chemokines including the monocyte chemoattractants CCL2, CCL7, and CCL22. Importantly, blockade of GPCR-dependent signaling, and thereby chemokine receptor activity, inhibited IL-33 driven monocyte recruitment. CCR2 deficiency prevented recruitment of monocytes to the lung extravascular space during allergic sensitization, and resulted in reduced eosinophilia after allergen challenge. Thus, IL-33 plays a critical role in the initiation of type 2 inflammatory responses by inducing upregulation of chemokines that promote monocyte recruitment to the lung interstitium.

## Introduction

Asthma is a chronic disease of the airways that is most commonly characterized by a type 2 inflammatory response (Locksley 2010, Aleman, Lim, and Nair 2016, Lambrecht and Hammad 2015). Multiple immune cell populations contribute to this response, but many unanswered questions remain about the role of antigen presenting cells (APCs) during allergic airway responses. In particular, there has been greater interest in understanding the role monocytes play during allergic airway inflammation. Studies in which circulating monocytes were depleted

demonstrated decreased allergic lung inflammation, suggesting that monocytes are important for initiating allergic lung responses (Zaslona et al. 2014, Tashiro et al. 2016, Jakubzick, Randolph, and Henson 2017).

Murine monocytes predominantly circulate through the vasculature at steady state and can be traditionally classified into two groups based on Ly6C expression. $Ly6C^-$ monocytes have been associated with patrolling blood vessel walls and playing an anti-inflammatory role during tissue injury (Auffray, Sieweke, and Geissmann 2009, Hanna et al. 2012). In contrast, $Ly6C^+$ monocytes are thought to rapidly infiltrate inflamed tissues where they can then differentiate into $CD11b^+$ moDCs or monocyte-derived macrophages (Ginhoux and Jung 2014). Other studies have also shown that $Ly6C^{hi}$ monocytes can act as precursors to $Ly6C^{lo}$ monocytes (Sunderkotter et al. 2004). Several investigations have elucidated key chemokines that promote monocyte trafficking. Chemokine receptors are differentially expressed between $Ly6C^-$ monocytes and $Ly6C^+$ monocytes (Shi and Pamer 2011). $Ly6C^-$ monocytes express high levels of $CX_3C$-chemokine receptor 1 ($CX_3CR1$), which binds to $CX_3CL1$ (Auffray et al. 2007). This ligand was found to be upregulated in human endothelial cells through a CD40-TNF receptor associated factor (TRAF) pathway (Greene et al. 2015). In contrast, $Ly6C^+$ monocytes have low expression of $CX_3CR1$ but high expression of CC-chemokine receptor 2 (CCR2). CCL2 and CCL7 have been shown to bind to CCR2 and promote monocyte recruitment (Serbina et al. 2008). Upregulation of these ligands can be found in various murine models including bacterial infections, peritonitis, autoimmune encephalitis, atherosclerosis, and asthma (Tsou et al. 2007, Roy, Wuthrich, and Klein 2012). While it has been shown that monocytes accumulate in response to upregulation of these chemokines, it is still unclear how early signals in allergic sensitization upregulate these monocyte chemoattractants in the lungs.

Early signals implicated in initiating type 2 lung inflammation include IL-33, which acts upon a variety of cell populations including both non-hematopoietic cells and hematopoietic cells. In response to IL-33, innate lymphoid cells type 2 (ILC2s), mast cells, basophils, and type 2 Th cells (Th2s) promote type 2 inflammation (Smith 2010). We previously demonstrated that immune complex (IC)-mediated and house dust mite (HDM)-mediated type 2 lung inflammation was dependent on the IL-33/ST2 pathway (Tjota et al. 2014, Tjota et al. 2013). Thus, our objective in this study was to investigate mechanisms by which IL-33 produced early after sensitization promotes allergic lung inflammation.

## Results

### *Localization of monocytes to the lung interstitium after allergen challenge is IL-33 dependent*

Monocytes are primarily present in the vasculature but are known to extravasate into tissues at the earliest stages of inflammation (Ingersoll et al. 2011). Our previous study had found that immune complex (IC) formation *in vivo* promoted type 2 inflammatory responses in the lung by upregulating IL-33 as early as 3 hours after administering allergen (Tjota et al. 2014, Tjota et al. 2013). To examine the influence of IL-33 in the early stages of this model, we have addressed whether OVA-IC-induced IL-33 affects the early migration of APCs into the lung tissue. To induce IC formation in vivo, mice are injected with OVA-specific serum on day 0, followed by an OVA i.t. sensitization on day 1 (Tjota et al. 2013). Utilizing fluorescently labeled OVA and intravascular staining of CD45 (Anderson et al. 2014), we identify APCs that take up antigen and whether they migrate into the extravascular space.

As IL-33 upregulation is seen as early as 3 hours after administering allergen (Tjota et al. 2014, Tjota et al. 2013), we investigated the early effects OVA-ICs had on monocytes at 30 minutes, 1 hour, and 3 hours post-sensitization. As expected, the $Ly6C^-$ and $Ly6C^+$ monocytes were primarily localized in the vasculature (Fig. 3-1). Although monocytes took up minimal amounts of OVA alone, antigen uptake was significantly enhanced in the presence of OVA-IC (Fig. 3-1, Fig. 3-2). Furthermore, OVA-ICs drove $Ly6C^+$ monocytes to localize to the lung interstitium as early as 3 hours after sensitization (Fig. 3-1B) and continued through to 18 hours (Fig. 3-2A). These findings demonstrate that OVA-IC enhanced antigen uptake by $Ly6C^-$ and $Ly6C^+$ monocytes, and promoted $Ly6C^+$ monocyte localization into the lung interstitium.

To determine if there is a role for IL-33 in the monocyte migration in the OVA-IC model, we sensitized WT and IL-$33^{-/-}$ mice. No significant difference was noted in the frequency of $Ly6C^-$ and $Ly6C^+$ monocytes at baseline between WT and IL-$33^{-/-}$ mice (data not shown). However, after challenge IL-$33^{-/-}$ mice displayed a significant decrease in the percentage of antigen-positive $Ly6C^-$ and $Ly6C^+$ monocytes localized to the lung interstitium (Fig. 3-2A, Fig. 3-2B). Given that our previous work found IL-33 upregulation to be downstream of FcγRIII, we analyzed whether a similar defect was present in FcγRIII$^{-/-}$ mice. Compared to WT mice, FcγRIII$^{-/-}$ mice had a significant decrease in the presence of antigen-positive monocytes in the lung interstitium (Fig. 3-4). These results demonstrated that during early sensitization, recruitment and localization of monocytes to the lung interstitium was IL-33 and FcγRIII dependent. Taken together with our previous investigations (Tjota et al. 2014, Tjota et al. 2013), these data suggest that augmentation of type 2 inflammatory responses through IL-33 upregulation may be driven by the early accumulation of monocytes.

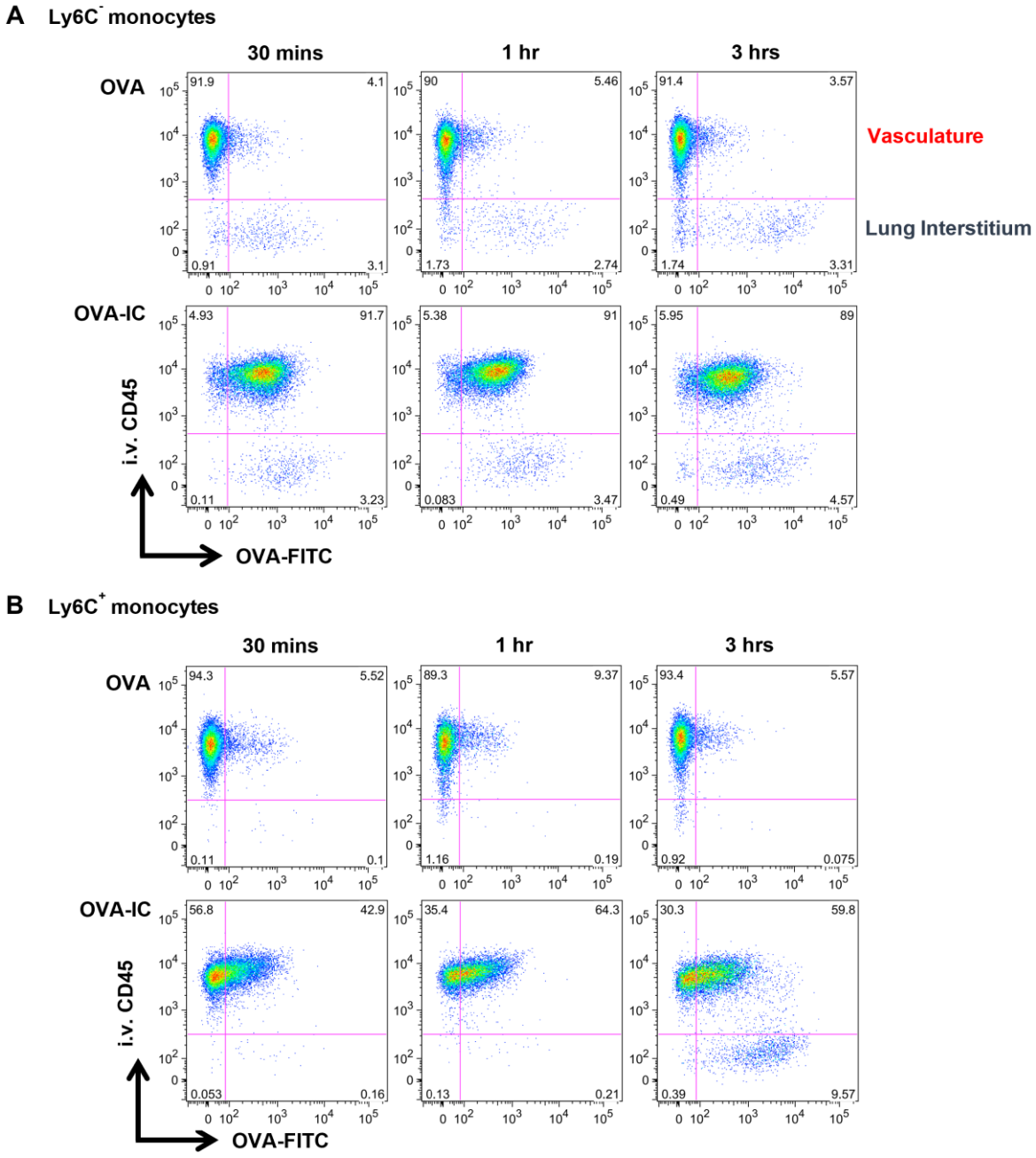


**Figure 3-1.** OVA-ICs enhance monocyte uptake of antigen and promote Ly6C+ monocyte accumulation in the lung extravascular space within 3 hours.

WT mice received α-OVA (OVA-IC) i.v. followed by a fluorescently labeled OVA (OVA-FITC) sensitization i.t. 24 hours later. Mice were sacrificed at 30 mins, 1 hr, and 3 hrs after sensitization. Representative flow plots of antigen uptake (OVA-FITC) versus localization (i.v. CD45) for (A) Ly6C- monocytes and (B) Ly6C+ monocytes. The data are from two independent experiments with at least six mice analyzed per group.

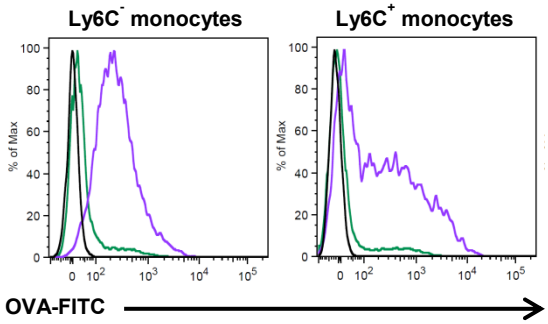


**Figure 3-2.** Antigen uptake in monocytes is enhanced by OVA-IC.

WT mice received α-OVA$^{depl}$ serum (OVA) or α-OVA serum (OVA-IC) i.v. followed by a fluorescently labeled OVA (OVA-FITC) challenge i.t. 24 hours later. 18 hours after challenge, the mice were sacrificed and FITC expression on monocytes was assessed by flow cytometry. (Black – unchallenged, Green – OVA, Purple – OVA-IC.) The data are representative plots with at least six mice analyzed per group. *Experiment conducted by Melissa Tjota.*

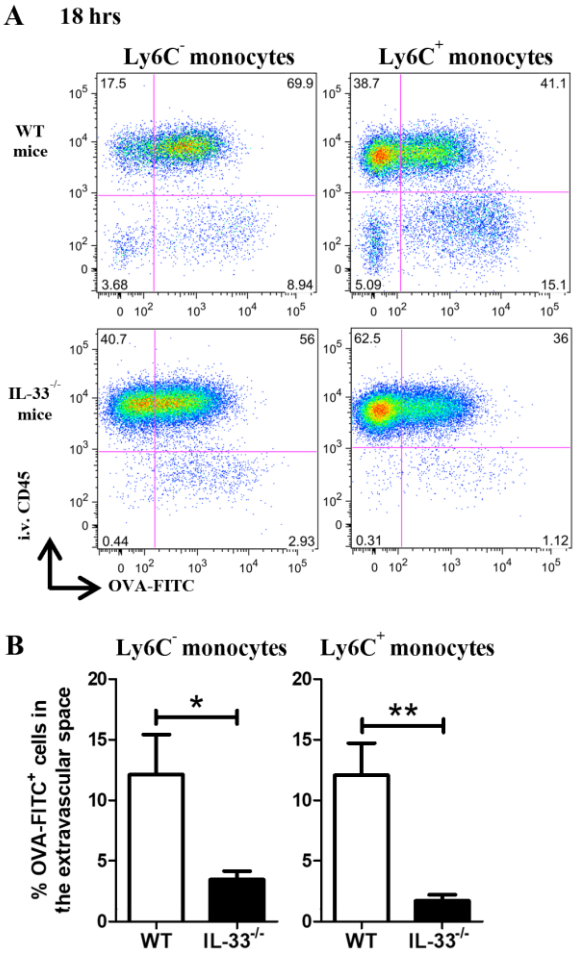


**Figure 3-3.** Monocyte migration from the vasculature to the interstitium is reduced in IL-33$^{-/-}$ mice.

WT and IL-33$^{-/-}$ mice received α-OVA serum i.v. followed by a fluorescently labeled OVA (OVA-FITC) sensitization i.t. 24 hours later. 18 hours after sensitization, the mice were sacrificed. Minutes prior to sacrifice, the mice received an i.v. injection of anti-CD45 to allow for identification of cell localization in the vasculature (i.v. CD45$^{+}$) or extravascular space (i.v. CD45$^{-}$). (A) Representative flow plots of antigen uptake (OVA-FITC) versus localization (i.v. CD45) for Ly6C$^{-}$ and Ly6C$^{+}$ monocytes. (B) Percentage of OVA-FITC$^{+}$ cells in the extravascular space is plotted. Data represent the mean ± SEM, and the combined data from at least two independent experiments with a total of at least six mice analyzed per group. *Experiment conducted by Melissa Tjota.*

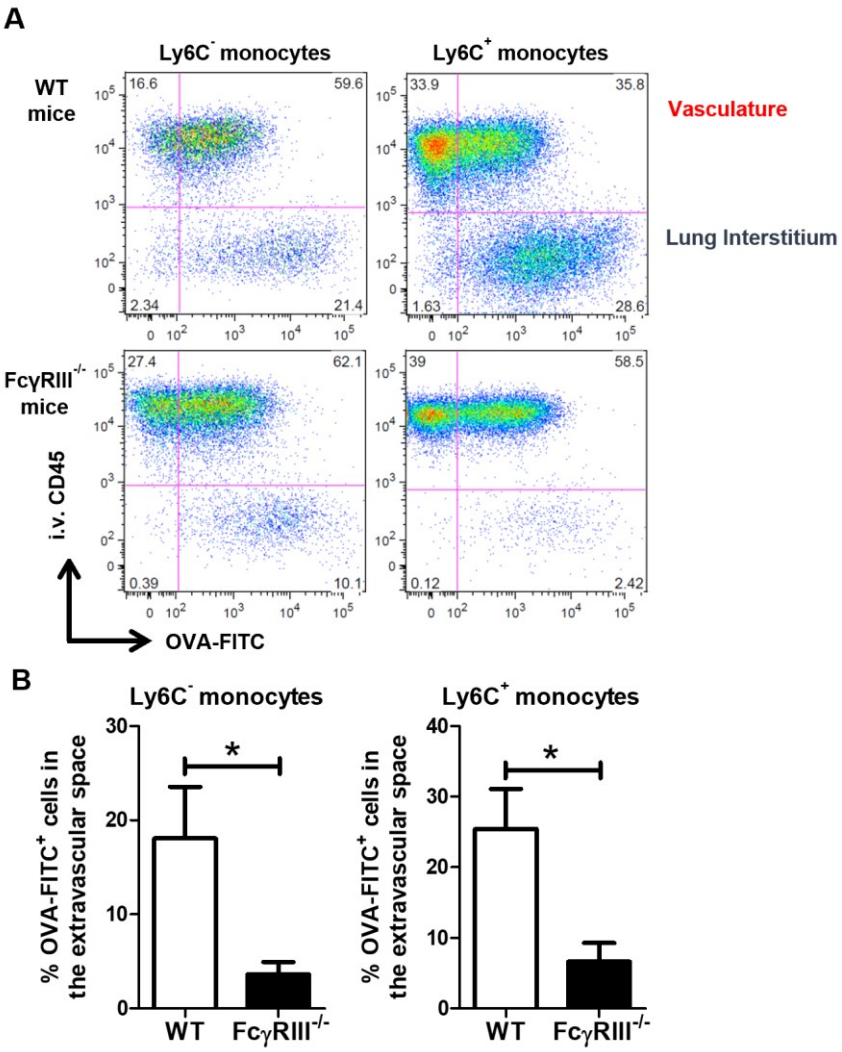


**Figure 3-4.** Monocyte migration from the vasculature to the interstitium is dependent on FcγRIII.

WT and FcγRIII$^{-/-}$ mice received α-OVA (OVA-IC) i.v. followed by a fluorescently labeled OVA (OVA-FITC) challenge 24 hours later. 18 hours after challenge, the mice were sacrificed. (A) Representative flow plots of antigen uptake (OVA-FITC) versus localization (i.v. CD45) for Ly6C$^{-}$ and Ly6C$^{+}$ monocytes. (B) Percentage of OVA-FITC$^{+}$ cells in the extravascular space is plotted. Data represent the mean ± SEM. The data are from two independent experiments with at least six mice analyzed per group. *Experiment conducted by Melissa Tjota.*

*CCR2-deficient mice have reduced eosinophilia after allergen sensitization and challenge*

CCR2 is an important chemokine receptor for monocyte egress from the bone marrow (Shi and Pamer 2011). Thus, $CCR2^{-/-}$ mice have a severe reduction in the number of circulating monocytes and are widely used as monocyte-deficient mice. We hypothesized that monocyte accumulation in the lung extravascular space during sensitization was required for inflammation during allergen challenge. To evaluate monocyte responses to allergen sensitization in these mice, WT or $CCR2^{-/-}$ mice were administered OVA-specific serum on day 0, followed by an OVA i.t. sensitization on day 1. As above, antigen uptake and localization of the monocytes were evaluated 18 hours after OVA sensitization. We were surprised to find that an almost significant proportion of $Ly6C^{+}$ monocytes took up antigen and were recruited to the lung extravascular space even in the absence of CCR2 (Fig. 3-5A). However, as expected, the total number of antigen-positive $Ly6C^{+}$ monocytes accumulated in the lung interstitium in the CCR2-deficient mice compared to in WT mice was greatly reduced (Fig. 3-5A).

To address whether the reduction in monocyte recruitment in the $CCR2^{-/-}$ mice affected IC-induced allergic inflammation, mice were sensitized as described above but were additionally challenged with OVA i.t. on days 8, 9, and 10. On day 11, type 2 airway inflammation was assessed by measuring the numbers of eosinophils and $CD4^{+}$ T cells in the BAL. Although CCR2-sufficient and CCR2-deficient mice developed equal degrees of BAL $CD4^{+}$ T cell infiltration, CCR2-deficient mice had reduced eosinophilia compared to their CCR2-sufficient counterparts (Fig. 3-5B). Importantly, others have reported the same defect in eosinophilia in a house dust mite model of allergic inflammation (Plantinga et al. 2013). Thus, monocyte accumulation in the lung interstitium after sensitization is required for optimal eosinophilia after challenge.

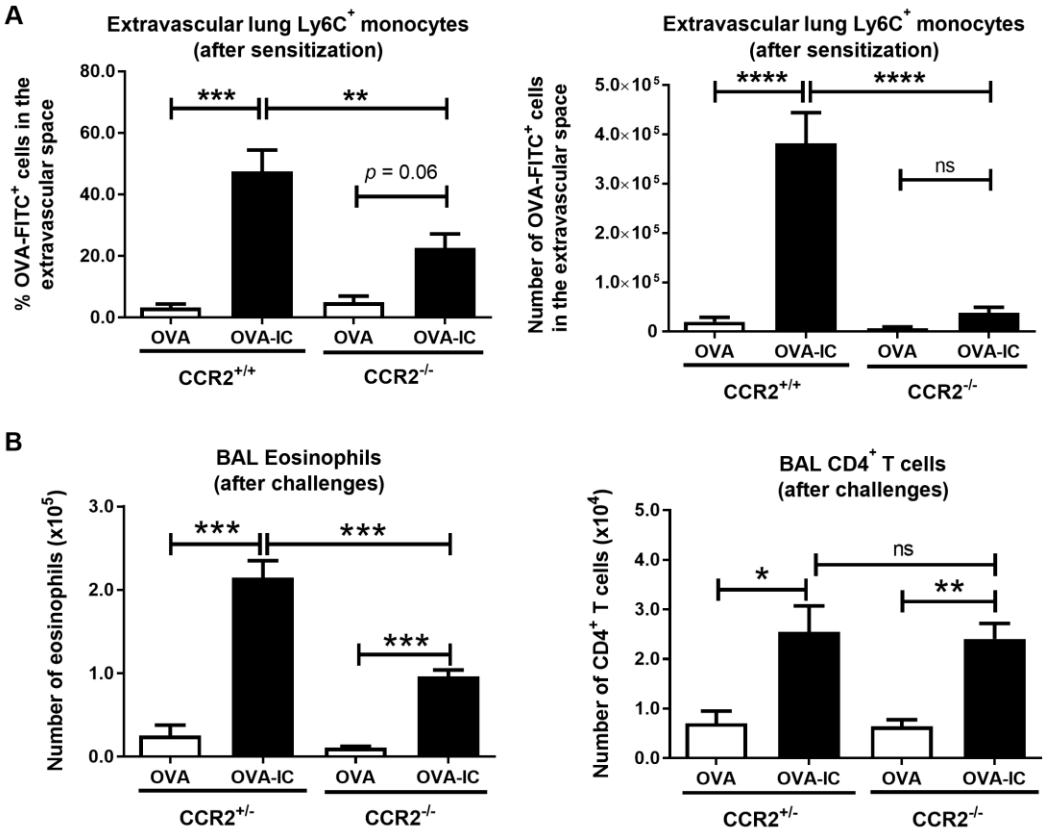


**Figure 3-5.** CCR2 is partially responsible for regulating Ly6C$^+$ monocyte migration from the vasculature to the interstitium after OVA-IC sensitization and lung eosinophilia after allergen challenge.

α-OVA serum (OVA-IC) was administered i.v. to naïve mice, whereas control mice received either (A) PBS or (B) α-OVA$^{depl}$. The next day the mice were challenged i.t. with (A) OVA-FITC or (B) OVA. (A) After 18 hours the mice were sacrificed. Minutes prior to sacrifice, the mice received an i.v. injection of anti-CD45 to allow for discrimination between cells in the vasculature and interstitium. Percentage of monocytes in the extravascular space is plotted. (B) On days 1, 8, 9, and 10 the mice were challenged i.t. with OVA. On day 11 the mice were sacrificed. Airway inflammation was assessed by determining the number of eosinophils and CD4$^+$ T cells in the BAL by flow cytometry. Data represent the mean ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant). The data represent the combined data from at least two independent experiments with a total of at least three mice analyzed per group. *Experiment in part B conducted by Melissa Tjota.*

*Administration of exogenous rIL-33 restores Ly6C$^{+}$ monocyte localization to the lung interstitium*

We had previously demonstrated that administration of exogenous rIL-33 during sensitization was sufficient to restore allergic lung inflammation in FcRγ$^{-/-}$ mice (Tjota et al. 2014). Since both monocyte recruitment to the lungs and IL-33 upregulation were early events in development of type 2 inflammatory responses in the lungs, we sought to determine if exogenous IL-33 was sufficient to restore lung interstitial localization of antigen-positive monocytes. Mice were sensitized with OVA-specific serum on day 0, and on day 1 mice received OVA-FITC with or without rIL-33. Notably, administration of rIL-33 to the IL-33$^{-/-}$ mice restored lung interstitial localization of antigen-positive Ly6C$^{+}$ monocytes (Fig. 3-6A). There was a trending increase in the Ly6C$^{-}$ monocytes, but it was not quite significant (Fig. 3-6A).

To determine whether IL-33 alone is sufficient to drive monocytes into the lung interstitium in the absence of antigen, PBS or rIL-33 alone was i.t. administered to IL-33$^{-/-}$ mice. The proportion of Ly6C$^{+}$ monocytes in the lung interstitium was significantly higher in the mice that had received rIL-33 compared to the PBS only controls (Fig. 3-6B). This effect was limited to Ly6C$^{+}$ monocytes as the localization of Ly6C$^{-}$ monocytes was unaltered by rIL-33 (Fig. 3-6B). Thus, exogenous rIL-33 was sufficient to promote Ly6C$^{+}$ monocyte accumulation independent of allergen.

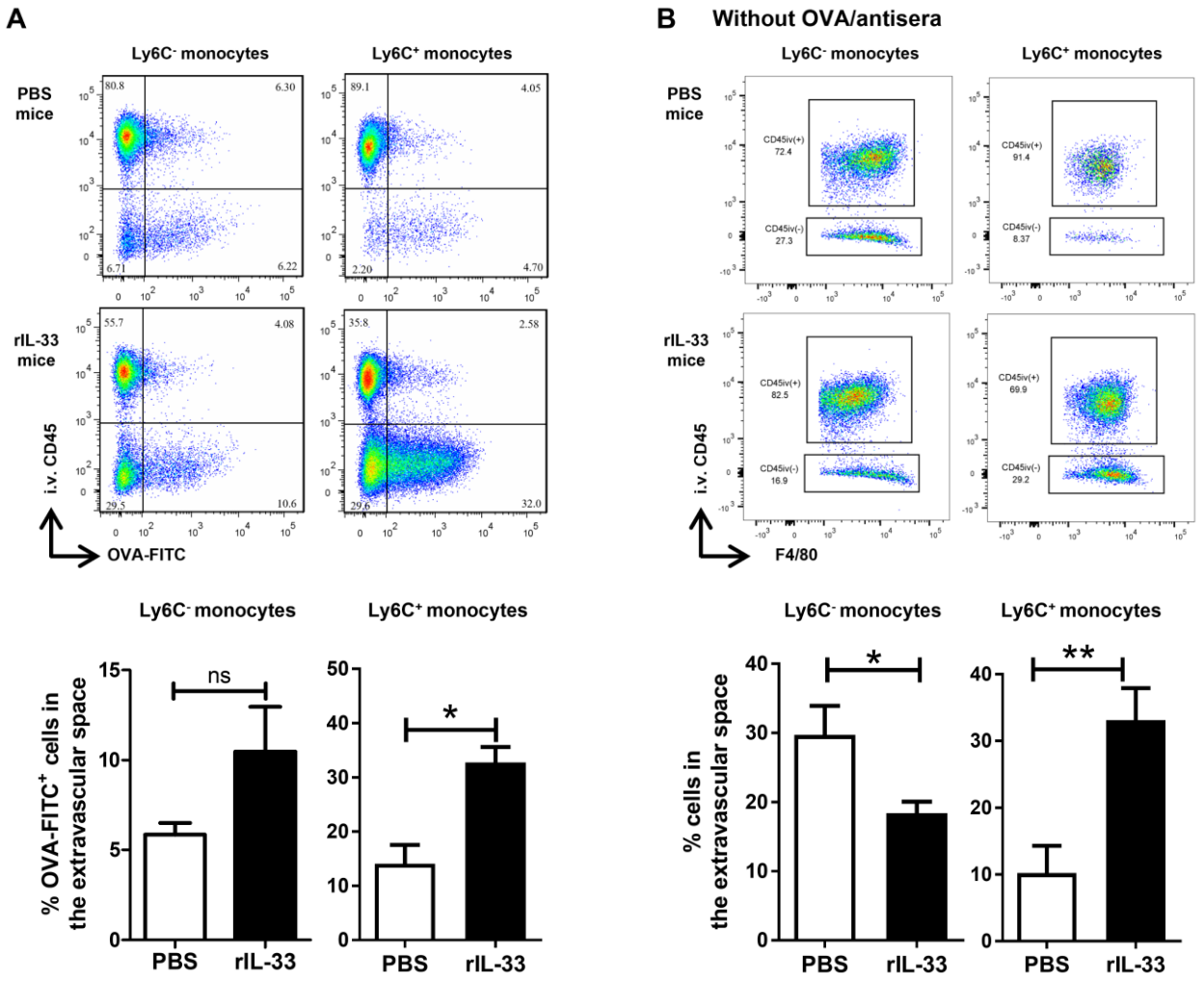


**Figure 3-6.** Administration of exogenous rIL-33 restores Ly6C$^+$ monocyte localization to the extravascular space in response to OVA-IC and is sufficient to promote monocyte extravasation without antigen.

(A) IL-33$^{-/-}$ mice received α-OVA i.v. followed by a fluorescently labeled OVA (OVA-FITC) sensitization with or without recombinant IL-33 (rIL-33) 24 hours later. Percentage of OVA-FITC$^+$ monocytes in the extravascular space is plotted. (B) In the absence of any antigen or antisera, IL-33$^{-/-}$ mice received i.t. PBS or rIL-33. Eighteen hours after challenge, the mice were sacrificed. Example flow plots of F4/80$^+$ monocytes (SSC$^{lo}$CD11c$^{lo/-}$MHC-II$^{lo/-}$CD11b$^+$). Percentage of monocytes in the extravascular space is plotted. Data represent the mean ± SEM combined from at least two independent experiments with a total of at least six mice analyzed per group.

*IL-33 promotes the expression of monocyte chemoattractants in the lungs*

IL-33 has previously been found to induce production of cytokines and chemokines in a variety of disease states, including asthma (Yagami et al. 2010, Nabe et al. 2015). As monocyte recruitment is sensitive to many chemokines, and because we showed that some monocyte recruitment occurs even in the absence of CCR2, we hypothesized that IL-33 promoted the upregulation of additional monocyte chemoattractants in the lungs. To determine whether IL-33 modulates chemokine expression, PBS or rIL-33 was administered to IL-33$^{-/-}$ mice, and the lungs were harvested six hours later for cDNA preparation. A qPCR array was performed to analyze the differential expression of 84 chemokines and their receptors. Notably, the mice that had received rIL-33 displayed increased expression of multiple monocyte chemoattractants such as CCL2, CCL4, CCL7, CCL8, CCL22, and CXCL10 (Fig. 3-7, Table 3-1). In particular, CCR2 is well known for its role in promoting recruitment of circulating monocytes to the lung (Shi and Pamer 2011). In addition, IL-33 promoted expression of several other chemokines including CCL11 and CCL24, which attract eosinophils expressing CCR3; CCL17, which recruits CCR4-expressing cells such as T cells; and CCL19, which chemoattracts dendritic cells, B cells, and T cells expressing the receptor CCR7. Thus, the introduction of IL-33 into the lungs altered the chemokine milieu to promote the recruitment of several inflammatory populations including monocytes.

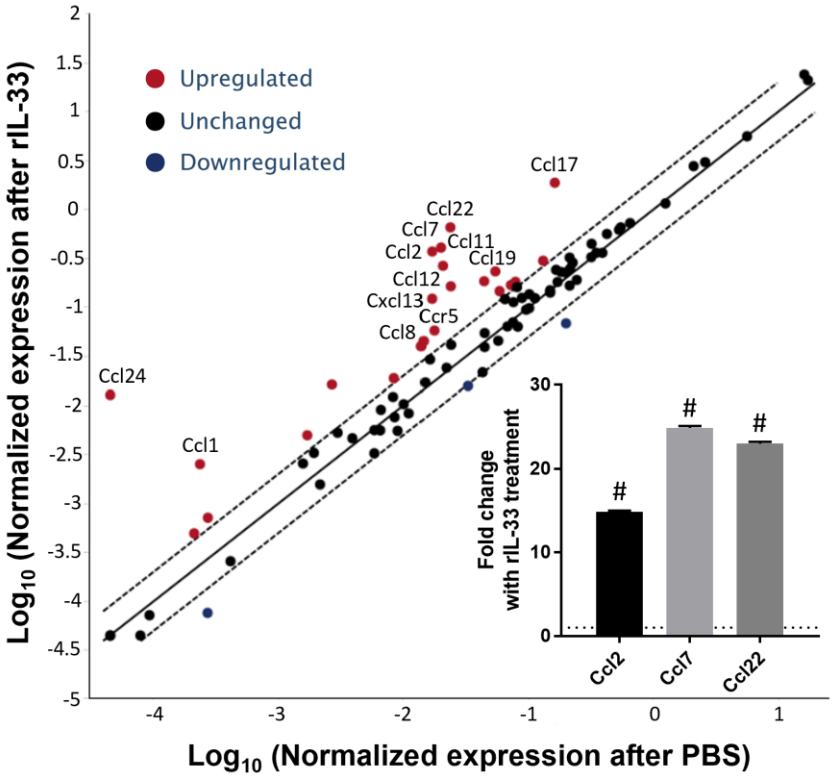


**Figure 3-7.** IL-33 upregulates chemokines in the lungs, including monocyte chemoattractants.

IL-33$^{-/-}$ mice received i.t. rIL-33 (n = 5) or PBS (n = 4). The mice were sacrificed six hours later and RNA was purified from total lung homogenate. cDNA was prepared and pooled for each group. A qPCR array was used to assess transcript abundance of genes encoding chemokines and chemokine receptors. Relative expression was calculated using the delta-delta $C_T$ method as compared to the housekeeping genes *Gusb* and *Hsp90ab1*. Inset: qPCR for *Ccl2, Ccl7,* and *Ccl22* was performed on individual mouse cDNA samples generated from mouse lung homogenates. Chemokine transcripts were normalized to the housekeeping gene *Hprt*. Dotted line represents expression levels of PBS-treated mice. rIL-33-treated mice (n = 5) were compared with PBS-treated mice (n = 4). # indicates $p < 10^{-10}$.

| Gene Symbol | Fold Difference rIL-33/PBS | Gene Symbol | Fold Difference rIL-33/PBS |
|---|---|---|---|
| Ccl24 | 284.9 | Cmklr1 | 1.2 |
| Ccl22 | 27.78 | Cxcr1 | 1.19 |
| Ccl7 | 21.97 | Ccl6 | 1.17 |
| Ccl11 | 20.46 | Tgfb1 | 1.17 |
| Ccl2 | 12.87 | Ccl25 | 1.15 |
| Ccl17 | 11.57 | Ccrl2 | 1.15 |
| Ccl1 | 10.7 | Mapk14 | 1.15 |
| Cxcl13 | 7.24 | Mapk1 | 1.12 |
| Ccl12 | 6.88 | Ccl9 | 1.1 |
| Ackr1 | 6.05 | Cxcl5 | 1.1 |
| Ccl19 | 4.29 | Ccl5 | 1.06 |
| Cxcl10 | 4.2 | Itgb2 | 1.04 |
| Ccr5 | 3.29 | Cxcr6 | 1.03 |
| Ccl8 | 3.11 | Cxcl12 | 1.02 |
| Il4 | 2.92 | Gusb | 1 |
| Ccl4 | 2.89 | Hsp90ab1 | 1 |
| Ccr8 | 2.62 | Ccr1l1 | 0.99 |
| Ccr1 | 2.5 | Cmtm2a | 0.99 |
| Cxcl9 | 2.34 | Slit2 | 0.99 |
| Itgam | 2.34 | Cx3cl1 | 0.97 |
| Ccr3 | 2.32 | Cxcr3 | 0.96 |
| Ccr2 | 2.29 | Cmtm4 | 0.95 |
| Tnf | 2.25 | Ccl20 | 0.94 |
| Cxcr2 | 1.99 | Cxcl2 | 0.94 |
| Ackr2 | 1.87 | Cxcl15 | 0.92 |
| Cxcl1 | 1.81 | Cxcr4 | 0.92 |
| Tymp | 1.77 | Cxcr5 | 0.89 |
| Ccl3 | 1.72 | Il16 | 0.88 |
| Ccr4 | 1.71 | Xcl1 | 0.85 |
| Ifng | 1.63 | Ccr7 | 0.8 |
| Cmtm6 | 1.51 | Ccl26 | 0.78 |
| Tlr2 | 1.5 | Ackr3 | 0.78 |
| Il6 | 1.48 | Fpr1 | 0.78 |
| Actb | 1.48 | Ppbp | 0.77 |
| Il1b | 1.45 | Gpr17 | 0.75 |
| C5ar1 | 1.41 | Ccr10 | 0.73 |
| Cxcl16 | 1.4 | Cmtm5 | 0.62 |
| Ccr6 | 1.37 | Ccr9 | 0.61 |
| Tlr4 | 1.34 | Ccl28 | 0.57 |
| Cxcl14 | 1.33 | Xcr1 | 0.56 |
| Gapdh | 1.32 | Ackr4 | 0.51 |
| Hif1a | 1.27 | Cx3cr1 | 0.48 |
| Cmtm3 | 1.26 | Cxcl3 | 0.34 |
| Pf4 | 1.23 | Cxcl11 | 0.28 |
| B2m | 1.22 | | |

**Table 3-1.** IL-33 alters chemokine expression in the lungs.

IL-33$^{-/-}$ mice received recombinant IL-33 (rIL-33) or PBS i.t. The mice were sacrificed six hours later and RNA was purified from total lung homogenate. cDNA was prepared and a qPCR array was used to assess transcript abundance of genes encoding chemokines and chemokine receptors, which were calculated using the delta-delta $C_T$ method.

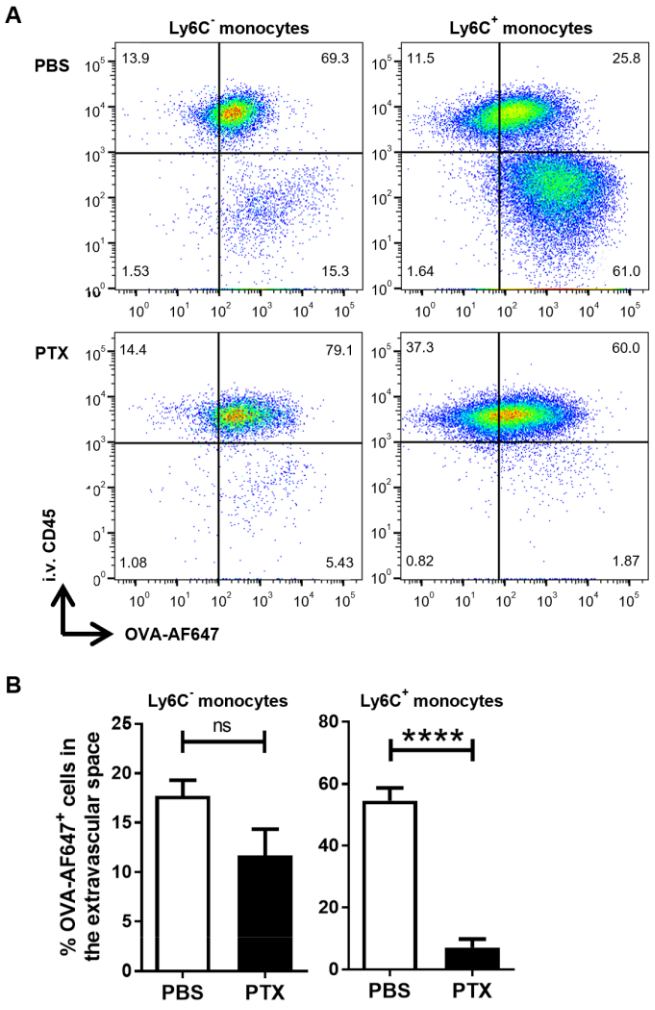


**Figure 3-8.** PTX treatment abolishes IL-33-dependent monocyte migration to the lung extravascular space in response to OVA-IC.

IL-33$^{-/-}$ mice received α-OVA i.v. and either PBS or PTX i.p. OVA-AF647 sensitization with rIL-33 was delivered i.t. 24 hours later. 18 hours after challenge, the mice were sacrificed. (A) Representative flow plots of antigen uptake (OVA-AF647) versus localization (i.v. CD45). (B) Percentage of OVA-AF647$^{+}$ cells in the extravascular space. Data represent the mean ± SEM combined from three independent experiments with a total of at least ten mice analyzed per group.

*Monocyte accumulation in the lung interstitium is G-protein coupled receptor dependent*

As we had demonstrated that IL-33 upregulated chemokines in the lungs, we questioned whether monocyte accumulation in response to OVA-ICs was chemokine-dependent. Pertussis toxin (PTX) has previously been shown to inhibit signaling of G-protein coupled receptors (GPCRs), including chemokine receptors (Sun and Ye 2012). Thus, treatment with PTX allowed us to inhibit all chemokine receptor activity simultaneously, as opposed to targeting a particular chemokine receptor. Mice were treated with PTX (or PBS control) on day 0 at the same time as administration of αOVA serum, followed by an OVA i.t. sensitization the next day. Notably, treatment with PTX inhibited extravascular accumulation of Ly6C+ monocytes after OVA-IC treatment with a trending decrease in Ly6C- monocytes (Fig. 3-8). These results demonstrated that the accumulation of monocytes in the lung interstitium after OVA-IC treatment occurred as a result of chemokine-dependent migration and is not a result of direct chemotactic effects of IL-33.

## Discussion

Using a murine model of asthma, we demonstrated that IL-33 was critical for the recruitment of Ly6C$^+$ monocytes from the vasculature to the lung parenchyma by upregulating monocyte chemoattractants including CCL2 and CCL7 (Serbina et al. 2008). Blocking GPCR-signaling inhibited Ly6C$^+$ monocyte localization to the extravascular space, demonstrating a critical role for IL-33 induced chemokines in monocyte migration. Monocyte infiltration has been shown to be important in promoting the development of type 2 inflammatory responses, either through direct effector functions or differentiation into CD11b$^+$ moDCs (Plantinga et al.

2013, Jakubzick, Randolph, and Henson 2017). Indeed, we found that CCR2$^{-/-}$ mice, in which monocyte accumulation is attenuated, had reduced eosinophilia after challenge. Studies have found that CD11b$^{+}$ moDCs can produce chemokines as well as migrate to draining lymph nodes to prime and activate naïve CD4$^{+}$ T cells (Plantinga et al. 2013). Thus, IL-33 induced monocyte recruitment during allergic sensitization may be critical to augmenting type 2 inflammatory responses in the lungs through multiple mechanisms.

As IL-33 was found to upregulate multiple chemokines, pharmacologic targeting of IL-33 may be able to broadly reduce type 2 inflammatory responses. Attempts to block chemokines and GPCR signaling in patients with asthma has been difficult to achieve as there is a diverse number of chemokines upregulated during allergic airway inflammation and there is much redundancy among chemokines and their receptors (Lukacs, Miller, and Hogaboam 2003, Adcock, Caramori, and Chung 2008). Pharmacologic approaches to block one particular mediator (i.e. CXCR2, IL-8/CXCL8, eotaxin/CCR3, and others) have met limited success and fail to improve or modify lung function (Durham et al. 2016). Identifying novel pathways to halt the disease early-on is critical because repeated exposure to allergens leads to cumulative tissue damage and pathological changes that affect respiratory function (Holt and Sly 2012). Our findings suggest that a more comprehensive strategy for inhibiting multiple downstream effectors may be achieved by targeting IL-33 to inhibit the development of type 2 inflammatory responses in the lungs.

In these studies, robust eosinophilic inflammation subsequent to allergen challenge required CCR2-dependent monocyte accumulation in the lung extravascular space following sensitization. However, T cells responded to allergen challenge independently of monocyte migration to the lungs following sensitization. Instead, T cell responses depend primarily upon

interactions with DCs, with the IRF4-dependent cDC2 subset displaying particular proficiency at the induction of $CD4^+$ T cell responses. The mechanisms regulating T cell responses to allergens will be the focus of the studies discussed in the next chapter, with particular attention to the role of DCs in mediating T cell activation.

# Chapter IV: IRF4 expression by lung dendritic cells drives a Th2-supporting program, but is dispensable during the memory response


## Abstract

Expression of the transcription factor IRF4 is required for the development of lung DCs that elicit Th2 responses, yet how IRF4 functions in lung DCs throughout the acute and memory allergic response is not clear. Here, we demonstrate that mice with IRF4-deficient DCs display impaired memory responses to allergen. This defect in the memory response is a direct result of ineffective Th2 induction and impaired recruitment of activated effector T cells to the lung after sensitization. Interestingly, while IRF4-deficient DCs exhibit minimal defects in the lungs, their migration to the draining lymph nodes is limited. Finally, T cells educated by IRF4-competent DCs mediate potent memory responses independently of IRF4-expressing DCs, demonstrating that IRF4-expressing DCs are not necessary specifically during the memory response. Thus,

IRF4 controls a program in mature DCs governing Th2 priming and effector responses, but IRF4-expressing DCs are dispensable during $T_{RM}$ cell-dependent memory responses.

## Introduction

Tissue-resident memory T cells ($T_{RM}$ cells) mediate inflammatory responses in various non-lymphoid tissues, but questions remain regarding the mechanisms underpinning the contribution of CD4(+) $T_{RM}$ cells to allergic airway disease. Like circulating memory T cells, $T_{RM}$ cells develop in response to initial antigen exposure at mucosal surfaces and persist beyond the contraction phase of the immune response. However, $T_{RM}$ cells maintain proximity to barrier tissues where they are poised to rapidly respond to subsequent antigen exposure (Nguyen et al. 2019). $T_{RM}$ cells can contribute to a "first-line" response until additional cells are recruited from the circulation and are often sufficient to control invading pathogens (Nguyen et al. 2019). However, how $T_{RM}$ cells are activated within the lung milieu and the role of DCs in $T_{RM}$ cell activation is not known. In settings where T cell responses are pathogenic, such as in allergic asthma, these sentinel $T_{RM}$ cells may serve as central mediators of disease. Thus, investigating how $T_{RM}$ cells are generated, maintained, and restimulated is critical to understanding chronic allergic lung disease.

Many questions remain unanswered regarding how long-lived $T_{RM}$ cells are maintained and how they are reactivated during subsequent responses, particularly with regard to CD4(+) $T_{RM}$ cells. While the pool of CD8(+) $T_{RM}$ cells remains stable in the skin for up to 200 days after its establishment (Mackay et al. 2012, Park et al. 2018, Jiang et al. 2012, Slutter et al. 2017), lung CD8(+) $T_{RM}$ cells decline more rapidly and rely on replenishment from the circulating memory

population (Slutter et al. 2017, Hogan et al. 2001, Liang et al. 1994, Wu et al. 2014) or reintroduction of the inflammatory stimulus. There is less evidence regarding the duration of CD4(+) $T_{RM}$ cell maintenance, but one study showed that maintenance of vaginal CD4(+) $T_{RM}$ cells against HSV2 was shorter than that of CD8(+) $T_{RM}$ cells (Shin and Iwasaki 2012). Whether this discrepancy depends on the particular tissue site or on the nature of the antigen is not known. However, the finding that antiviral CD8(+) $T_{RM}$ cells in the lung diminish over time does seem to apply to CD4(+) populations as well. Two studies have demonstrated declining allergen-specific CD4(+) $T_{RM}$ cells in the lungs in the 70- to 90-day period following allergen challenge (Yeon et al. 2017, Hondowicz et al. 2016). During this time, several cytokines support the development and maintenance of lung CD4(+) $T_{RM}$ cells in type 2 immunity. The development of $T_{RM}$ cells in response to allergen has been shown to require IL-2 signaling through CD25 (Hondowicz et al. 2016), and the development of CD4(+) $T_{RM}$ cells in response to helminth infection requires signaling through IL-4Rα (Thawer et al. 2014). Further, the maintenance of CD4(+) $T_{RM}$ cells in response to allergen requires IL-7 signaling through CD127 (Yeon et al. 2017). Thus, cytokine signals can support the development and maintenance of CD4(+) lung $T_{RM}$ cells in type 2 immunity, but the source of these cytokines and whether these signals are sufficient remains to be elucidated. Although APCs have been shown to play important roles during the initiation of the adaptive immune response, whether a similar division of labor occurs during $T_{RM}$ cell-dependent recall response is not known. Apart from requiring antigen presentation from a specific DC subset, it is possible that $T_{RM}$ cells are fully licensed to respond when antigen is presented from any source, or $T_{RM}$ cells may be more reliant on innate or cytokine signals to prompt a response (Harrison et al. 2019, Guo et al. 2015).

As in all mucosal tissues, numerous DC populations exist in the lungs. Recently it has become evident that specific transcription factors regulate DC development and function in acute immune responses. Conventional dendritic cells (cDCs) in the lung are broadly divided into two groups: cDC1 which express CD103 and cDC2 which express CD11b. The cells known as cDC1, which depend on the transcription factors BATF3 and IRF8, are proficient at cross-presentation as well as priming Th1 and Treg responses (Khare et al. 2013, Sichien et al. 2016, Hildner et al. 2008, Fuertes et al. 2011). Roles for cDC2, which are dependent upon IRF4 for development, include promoting Th2, Th17, and Treg responses (Williams et al. 2013, Gao et al. 2013, Plantinga et al. 2013, Scott et al. 2015, Tussiwand et al. 2015, Vander Lugt et al. 2014, Vander Lugt et al. 2017, Schlitzer et al. 2013). The Th2-promoting cDC2 subset is dependent on the transcription factor KLF4 and expresses CD24, while the Th17-promoting cDC2 subset is dependent on Notch2 and lacks CD24 (Ref. (Tussiwand et al. 2015) and Fig. 5-1). *Irf4*$^{-/-}$ mice are severely deficient in their splenic cDC2 population and in vitro DC culture from bone marrow progenitors is defective (Suzuki et al. 2004, Tamura et al. 2005). Mice lacking IRF4 in both CD11c$^{int}$ pre-cDC and CD11c$^{high}$ mature DC compartments fail to develop cDC2 populations in the lungs, lung-draining lymph nodes, small intestine, mesenteric lymph nodes, and spleen (Bajana et al. 2016, Schlitzer et al. 2013, Vander Lugt et al. 2014). The remaining cDC2 show reduced migration to tissue-draining lymph nodes during the initiation of type 2 immunity (Bajana et al. 2012), and in vitro DC cultures show a diminished capacity for antigen processing and presentation (Vander Lugt et al. 2014). Because of systemic failure of cDC2 development in the absence of IRF4, it has been difficult to study the ongoing role for IRF4 in mature DCs during inflammatory responses in vivo. To circumvent this, many studies have instead focused on the functional capacity of cultured BMDCs. We have previously developed a mouse model

which allows for IRF4 knockdown in only CD11c$^{high}$ mature DCs, with normal development during the pre-cDC stage (Williams et al. 2013). This has enabled us to isolate IRF4-dependent DC functions during the adaptive immune response in vivo.

Whether there is a role for tissue DCs in maintaining or reactivating $T_{RM}$ cells in the lungs has yet to be determined. In a vaginal infection model, CD8(+) $T_{RM}$ cells were still capable of proliferation in response to infection despite depletion of CD11c(+) cells, indicating that antiviral $T_{RM}$ cells are able to mediate a response in the absence of DCs (Beura et al. 2018). However, another vaginal infection model in which CD8(+) $T_{RM}$ cells were generated using the prime and pull technique, demonstrated that mice in which MHC-I-expressing CD301b(+) cDC2s were eliminated from the vaginal lamina propria were much more susceptible to infection compared to mice possessing this DC subset (Shin et al. 2016). One study focusing on lung CD4(+) $T_{RM}$ cells demonstrated that the proportion of lung cDC2s is increased during an allergic memory response, with more DCs expressing CD86 (Turner et al. 2018). The activation of these DCs during memory inflammation suggests a possible unexplained role for cDC2 in the memory recall response. Thus, a more detailed understanding of how DCs support memory $T_{RM}$ cell responses is needed.

In this study, we address fundamental questions regarding whether IRF4-expressing DCs regulate the development and recall response of type 2 $T_{RM}$ cells. We demonstrate that mice having IRF4-deficient DCs during sensitization exhibit diminished $T_{RM}$ cell-dependent memory responses to allergen. In addition, we confirm and expand upon previous findings regarding critical functions of IRF4 in cDC2s earlier in the course of type 2 lung immunity in vivo. In particular, we demonstrate that IRF4 controls DC expression of OX40L, IL-10, and IL-33 as well as migration to the draining lymph node during allergic sensitization, without which there

are diminished type 2 effector responses in the lungs following allergen challenge. As IRF4-expressing DCs are necessary during sensitization, we investigated the ongoing role for IRF4-expressing DCs in maintaining $T_{RM}$ cells that were educated by IRF4-competent DCs in wild-type hosts. T cells educated by IRF4-competent DCs are able to seed the lungs and mediate potent memory responses independently of IRF4-expressing DCs. In total, we find that IRF4 controls a program in mature DCs that governs Th2 priming during sensitization and Th2 effector responses during challenge, but that mitigated $T_{RM}$ cell-dependent responses stem from defects in earlier education.

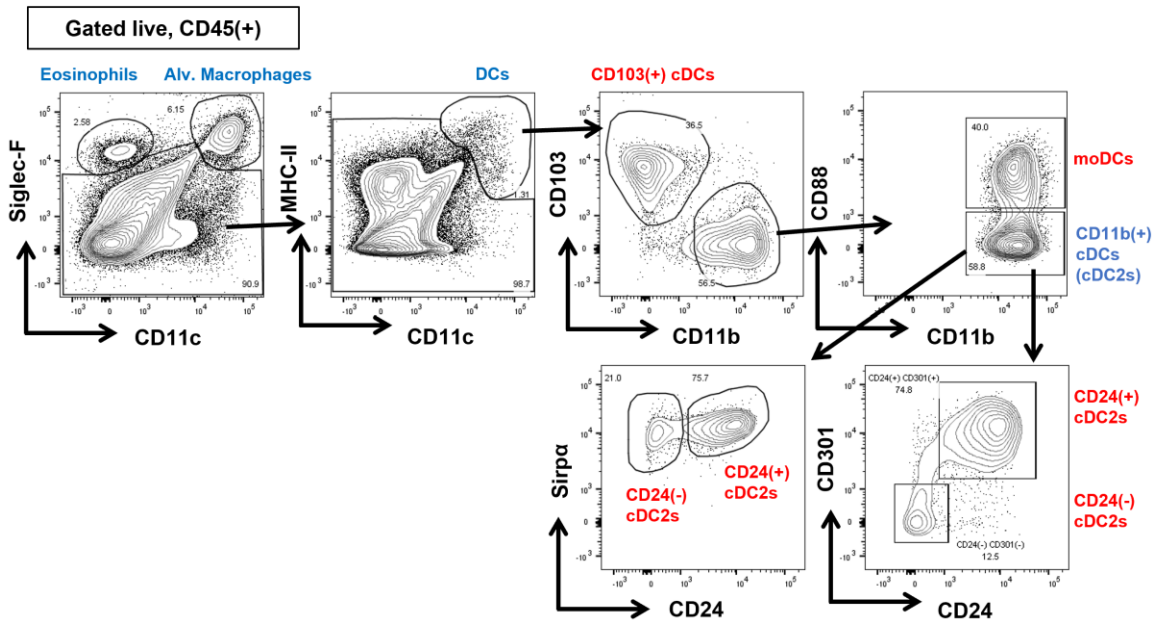


**Figure 4-1.** Lung DC gating.

After exclusion of Siglec-F(+) eosinophils and alveolar macrophages, lung DCs are defined as CD11c(+) and MHC-II high. This excludes interstitial macrophages, which are CD11c(-). DCs in the lung either express CD103 or CD11b, apart from a small population of pDCs expressing neither. The CD103(+) population are cDC1s. The CD11b(+) DCs include populations of CD88(+) monocyte-derived DCs, CD24(-) conventional DC2s, and CD24(+) conventional DC2s.

## Results

### *IRF4-expressing dendritic cells regulate the $T_{RM}$ cell-restricted type 2 inflammatory memory response to HDM rechallenges*

We previously reported that mice lacking the transcription factor IRF4 in mature DCs do not develop robust type 2 effector responses in the lungs following house dust mite (HDM) sensitization and challenge (Williams et al. 2013). Whether these defects persist throughout subsequent memory recall responses, or if the defective development of the type 2 response resolves over time remains unknown. We sensitized and challenged *Irf4$^{fl/fl}$ or Irf4$^{fl/fl}$*CD11cCre mice with HDM, and rechallenged the mice 4-5 weeks later. Rechallenges were performed while treating the mice with FTY720 to limit the recall response to lung $T_{RM}$ cells (Fig. 4-2A, ref. (Hondowicz et al. 2016)), as others have shown that treatment with this sphingosine-1-phosphate receptor agonist downregulates $S1P_1$ and thus retains circulating T cells in lymphoid organs (Matloubian et al. 2004). We confirmed that FTY720 treatment does not affect the lung $T_{RM}$ cell population, but does effectively deplete most circulating T cells (Fig. 4-3). Mice lacking IRF4 in DCs exhibit a severely mitigated type 2 inflammatory $T_{RM}$ cell response to HDM rechallenge compared to their WT littermates, with fewer eosinophils and CD4(+) T cells infiltrating the airways (as measured by cells in the bronchoalveolar lavage; BAL) or present in the lungs (Fig. 4-2B). Indeed, evaluation of H&E-stained histological sections revealed that mice with IRF4-expressing DCs mount robust inflammation in response to HDM compared to PBS, whereas the lungs of mice with IRF4-deficient DCs become less inflamed (Fig. 4-2C).

An analysis of the lung DCs and their expression of costimulatory molecules revealed that IRF4-deficient CD24(+) cDC2s and CD24(-) cDC2s are present to an equal extent compared to their IRF4-expressing counterparts (Fig. 4-2D), yet they express less CD86 (Fig. 4-2E). None

of the APCs examined exhibit IRF4-dependent expression of CD80 (data not shown). However, all APCs examined appear to express reduced OX40L in the lungs of *Irf4*$^{fl/fl}$CD11cCre mice (Fig. 4-2E and Fig. 4-4B). This demonstrates that mice with IRF4-deficient DCs fail to mount memory type 2 responses and that their DCs are not appropriately activated during the recall response. Thus, when taken together with our previous findings that *Irf4*$^{fl/fl}$CD11cCre mice display diminished effector responses to HDM (Williams et al. 2013), we find that IRF4 regulates DC control of type 2 inflammatory responses at multiple timepoints following allergic exposure.

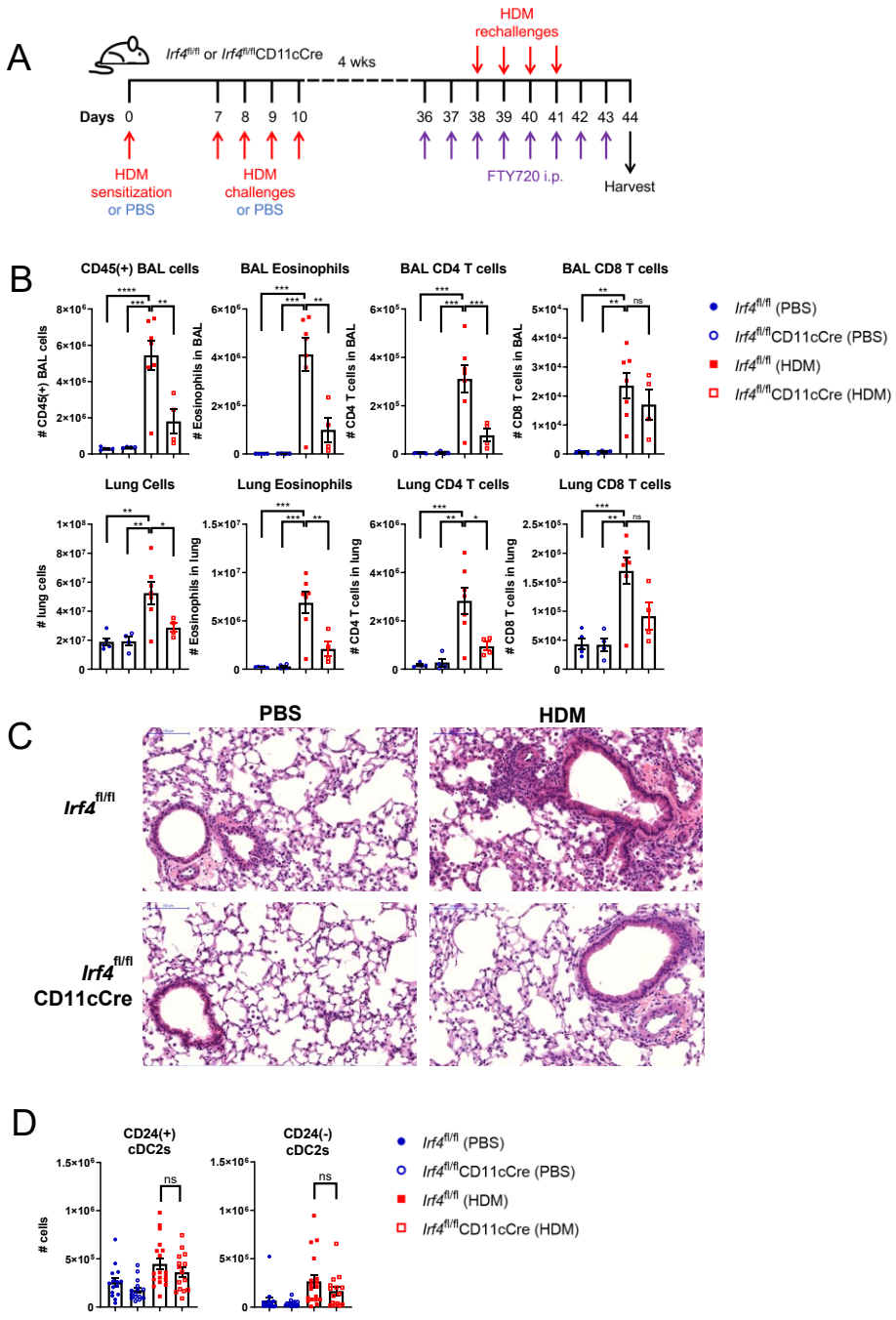


**Figure 4-2.** IRF4-expressing dendritic cells regulate the $T_{RM}$ cell-restricted type 2 inflammatory response to HDM rechallenges.

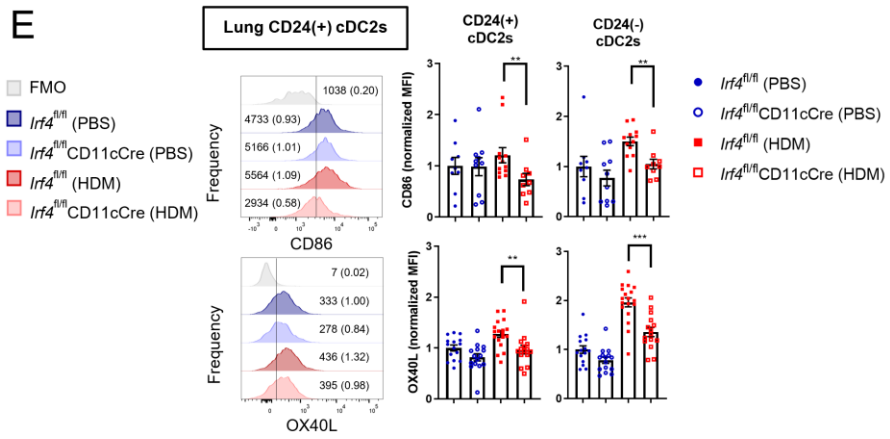


**Figure 4-2, continued.** IRF4-expressing dendritic cells regulate the $T_{RM}$ cell-restricted type 2 inflammatory response to HDM rechallenges.

(a) Schematic of experimental protocol for $T_{RM}$ cell-restricted memory response to HDM (b) Plots of total cellularity, eosinophils, CD4(+) T cells, and CD8(+) T cells in the airways (top) or lungs (bottom) (c) H&E staining of the lungs confirms that mice with IRF4-deficient DCs are protected from allergic airway inflammation during the memory recall response to HDM (d) Number of CD24(+) cDC2s and CD24(-) cDC2s in the lungs (e) For lung CD24(+) cDC2s and CD24(-) cDC2s, MFI of CD86 (top) or OX40L (bottom) normalized to the mean of the PBS-treated *Irf4*$^{fl/fl}$ group, with representative flow plots (left). Data are representative of (b, c) or combined from (d, e) 3 independent experiments with $n \geq 4$ mice per group in each experiment, and statistics (b: ordinary one-way ANOVA with Tukey's multiple comparisons test; c, d: Mann-Whitney test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant).

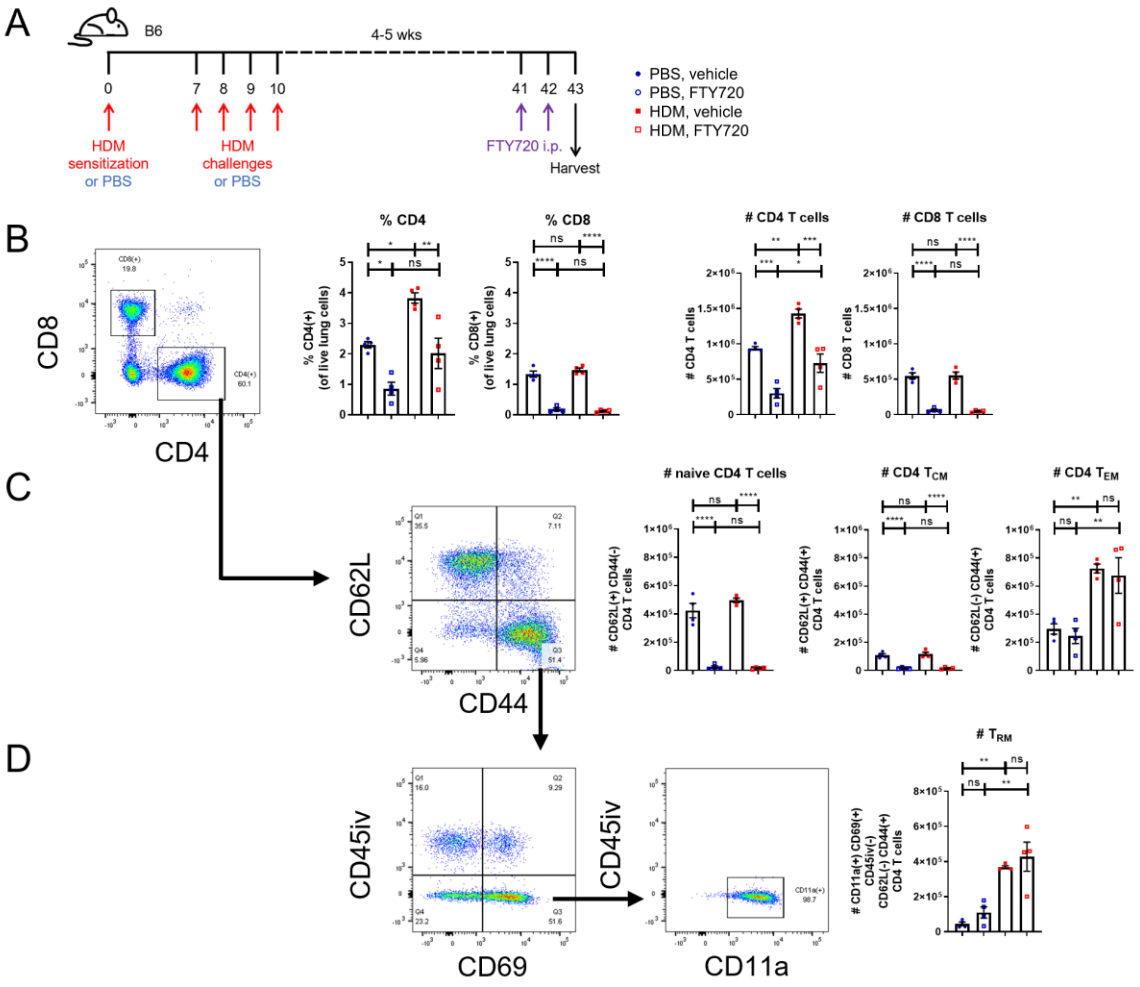


**Figure 4-3.** Systemic treatment with FTY720 depletes circulating T cell populations but leaves lung $T_{RM}$ cells intact.

(a) Schematic depicting experimental protocol. After sensitization and challenge with HDM and resolution of the acute response, mice underwent treatment with 25 ug FTY720 administered intraperitoneally daily until sacrifice. (b) Examination of the lung T cell populations revealed considerable depletion of the CD4 compartment and near-total depletion of the CD8 compartment. (c) CD4(+) T cells were defined as naïve (CD44(low)CD62L(high)), central memory ($T_{CM}$, CD44(high)CD62L(high)), or effector memory ($T_{EM}$, CD44(high)CD62L(low)). FTY720 depleted the naïve and $T_{CM}$ cells, but not $T_{EM}$ cells. (d) CD4(+) $T_{RM}$ cells were defined as CD4(+) $T_{EM}$ cells which expressed CD69 and CD11a, but did not display intravascular CD45 staining. CD4(+) $T_{RM}$ cells were not depleted by FTY720 treatment. Data represent one experiment with n = 4 mice per group, and statistics (ordinary one-way ANOVA with Tukey's multiple comparisons test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$; ns, not significant).

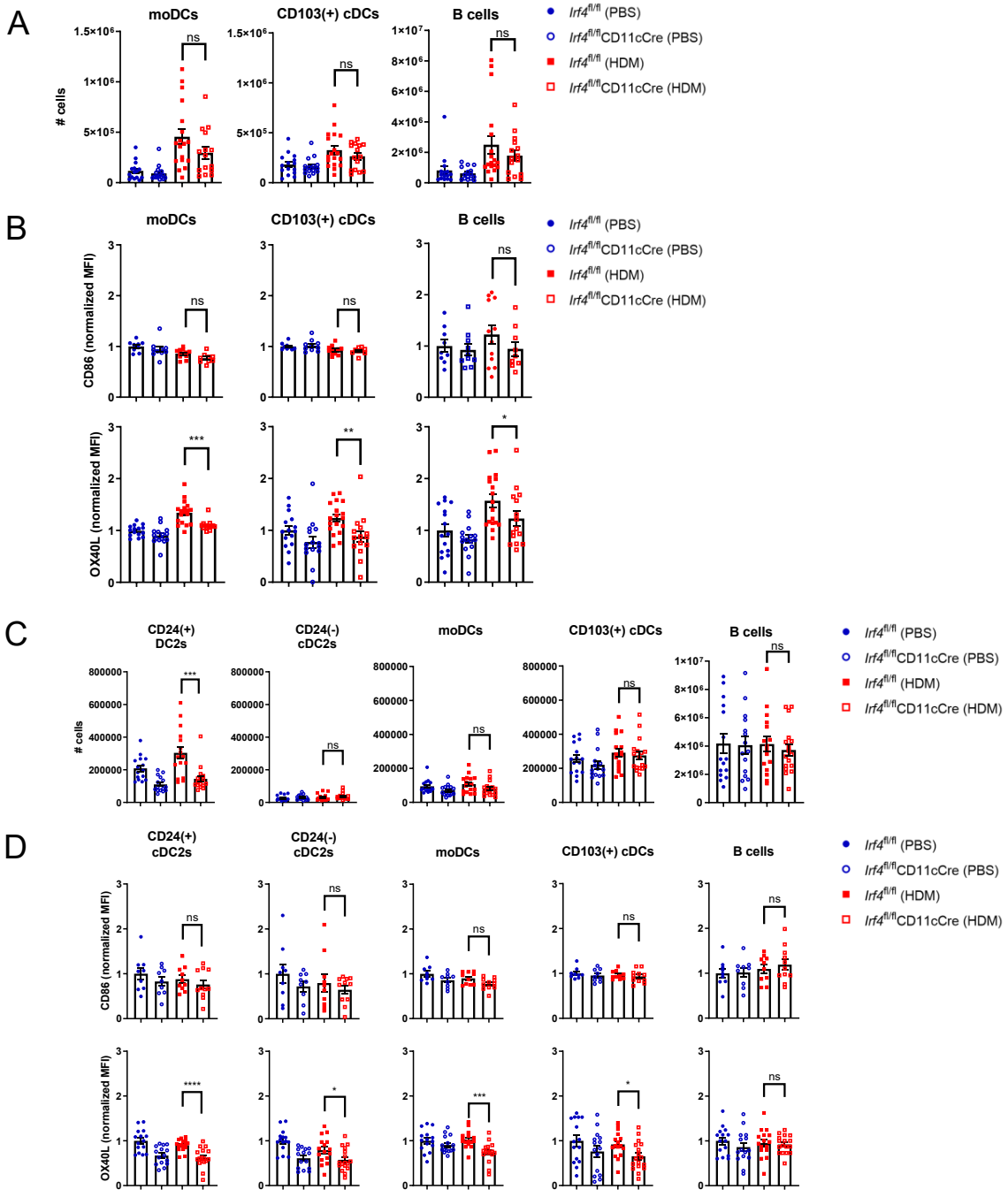


**Figure 4-4.** Number and activation states of other DC subsets during memory responses to HDM.

(a, b) During memory HDM rechallenges, the (a) number of moDCs, CD103(+) cDCs, and B cells in the lungs and (b) their expression of CD86 (top) and OX40L (bottom). Expression of these markers was normalized to the mean expression by the *Irf4*$^{fl/fl}$ (PBS) group. (c, d) During the resting memory phase (c) numbers of all examined DC subsets the lungs and (d) their expression of CD86 (top), and OX40L (bottom). Data are combined from 3 experiments for a

**Figure 4-4, continued.**

total of $n \geq 15$ mice per group, and statistics (Mann-Whitney test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

*Lungs of mice with IRF4-deficient DCs contain fewer Der p 1-specific $Th2_{RM}$ cells during the memory phase*

We hypothesized that the reduced memory response to HDM in *Irf4*$^{fl/fl}$CD11cCre mice was due to a defect in the underlying $T_{RM}$ cell pool that develops in response to sensitization and challenge. Without rechallenging the mice, we harvested the lungs 4-5 weeks after sensitization and d7-10 challenge of *Irf4*$^{fl/fl}$ *or Irf4*$^{fl/fl}$CD11cCre mice to HDM or PBS vehicle control (Fig. 4-5A). To identify tissue resident cells in the lung parenchyma, such as the $T_{RM}$ cells, we labeled the cells in circulation by intravenously injecting the mice with fluorescent αCD45 minutes prior to sacrifice (Anderson et al. 2014). While no significant difference in the total number of $T_{RM}$ cells was evident in the absence of IRF4-expressing DCs, the $T_{RM}$ cells in *Irf4*$^{fl/fl}$CD11cCre mice were less likely to express the IL-33 receptor, ST2, which is expressed on Th2 cells and on a subset of Tregs (Fig. 4-5B), suggesting a reduction in $T_{RM}$ cells of a Th2 phenotype.

Using tetramers to identify antigen-specific cells, we found that *Irf4*$^{fl/fl}$CD11cCre mice displayed fewer GATA3(+) ST2(+) Der p 1-specific $T_{RM}$ cells (Fig. 4-5C), suggesting that decreased antigen-specific $T_{RM}$ cells may limit memory Th2 responses. Further, mice without IRF4-expressing DCs did not show significant differences in the development of Foxp3(+) Der p 1-specific $T_{RM}$ cells (Fig. 4-5C). The IRF4-deficient lung DCs displayed reduced expression of OX40L during rechallenge, but expression of CD86 (Fig. 4-4D) and CD80 (data not shown) were normal. Taken together, these data suggest that allergen-specific $Th2_{RM}$ cell defects in mice whose DCs lack IRF4 give rise to a reduced $T_{RM}$ cell-restricted response.

To determine the stage at which Th2 defects begin, we sensitized *Irf4*$^{fl/fl}$ *or* *Irf4*$^{fl/fl}$CD11cCre mice to HDM and evaluated the T cells in the lung seven days later (Fig. 4-6A).

This revealed a reduced proportion of lung parenchymal T effector/memory cells and reduced expression of CD69 after HDM sensitization (Fig. 4-6B), demonstrating a defect in the recruitment of activated T cells to the lungs after sensitization. In the lung-draining lymph nodes four days post-sensitization (Fig. 4-6C), we examined the expression of lineage-specifying transcription factors and found that the number of conventional Th2 cells was reduced in the lymph nodes of *Irf4*$^{fl/fl}$CD11cCre mice by this timepoint (Fig. 4-6D). Further, the number of Der p 1-MHC-II tetramer-positive CD4(+) T cells was also reduced at this timepoint, demonstrating a diminished antigen-specific response (Fig. 4-6E). These data indicate that IRF4 is acting in DCs during the earliest stages of HDM sensitization to initiate type 2 lung responses.

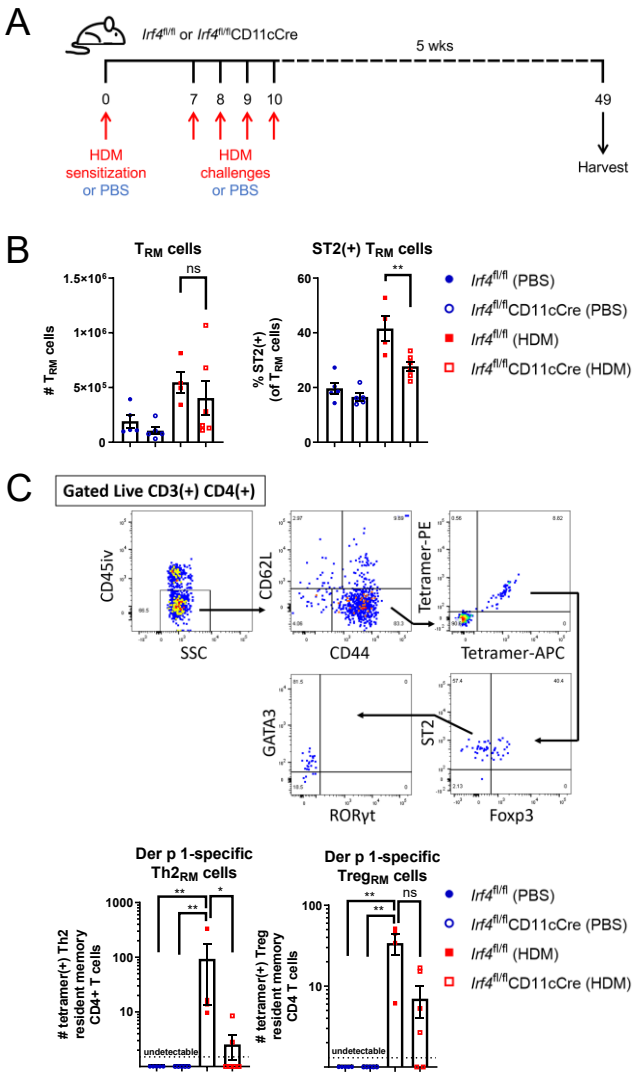


**Figure 4-5.** Lungs of mice with IRF4-deficient DCs contain fewer Der p 1-specific $Th2_{RM}$ cells during the memory phase.

(a) Schematic of experimental protocol for resting memory lung analysis. (b) Plotted is the number of lung $T_{RM}$ cells and the proportion expressing ST2. (c) Gating of antigen-specific T cells and their expression of lineage-defining transcription factors. Graphed flow plots show the number of tetramer(+) CD4(+) $T_{RM}$ cells expressing GATA3 or Foxp3. Data are representative of at least 2 independent experiments with $n \geq 4$ mice per group in each experiment, and statistics (b: ordinary one-way ANOVA with Tukey's multiple comparisons test; c: Kruskal-Wallis test with Dunn's multiple comparisons test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ns, not significant).

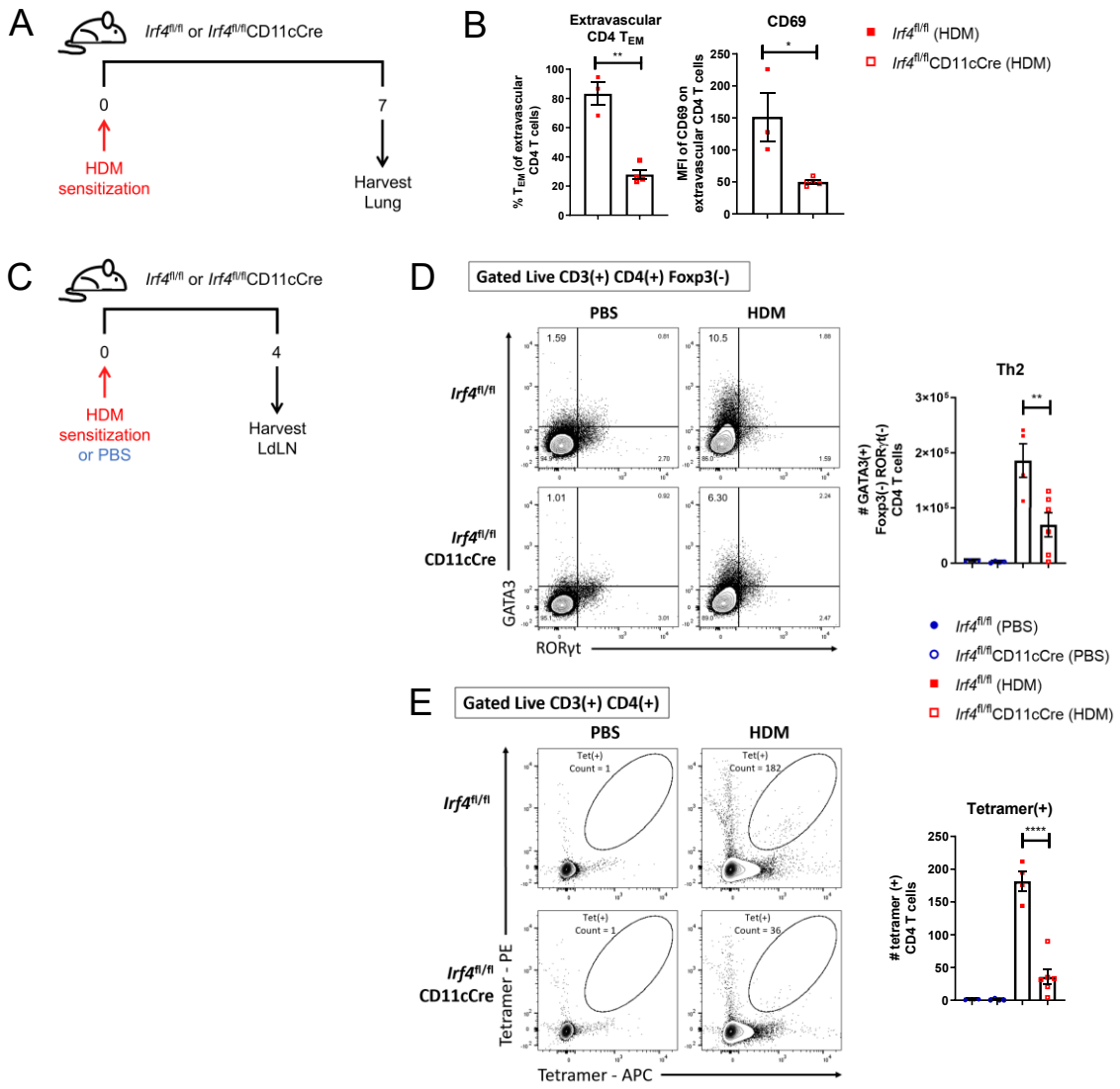


**Figure 4-6.** Mice with IRF4-deficient DCs are unable to produce Th2 cells nor tetramer(+) T cells in the LLN, nor adequately recruit $T_{EM}$ cells to the lungs after HDM sensitization.

(a) Lungs were harvested on day 7 following HDM sensitization (b) Depicted is the proportion of $T_{EM}$ cells out of lung extravascular CD4(+) T cells and CD69 expression by extravascular lung T cells. (c) LdLN were harvested on day 4 following HDM sensitization (d) GATA3 and RORγt expression by LdLN $T_{conv}$ cells on day 4 after HDM sensitization, (c) Number of tetramer(+) LdLN CD4(+) T cells. Data are representative of at least 2 independent experiments with n ≥ 3 mice per group, and statistics (b: unpaired t test with Welch's correction; c,d: ordinary one-way ANOVA with Tukey's multiple comparisons test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$).

*IRF4 regulates allergen phagocytosis, DC migration, and priming of naïve T cells during HDM sensitization*

We hypothesized that IRF4 regulates particular DC processes required to successfully initiate Th2 responses during sensitization *in vivo*. An initial step in initiating the immune response is uptake of allergens by DCs. To assess allergen uptake, we sensitized mice with fluorescently-labeled HDM and evaluated lung DCs the next day. There was a slight but statistically-significant reduction in the proportion and number of HDM-bearing CD24(+) cDC2s in the lungs of *Irf4*$^{fl/fl}$CD11cCre mice compared to *Irf4*$^{fl/fl}$ mice (Fig. 4-7A). There was no difference in the number of any DC subset overall (Fig. 4-7A, Fig. 4-8A). The CD24(-) cDC2 subset, which is also IRF4-dependent, had a slight but statistically-significant reduction in the proportion that were HDM-positive (Fig. 4-8B), but no reduction in cell number (Fig. 4-8C) or MFI (Fig. 4-8D). Thus, allergen uptake is largely independent of IRF4.

It has been previously described that IRF4 is needed for DC expression of CCR7 and subsequent migration to the tissue-draining lymph nodes in the skin (Bajana et al. 2012, Bajana et al. 2016). Indeed, we confirmed that CD24(+) cDC2s from the lungs of *Irf4*$^{fl/fl}$CD11cCre mice had reduced CCR7 following sensitization in vivo (Fig. 4-9). Thus, we found an expected reduction in the number of migratory CD24(+) cDC2s in the lung-draining lymph nodes of *Irf4*$^{fl/fl}$CD11cCre mice (Fig. 4-7B). Nevertheless, a number of HDM-bearing cDC2s could be detected in the lung-draining lymph nodes of *Irf4*$^{fl/fl}$CD11cCre mice (Fig. 4-7B). In fact, the proportion of CD24(+) cDC2s that were HDM-positive was equal in the lung-draining lymph nodes between *Irf4*$^{fl/fl}$ *or Irf4*$^{fl/fl}$CD11cCre mice, despite the reduction in the number of HDM+ DCs in the *Irf4*$^{fl/fl}$CD11cCre mice (Fig. 4-7B). Consistent defects were not seen for other APC subsets lacking IRF4 in the LdLN (Fig. 4-8E-H). Thus, as was previously described for skin DCs

(Bajana et al. 2012, Bajana et al. 2016), the migration of lung CD24(+) cDC2s to the lymph node is regulated by IRF4. However, some allergen-bearing DCs are nevertheless capable of reaching the lymph nodes.

These findings raised the question of whether reduced Th2 responses in *Irf4*$^{fl/fl}$CD11cCre mice are solely due to a reduced quantity of DCs reaching the lymph nodes. Using in vitro-derived BMDCs, we previously showed that IRF4-deficient DCs display a reduced capacity to promote Th2 differentiation in vitro (Williams et al. 2013). Thus, we hypothesized that IRF4 regulates DCs processes beyond migration. One such function is the ability to process antigens upon phagocytosis. To assess this capability, we sensitized mice to HDM in combination with the surrogate reagent DQ Red BSA, which becomes fluorescent upon proteolytic cleavage. IRF4-deficient CD24(+) cDC2s were still capable of processing antigens both in the lungs (Fig. 4-7C) and in the LdLN (Fig. 4-7D). To address whether there is a cell-intrinsic defect in the ability of IRF4-deficient DCs to prime T cells, we turned to ex vivo cultures where the number of DCs can be normalized. After HDM plus OVA sensitization, lung and LdLN DCs from *Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice were sorted and used to stimulate CFSE-labeled T cells from OTII transgenic mice in vitro (Fig. 4-10A). We found that IRF4-deficient CD24(+) cDC2s were less effective at inducing T cell proliferation (Fig. 4-10B), as cultures with IRF4-deficient DCs produced fewer OTII cells with a greater proportion of undivided cells, leading to a reduced division index. The proliferation index, which indicates the number of divisions undergone by cells that have entered cell division, was unchanged, suggesting that IRF4-expressing DCs are important for prompting T cell division but that once T cells divide, they do so to an equal extent. While CD24(-) cDC2s had similar trends in these measures, their ability to induce cell division was inferior to that of CD24(+) cDC2s (number of OTII cells from coculture with WT

CD24(+) vs WT CD24(-) cDC2s, p = 0.0002), suggesting that CD24(-) cDC2s are not well-suited to T cell priming in response to HDM (Fig. 4-11). That IRF4-deficient CD24(+) cDC2s are intrinsically less capable of priming T cells suggests that there are downstream effectors of IRF4 in DCs responsible for driving allergic T cell responses.

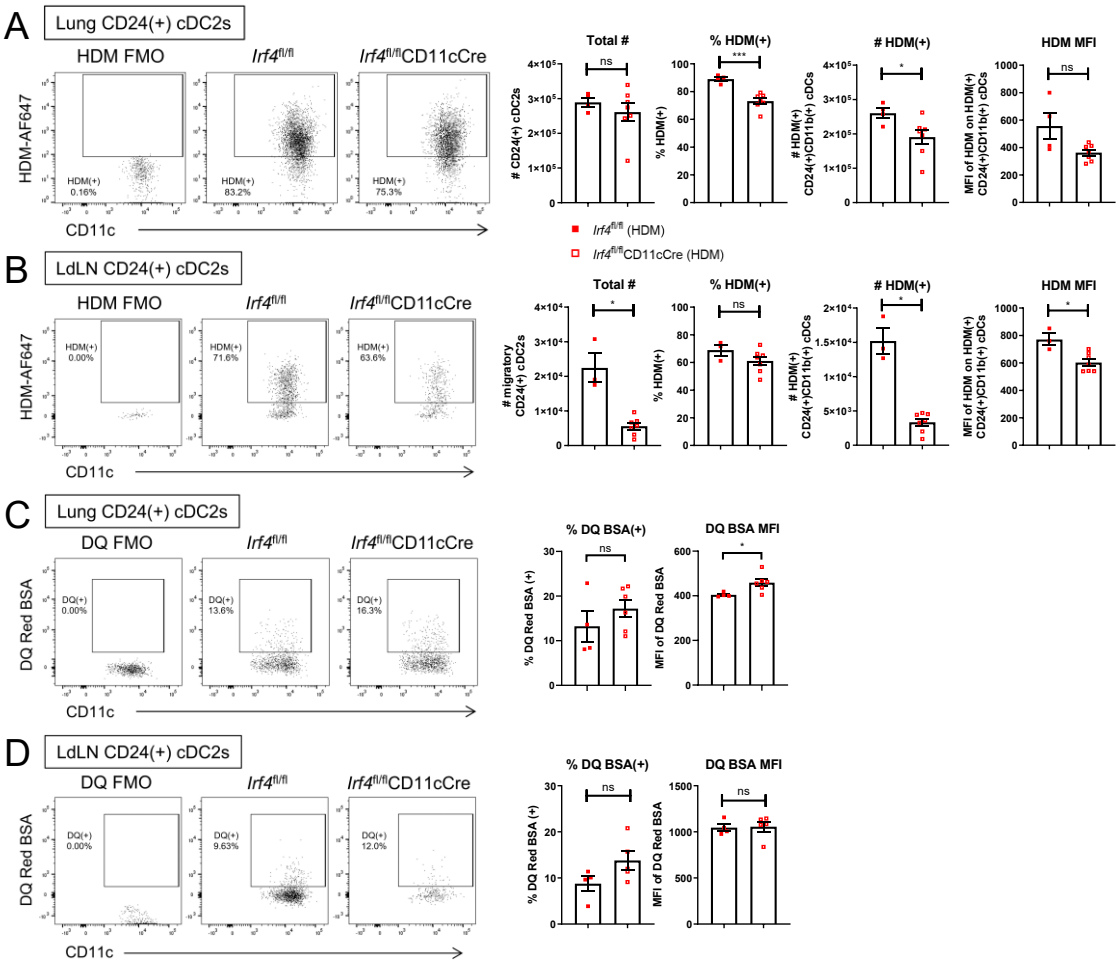


**Figure 4-7.** IRF4 regulates allergen phagocytosis and migration of CD24(+) cDC2s to the lung-draining lymph nodes during HDM sensitization.

(a, b) Flow cytometry plots depict fluorescently-labeled HDM in CD24(+) cDC2s, quantified number of CD24(+) cDC2s, proportion and number that are HDM(+), and MFI of HDM in either the lungs (a) or LdLN (b). (c, d) Flow cytometry plots depicting fluorescence of processed DQ Red BSA in CD24(+) cDC2s, proportion DQ(+), and MFI of DQ in either the lungs (c) or LdLN (d). Data are representative of at least 2 independent experiments with $n \geq 4$ mice per group, and statistics (unpaired t test with Welch's correction) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; ***, $p < 0.001$; ns, not significant).

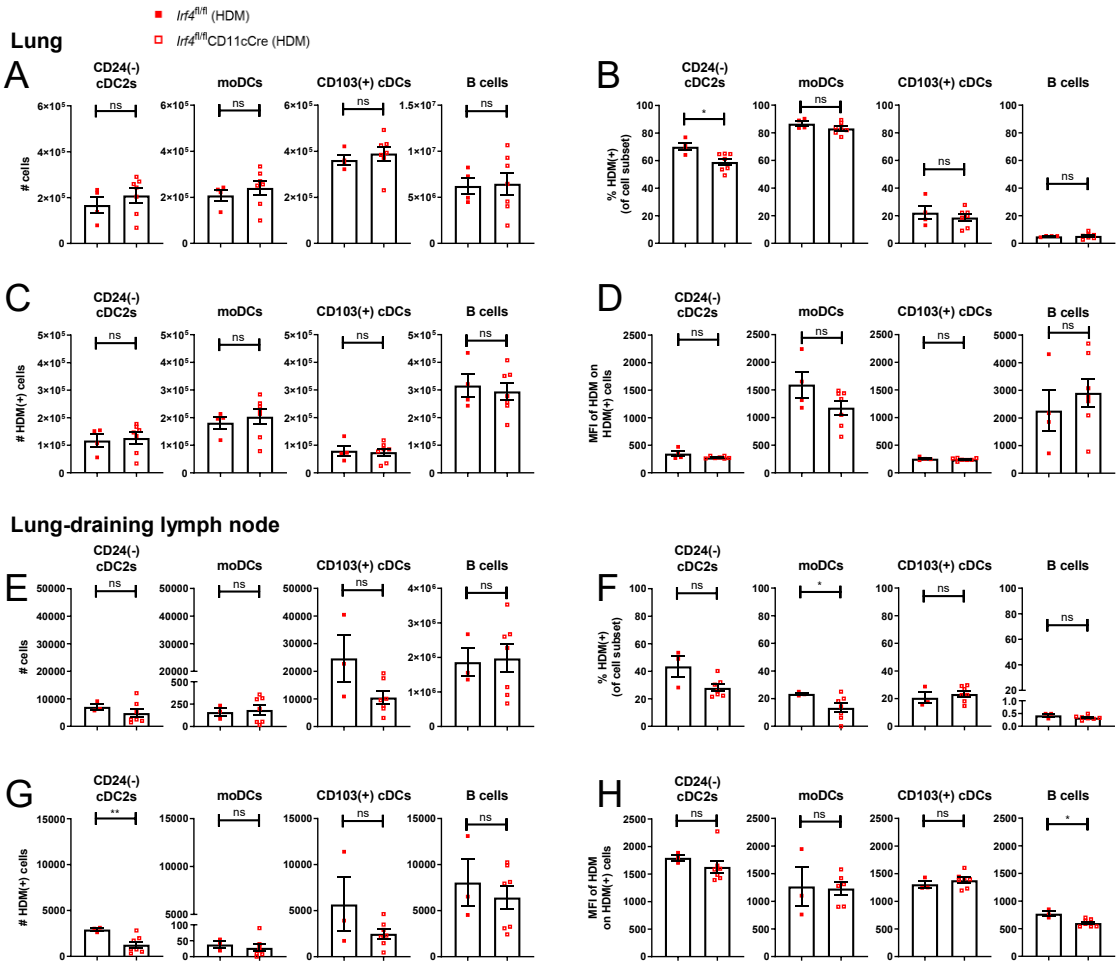


**Figure 4-8.** Number, allergen uptake, and allergen processing by other lung and LdLN DCs during sensitization.

(a) Number of lung CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells (b) HDM uptake by lung CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells, (c) Number of HDM-bearing lung CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells (d) MFI of HDM on HDM-bearing lung CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells (e) Number of LN CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells (f) HDM uptake by LN CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells, (g) Number of HDM-bearing LN CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells, (h) MFI of HDM on HDM-bearing LN CD24(-) cDC2s, moDCs, CD103(+) cDCs, and B cells. Data are representative of at least 2 independent experiments with $n \geq 4$ mice per group, and statistics (unpaired t test with Welch's correction) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ns, not significant).

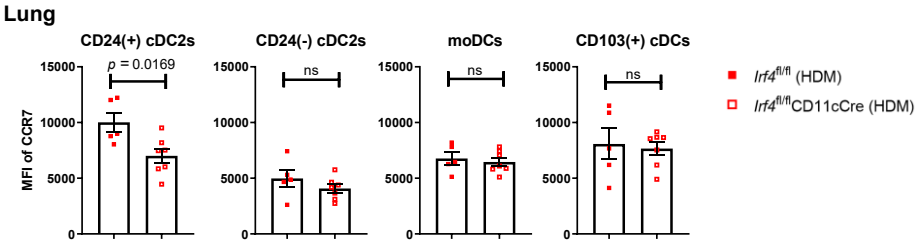


**Figure 4-9.** IRF4-dependent expression of CCR7 on DCs following HDM sensitization.

MFI of CCR7 on the indicated DC subset either from the lungs of mice sensitized with HDM + DQ Red BSA overnight. Data represent one experiment with n ≥ 4 mice per group, and statistics (unpaired t test) were performed in GraphPad Prism. Bar represents the mean ± SEM (ns, not significant).

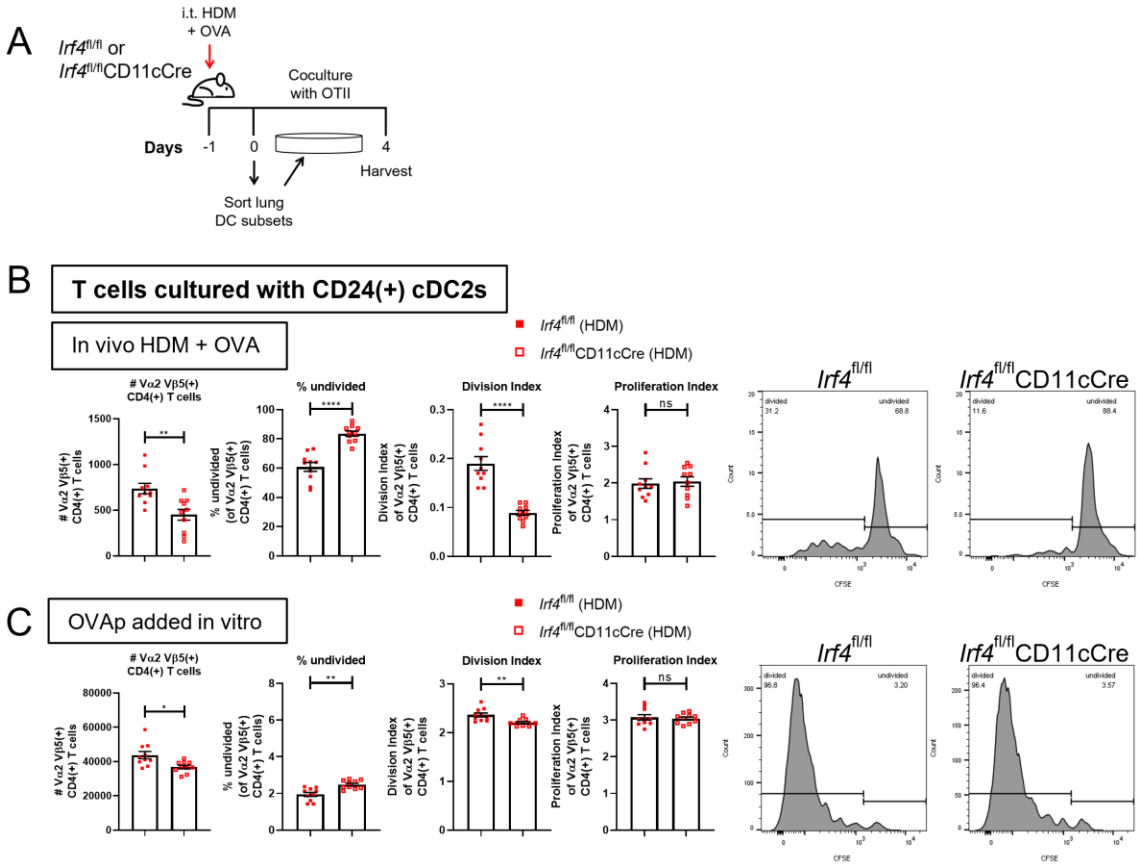


**Figure 4-10.** Ex-vivo sorted CD24(+) cDC2s require IRF4 for robust T cell priming in vitro.

(a) Schematic of experimental protocol for in vivo sensitization to HDM + OVA, DC sorting, and in vitro coculture with CFSE-labeled T cells from naïve OTII mice (b, c) Plotted are the number of OTII cells after culture, % undivided, division index, proliferation index, and CFSE dilution histograms for (b) in vivo HDM+OVA sensitized CD24(+) cDC2s or (c) with $OVA_{323-339}$ peptide added. Data are representative of at least 2 independent experiments with $n \geq 4$ wells per group, and statistics (unpaired t test with Welch's correction) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant).

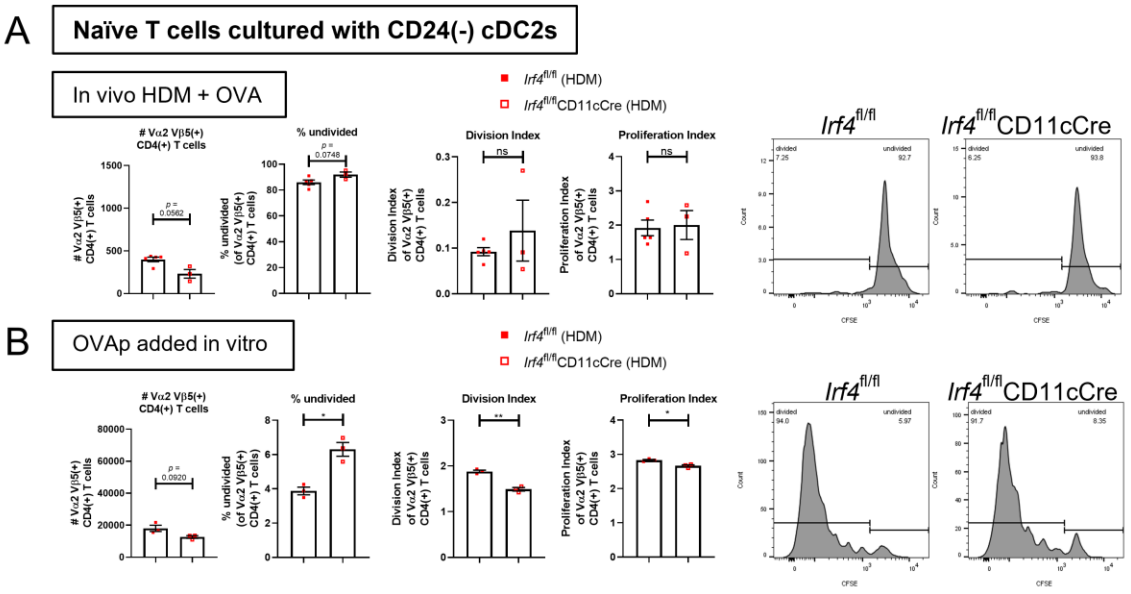


**Figure 4-11.** CD24(-) cDC2s are poor stimulators of naïve T cells in response to HDM.

CD24(-) cDC2s were sorted from the lungs on day 1 following in vivo sensitization to HDM + OVA, then were cocultured with CFSE-labeled T cells from naïve OTII mice. Depicted are the number of OTII cells after culture, % undivided, division index, proliferation index, and CFSE dilution histograms for (a) in vivo HDM + OVA sensitized CD24(-) cDC2s or (b) with $OVA_{323-339}$ peptide added. Data are representative of at least 2 independent experiments with $n \geq 3$ wells per group, and statistics (unpaired t test with Welch's correction) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ns, not significant).

*IRF4 regulates a pro-Th2 program in cDC2s*

Numerous DC-surface and secreted factors have been implicated in promoting the differentiation of Th2 cells, though the role for IRF4 in maintaining this program in mature DCs has not been addressed. OX40L expression by DCs is known to play an important role in the induction of Th2 differentiation (Salek-Ardakani et al. 2003, de Kleer et al. 2016). In silico analysis demonstrated that the OX40L locus contains multiple IRF4 binding sites suggesting that OX40L may be a downstream target of IRF4 (Fig. 4-12A). We confirmed using flow cytometry that lung CD24(+) cDC2 expression of OX40L after HDM sensitization is reduced in the absence of IRF4 (Fig. 4-12B). In addition, *Il33* and *Il10* expression by lung cDC2s, as assessed by qPCR, is dependent on IRF4 during in vivo HDM sensitization (Fig. 4-13A). This suggests that IRF4 controls a pro-Th2 program involving these factors which culminate in Th2 polarization.

We hypothesized that reintroduction of IL-10 and IL-33 to the lungs during sensitization would reconstitute the type 2 effector response in vivo. We sensitized $Irf4^{fl/fl}$ or $Irf4^{fl/fl}$CD11cCre mice to HDM, with some mice receiving additional recombinant IL-33 and IL-10. We then challenged the mice with HDM and evaluated the type 2 effector response (Fig. 4-13B). Compared to LdLN CD4(+) T cells from $Irf4^{fl/fl}$ mice given only HDM, the T cells from $Irf4^{fl/fl}$CD11cCre mice displayed a reduced capacity for the production of the type 2 cytokines IL-4, IL-5, and IL-13 (Fig. 4-13C). This indicates that IRF4-expressing DCs must be present for the robust development of cytokine-producing Th2 effector cells. However, $Irf4^{fl/fl}$CD11cCre mice receiving exogenous IL-33 and IL-10 during sensitization no longer exhibited IRF4-dependent differences in IL-4 and IL-5. Differences in IL-13 production remained. Thus, IL-33 and IL-10 add-back during sensitization synergizes to promote IL-4 and IL-5 expression by T

cells in the LdLN (Fig. 4-13C), consistent with previous findings in vitro (Williams et al. 2013), but does not restore IL-13 production. When we evaluated airway inflammation, we were interested to see that addition of IL-33 to HDM sensitization amplified eosinophilia ($p < 0.0001$) and CD4(+) T cell infiltration ($p = 0.01$) of the airways in *Irf4*$^{fl/fl}$ mice (Fig. 4-13D), but these responses remained IRF4-dependent. Reintroduction of IL-33 and IL-10 together in *Irf4*$^{fl/fl}$CD11cCre mice did not restore type 2 inflammation to the airways (Fig. 4-13D), despite the partial restoration of Th2 cytokine expression seen in the LdLN. These data suggest that other IRF4-dependent factors are required to fully reconstitute the allergic response to the airways.

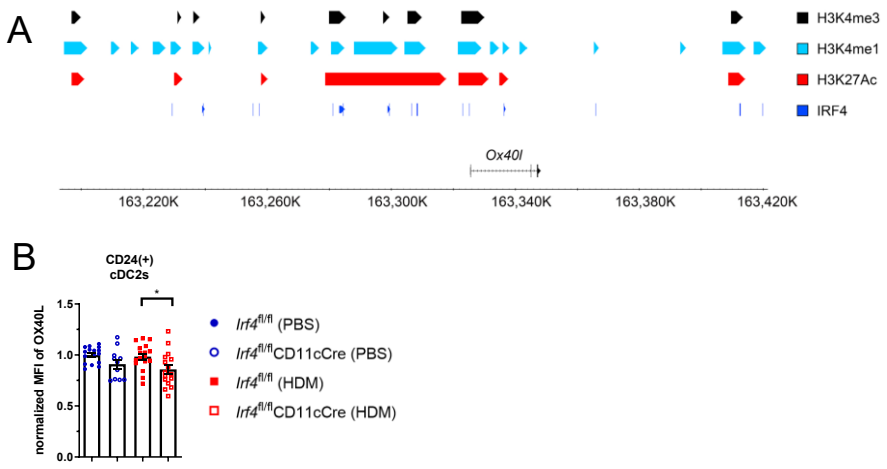


**Figure 4-12.** IRF4 binding sites are present at the *Ox40l* locus, and IRF4 regulates OX40L expression during HDM sensitization.

(a) IRF4 ChIP-seq at the *Ox40l* locus, (b) OX40L expression by flow cytometry on CD24(+) cDC2. Data are combined from 4 experiments for a total of $n \geq 11$ mice per group, and statistics (Mann-Whitney test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$).

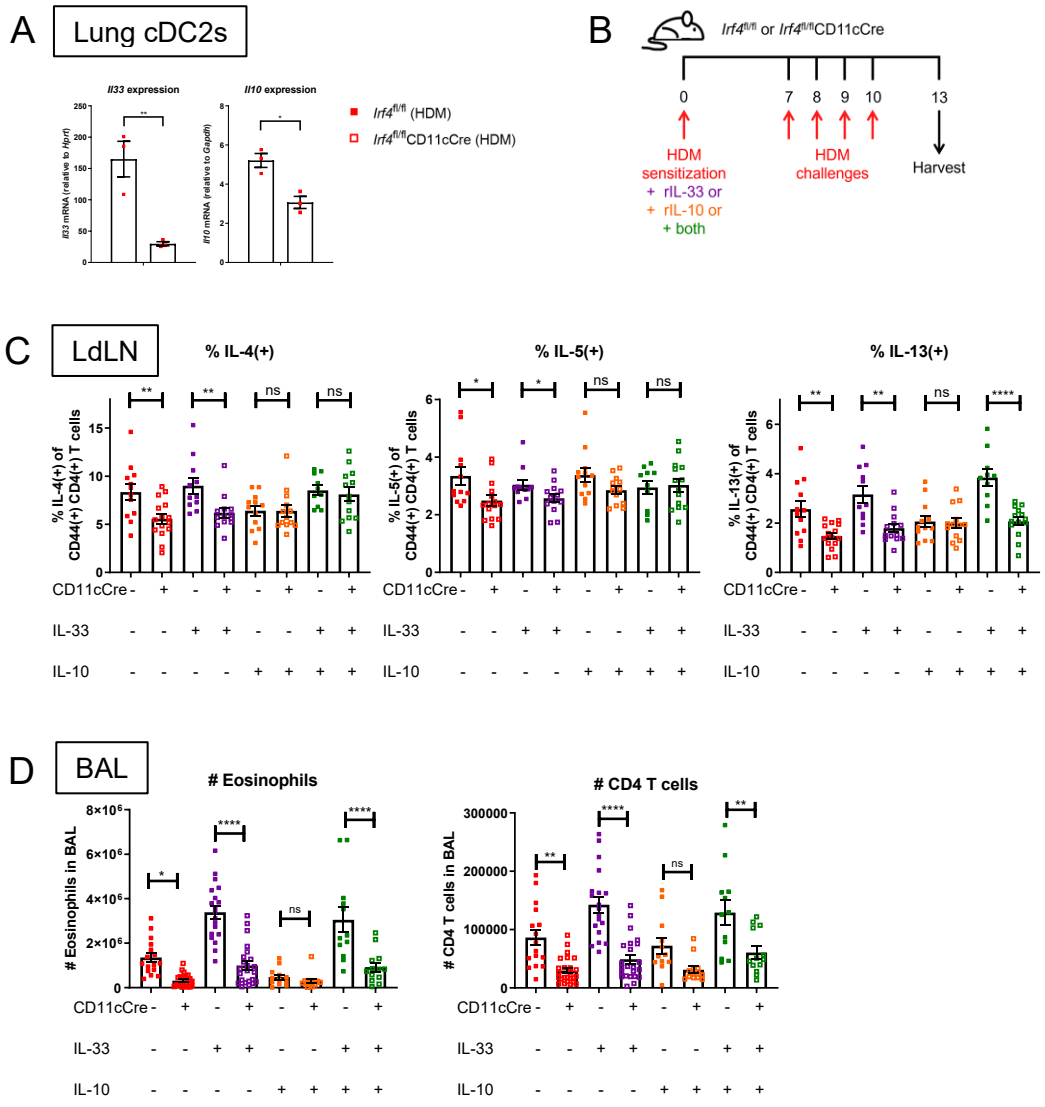


**Figure 4-13.** IRF4 controls a pro-Th2 program in DCs responding to HDM which includes expression of OX40L, IL-10, and IL-33.

(a) IL-33 and IL-10 expression by qPCR of sorted lung cDC2s after in vivo HDM sensitization. Data represent 1 experiment with n = 3 mice per group, and statistics (unpaired t test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$). (b) Experimental schematic depicting protocol in which rIL-10 and/or rIL-33 were added to the lungs during sensitization and the type 2 inflammatory response was assessed after challenge. (c) Proportion of CD44(+) CD4(+) T cells expressing IL-4, IL-5, and IL-13 in the LdLN after challenge, (d) Number of eosinophils and CD4(+) T cells infiltrating the airways. Data represent 3 combined experiments with n ≥ 4 mice per group, and statistics (unpaired t tests or ordinary two-way ANOVA with Sidak's multiple comparisons test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant).

*IRF4 regulates a subset of genes/pathways involved in the cDC2 response to HDM*

To gain a broad understanding of the IRF4-dependent DC transcriptional response to HDM, we conducted RNAseq on CD24(+) cDC2s isolated from the lungs of *Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice nine hours following HDM sensitization or PBS vehicle control. Our analysis revealed that WT CD24(+) cDC2s differentially expressed 656 genes in response to HDM, 275 of which were upregulated and 381 which were downregulated (Table 4-1). HDM-activated DCs required IRF4 expression for regulation of 372 genes, 219 of which increased in the absence of IRF4 and 153 which appeared to require IRF4 for their expression (Table 4-1). Further analysis of this dataset is ongoing and will detail which pathways are positively and negatively regulated by IRF4 in CD24(+) cDC2s responding to HDM.

| Comparison | DEG | Up | Down |
|---|---|---|---|
| **Lung CD24(+) cDC2s** | | | |
| *Irf4*$^{fl/fl}$: HDM-treated compared to PBS | 656 | 275 | 381 |
| *Irf4*$^{fl/fl}$CD11cCre: HDM-treated compared to PBS | 1378 | 687 | 691 |
| HDM-treated: *Irf4*$^{fl/fl}$CD11cCre compared to *Irf4*$^{fl/fl}$ | 372 | 219 | 153 |

**Table 4-1.** IRF4 regulates genes involved in the cDC2 response to HDM.

Lung CD24(+) cDC2s were sorted from *Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice nine hours following HDM sensitization or PBS vehicle control. RNAseq was conducted on these populations and differentially-expressed genes (DEG) were evaluated.

*$T_{RM}$ cells do not require IRF4-expressing DCs to persist during memory maintenance, nor to mount a $T_{RM}$ cell-restricted type 2 response to HDM*

Having demonstrated early defects in the immune response when DCs lack IRF4, the question remained of whether the impaired memory response observed was solely attributable to these early defects or whether IRF4-expressing DCs played an ongoing role in sustaining the allergic response. We circumvented early defects in T cell education and differentiation by sensitizing and challenging CD45.1(+) WT mice to HDM, isolating CD4(+) T cells from the inflamed lungs, and then parking the T cells in *Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice (Fig. 4-14A). Recruitment of the adoptively transferred cells was assisted by "pulling" with intratracheal instillation of rIL-33, which induces lung expression of chemokines (Tjota et al. 2017). In this previous study, the Th2 chemoattractants CCL17 and CCL22 were among the chemokines induced by IL-33 in the lungs (Table 3-1). Co-administration of IL-33 to the airways during intravenous adoptive transfer had the best ability to pull the HDM-specific $T_{EM}$ cells to the lungs for development into $T_{RM}$ cells compared to i.t. co-administration of these particular chemokines, systemic blockade of CD62L, or direct instillation of the T cells into the airways (Fig. 4-15). Once parked, the mice rested for 4-5 weeks to allow for the contraction of the adoptively transferred effector cells and consolidation into memory maintenance. We found that after 4-5 weeks of rest, recipient mice with IRF4-deficient DCs had fewer $T_{RM}$ cells in their lungs compared to *Irf4*$^{fl/fl}$ controls (Fig. 4-15B). However, this was not due to a difference in donor $T_{RM}$ cells, but a diminished number of host $T_{RM}$ cells (Fig. 4-14B). Polyclonal and tetramer-positive donor $T_{RM}$ cells were present to an equal extent in the lungs of both *Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice, demonstrating that T cells educated by IRF4-expressing DCs were

capable of taking up residence and surviving for many weeks without the continued presence of IRF4-expressing DCs.

The mice were then challenged with HDM during simultaneous FTY720 treatment to restrict the memory response to the lung $T_{RM}$ cells (Fig. 4-14C). In this case, the only T cells that had been previously educated were those that were adoptively transferred. Upon challenge, *Irf4*$^{fl/fl}$CD11cCre mice displayed allergic responses equal in magnitude to those mounted by *Irf4*$^{fl/fl}$ littermates, as they are equally capable of recruiting eosinophils and CD4(+) T cells to the airways (Fig. 4-14D). While there were fewer recipient CD4(+) T cells participating in the response (Fig. 4-14D), mirroring the reduced number of recipient $T_{RM}$ cells at rest (Fig. 4-14C), the donor CD4(+) T cells were fully capable of responding even in mice with IRF4-deficient DCs. During this response, the DCs expressed similar levels of CD86 and OX40L regardless of their ability to express IRF4 (Fig. 4-16). Thus, T cells educated by IRF4-expressing DCs are able to mediate a robust type 2 response even when only IRF4-deficient DCs are present during the recall response. Taken together, our data demonstrate a role for IRF4-expressing DCs in the development of the effector Th2 response but show that they are not necessary for reactivating resting $T_{RM}$ cells that were sufficiently educated during the effector response.

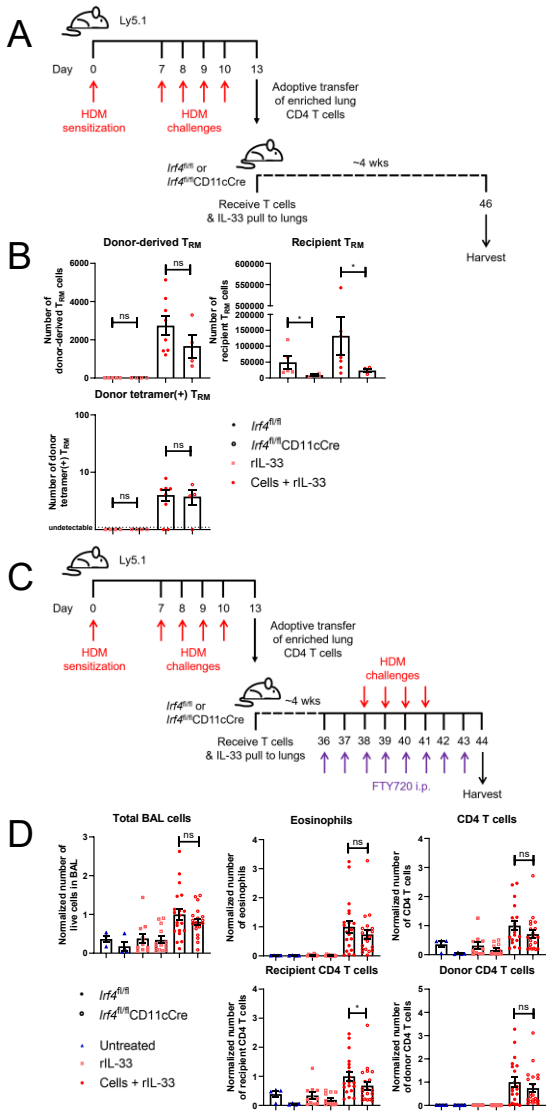


**Figure 4-14.** $T_{RM}$ cells do not require IRF4-expressing DCs to persist during memory maintenance, nor to mount a $T_{RM}$ cell-restricted type 2 response to HDM.

(a) Ly5.1 mice were sensitized and challenged with HDM. Lung $T_{EM}$ cells were adoptively transferred to *Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice and pulled to the lungs with intratracheal rIL-33 in the "Cells + rIL-33" group. Control groups received no cells and either rIL-33 alone or no treatment at all. Lungs were harvested after 4 weeks. (b) Quantified number of donor-derived $T_{RM}$ cells, host $T_{RM}$ cells, and donor-derived tetramer(+) $T_{RM}$ cells. Data represent 1 experiment with $n \geq 4$ mice per group, and statistics (Mann-Whitney test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; ns: not significant). (c) As in (a), but followed by HDM challenges during FTY720 treatment, (d) Number of total cells, eosinophils, CD4(+) T cells, donor CD4(+) T cells, and recipient CD4(+) T cells in the airways normalized to the mean of the *Irf4*$^{fl/fl}$ group receiving donor cells. Data represent 4 combined experiments with $n \geq 4$ mice per group, and statistics (Mann-Whitney test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, $p < 0.05$; ns: not significant).

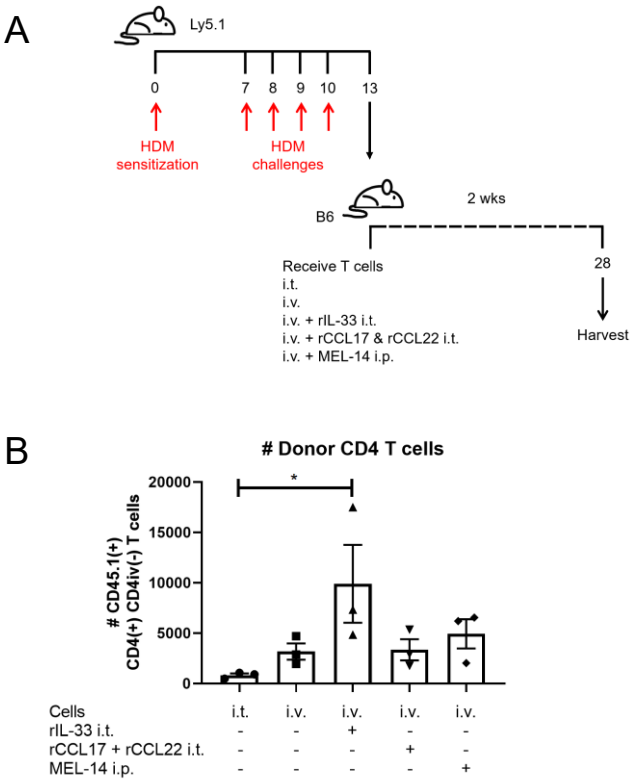


**Figure 4-15.** Comparison of adoptive transfer strategies for HDM-specific lung $T_{EM}$ recruitment to the lungs.

(a) Experimental schematic. (b) Number of donor CD4(+) T cells present in the lungs of recipient mice 2 weeks after adoptive transfer. Data are representative of one experiment with n = 3 mice per group, and statistics (one-way ANOVA with Kruskal-Wallis post-test) were performed in GraphPad Prism. MEL-14 is the clone of antibody used to block CD62L. Bar represents the mean ± SEM (*, $p < 0.05$). ANOVA p value = 0.0106.

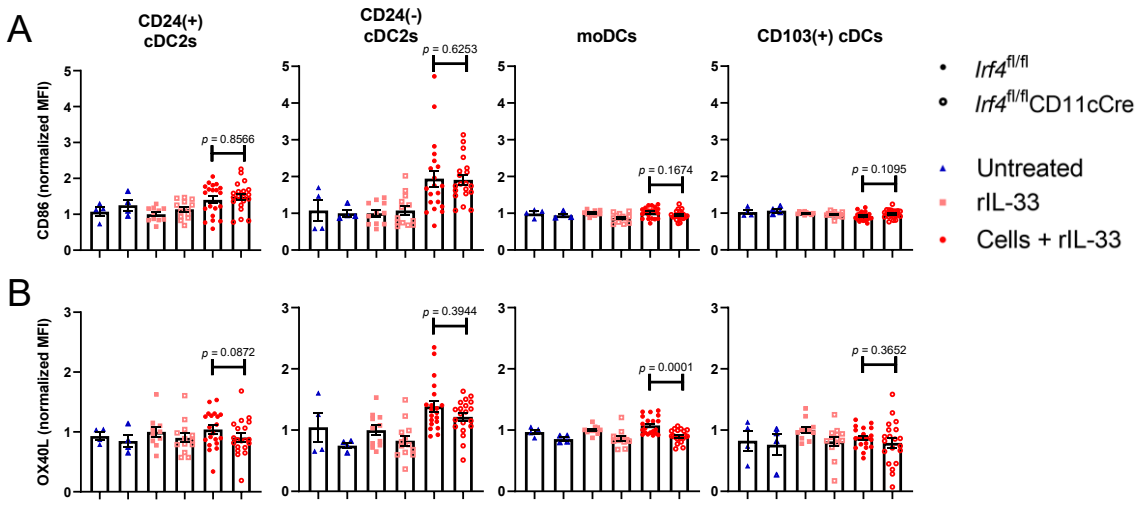


**Figure 4-16.** Lung DCs in naïve recipients of $T_{EM}$ do not display large IRF4-dependent alterations in activation markers during HDM treatment.

Ly5.1 mice were sensitized and challenged with HDM. Lung $T_{EM}$ cells were adoptively transferred to *Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice and pulled to the lungs with intratracheal rIL-33 in the "Cells + rIL-33" group. Control groups received no cells and either rIL-33 alone or no treatment at all. Mice were then treated with HDM while receiving FTY720. Expression of (a) CD86 or (b) OX40L by flow cytometry on all examined lung DC subsets in adoptive transfer rechallenged experiments. Data are combined from 4 experiments for a total of $n \geq 3$ mice per group, and statistics (Mann-Whitney test) were performed in GraphPad Prism. Bar represents the mean ± SEM.

## Discussion

In this study, we find that mature IRF4-expressing CD24(+) cDC2s play numerous crucial roles in the education of naïve T cells in response to HDM in vivo. IRF4-deficient CD24(+) cDC2s display minor defects in their ability to phagocytose inhaled allergens, which they are subsequently capable of processing, but these DCs have a reduced capacity for migration to lymph nodes. Beyond this quantitative deficiency, IRF4-deficient CD24(+) cDC2 display defects in their capacity to prime naïve T cells and deliver additional signals such as IL-10, IL-33, and OX40L during sensitization. Intriguingly, we find that IRF4 need not be expressed by mature DCs during the homing of $Th2_{EM}$ cells to the lungs, their consolidation into $Th2_{RM}$ cells, or their subsequent survival for as long as five weeks when the T cells were previously educated by IRF4-expressing DCs. These $Th2_{RM}$ cells that have been educated by IRF4-expressing DCs are able to orchestrate the infiltration of eosinophils and CD4(+) T cells to the airways despite the absence of IRF4-expressing CD24(+) cDC2s during the recall response.

Our study pinpoints numerous IRF4-dependent functions for mature lung DCs in educating Th2 cells during in vivo sensitization. We were able to study these effects in vivo for the first time because, in contrast to previous mouse models where deletion of IRF4 globally or in the CD11c(+) compartment causes the absence of lung, intestine, lymph nodes, and spleen CD24(+) cDC2s (Bajana et al. 2012, Bajana et al. 2016, Persson et al. 2013, Schlitzer et al. 2013, Zhou et al. 2014), we find that IRF4-deficient CD24(+) cDC2s are present to a normal extent in the lungs and spleen of our mice (Fig. 4-7 and ref. (Williams et al. 2013)). This is because cre-mediated excision of IRF4 is not taking place until after the pre-cDC stage (Williams et al. 2013), allowing unfettered development of the lung and spleen cDCs. While these other models have not been useful for studying lung DCs in vivo, dermal DCs were still capable of developing

independently of IRF4. In fact, there were increased numbers of CD11b(+) DCs, attributed to IRF4-dependent deficiency in CCR7 and thus reduced drainage to the skin-draining LNs (Bajana et al. 2012). In our mouse model, lymph node homing of migratory CD24(+) cDC2s was severely limited in the context of IRF4-deficiency. However, this did not appear to cause an increase in the number of these DCs in the lungs (Fig. 4-7).

It was previously described that IRF4-deficient bone marrow-derived DCs (BMDCs) could have inferior T cell priming capabilities due to defects in the antigen processing and presentation pathway, with IRF4-dependent expression of genes such as *Ctss* (cathepsin S), *H2-Oa*, *H2-DMb2*, *Ciita*, and *Cst3* (Vander Lugt et al. 2014). Our transcriptomic analysis of in vivo stimulated CD24(+) cDC2s did not reveal IRF4-dependent expression of these transcripts, and actually found increased expression of *Ctsd* and *Ctsl* which encode cathepsin D and cathepsin L, upon deletion of IRF4 (data not shown). Further in our study, IRF4-deficient DCs were capable of processing exogenous antigen and, in fact, IRF4-deficient lung DCs were found to have higher levels of antigen processing as measured by BSA-DQ fluorescence (Fig. 4-7). Another study examining splenic cDC1s deficient in BATF3, a lineage-defining transcription factor for cDC1, similarly found that this genetic deletion led to increased OVA-DQ processing (Chandra et al. 2017). This may suggest that the antigen processing pathway is dysregulated when cDC2s lack IRF4 during development, but that cDC2s capable of expressing IRF4 during development process antigens independently of IRF4 once mature. Alternatively, this may highlight a difference between in vitro-generated DC cultures and in vivo tissue-derived DCs.

Our findings demonstrate particular roles for IL-10 and IL-33 in Th2 differentiation, and suggest that OX40L is also regulated by IRF4 in mature DCs throughout the effector and memory responses. A recent report has corroborated the IRF4-dependent production of IL-10 by

lung DCs (Ainsua-Enrich et al. 2019). While other cells may be capable of producing these factors in response to a type 2 inflammatory stimulus, DCs are uniquely capable of migrating to particular microanatomic areas of the tissue-draining LN (Eisenbarth 2019). Their ability to position themselves at the T cell – B cell border, a site for the education of Th2 cells, suggests that they are particularly unique messengers for delivering these signals (Krishnaswamy et al. 2017). This may be especially important for short-range delivery of the cytokine IL-33, which is otherwise entirely bound by the soluble decoy receptor sST2 (Hayakawa et al. 2007, de Kleer et al. 2016) or is inactivated by oxidation (Cohen et al. 2015). Previous studies have indicated that IRF4-expressing DCs play a role in the early education of Th2 cells, Treg cells, and CD8(+) $T_{RM}$ cells (Williams et al. 2013, Vander Lugt et al. 2014, Vander Lugt et al. 2017, Ainsua-Enrich et al. 2019). It was previously demonstrated that HDM generates a pool of CD4(+) $T_{RM}$ cells, but is a less potent inducer of CD8(+) $T_{RM}$ cells (Turner et al. 2018). We find that HDM sensitization promotes allergen-specific CD4(+) $T_{RM}$ cells with both Th2 and Treg subsets, but that IRF4-deficiency in DCs results in a more significant loss of the Th2 subset. This loss of Th2 cells may be a result of diminished downstream mediators of IRF4 such as IL-33, IL-10, and/or OX40L.

We have demonstrated that IRF4 expression by DCs is not necessary for the maintenance and recall response of Th2 cells that were previously educated by IRF4-expressing DCs. In particular, adoptively transferred $T_{EM}$ raised against HDM antigens were capable of homing to the lungs and surviving as memory cells for five weeks in the absence of allergen or IRF4-expressing DCs. Accordingly, in these otherwise naïve recipient mice, levels of CD86 and OX40L on CD24(+) cDC2 subset were not IRF4-dependent, suggesting that IRF4-dependent expression of costimulatory molecules does not emerge until after antigen experience. Upon reintroduction of allergen, these $T_{RM}$ cells were able to mediate a type 2 response without

assistance from circulating cells nor IRF4-expressing DCs. It was previously shown that antigen-specific CD8(+) $T_{RM}$ cells were capable of proliferating in response to an LCMV peptide in the female reproductive tract when CD11c(+) cells were depleted (Beura et al. 2018). These findings leave open the possibilities that other APCs are sufficiently capable of presenting antigen and stimulating appropriately-educated $T_{RM}$ cells, or that these $T_{RM}$ cells are licensed to conduct an allergic recall response in the presence of allergen-triggered cytokines. The latter possibility is consistent with previous investigations pointing to tissue-derived signals such as TSLP, IL-25, and IL-33 as important for licensing of lung Th2 cells (Van Dyken et al. 2016), and in particular the requirement and sufficiency of IL-33 signaling in memory Th2 cells for IL-5-mediated eosinophilic responses (Endo et al. 2015). Indeed, our data indicate that the Der p 1 tetramer-specific T cells display nearly uniform expression of the IL-33 receptor ST2. Although we tracked the T cell response to the immunodominant epitope of HDM, there remain unexamined tetramer negative cells with other HDM-relevant specificities which may follow similar patterns, as nearly all microbe-specific clonal effector populations give rise to their own pool of long-lived memory cells (Tubo et al. 2016). As such, it would be of interest to evaluate whether the ST2(+) T cell compartment harbors the allergen-specific T cells of specificities other than for Der p 1 and whether these cells conduct an allergic response to IL-33 alone.

Our study in particular has evaluated the requirement for *mature* IRF4-expressing DCs during the allergic memory recall response, but loss of IRF4 earlier in development appears to result in the alteration of different DC processes. It is possible that loss of IRF4 earlier in DC development could reveal a differential requirement for IRF4-expressing DCs during the memory response. This would be difficult to examine in vivo as mice that delete IRF4 earlier during CD11c(+) cell development lack lung CD24(+) cDC2s altogether, and inducible

CD11cCre mice exhibit very ineffective recombination (Probst et al. 2003). Thus, further study should focus on manipulation of the identified targets and pathways downstream of IRF4.

Together, these findings demonstrate that IRF4 controls a program in mature CD24(+) cDC2s that governs Th2 priming during sensitization with profound implications for Th2 effector responses during challenge, but that mitigated $T_{RM}$ cell-dependent memory responses when DCs lack IRF4 stem from defects in earlier T cell education.

# Chapter V: IRF4-expressing intestinal DCs contribute to the Th22 response against *Citrobacter rodentium*

## Introduction

Intestinal infection with attaching and effacing bacterial pathogens remains a major global health threat (Gomes et al. 2016). Attaching and effacing pathogens colonize the intestinal mucosa by physically attaching to the epithelium, destroying microvilli, and causing the formation of characteristic actin-rich pedestal structures (Nataro and Kaper 1998, Frankel and Phillips 2008). Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) are agents of human disease that employ these mechanisms. While mice are resistant to infection with these particular species, their natural pathogen *Citrobacter rodentium* is also an attaching and effacing pathogen which shares many common virulence mechanisms and lends itself to experimental study (Collins et al. 2014). Colitis pursuant to murine infection

with *C. rodentium* has also been used for modeling inflammatory bowel disease pathogenesis (Eckmann 2006).

The host response to infection with *C. rodentium* is complex, and CD4(+) T cells are critical for immunity to this pathogen (Simmons et al. 2003, Bry and Brenner 2004). Among the numerous T cell responses induced are γδ T cells, Th1 cells, Th17 cells, and Th22 cells (Collins et al. 2014). The Th17 and Th22 components of this response are considered most protective, though controversy remains regarding the relative importance of their particular contributions. While both IL-17 and IL-22 have been shown to promote the production of antimicrobial peptides, IL-17 promotes a strong neutrophilic response, whereas IL-22 promotes tissue repair by the epithelium (Ishigame et al. 2009, Basu et al. 2012).

Dendritic cells (DCs) are required for protection against *C. rodentium* (Satpathy et al. 2013). The gut contains numerous DC populations including moDCs, cDC1s, and cDC2s. Intestinal cDCs uniformly express high levels of MHC-II, CD11c, and CD103. The IRF4-dependent cDC2s express CD11b, whereas the BATF3-dependent cDC1s do not. Total $IRF4^{-/-}$ mice, in which CD103(+) CD11b(+) DCs are reduced in number, lose weight and succumb to infection (Satpathy et al. 2013). Deletion of IRF4 in the CD11c compartment during the pre-cDC stage mice reduces the small intestine population of CD11b(+) CD103(+) cDC2s by 50% and the number of the migratory CD11b(+) CD103(+) cDCs in the mesenteric LNs by 90%. In these mice, T cells display reduced propensity for IL-17 expression in the small intestine and colon, but regular expression of Foxp3 and IFNγ, during treatment with a Th17 stimulus. A suggested mechanism for how these DCs promote Th17 responses stems from the finding that IRF4-deficient CD11b(+) CD103(+) cDC2s are a poor source of IL-6 compared to their IRF4-expressing counterparts (Persson et al. 2013).

How the response to *C. rodentium* depends on the overall population of IRF4-expressing DCs is not entirely understood, however elimination of the IL-23-producing Notch2-dependent subset of IRF4-dependent DCs is detrimental for survival (Satpathy et al. 2013). In this study, we examined the role of mature IRF4-expressing DCs in a *C. rodentium* model of enterocolitis using mice that do not delete IRF4 until after the pre-cDC stage. While IRF4-expressing DCs were not required for survival during infection, mice with IRF4-expressing DCs cleared infection more rapidly than did mice with IRF4-deficient DCs. This reduced clearance was accompanied by fewer IL-22-producing T cells but an unimpaired capacity to elicit IL-17-producing T cells. Our study suggests that there is an ongoing role for IRF4 in mature DCs during the host response to attaching and effacing pathogens.

## Results and Discussion

### *Survival and weight maintenance during* C. rodentium *infection are not dependent upon IRF4-expressing DCs*

To determine whether there is a role for IRF4 in mature DCs during intestinal infection with *C. rodentium*, we infected *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$CD11cCre mice with 2 x $10^9$ CFU via oral gavage. Survival and weight of the mice were monitored daily. The survival rate was slightly worse for *Irf4*$^{fl/fl}$CD11cCre mice; however, this was not statistically significant (Fig. 5-1A). Further, mice with IRF4-deficient DCs did not display any significant differences in weight maintenance compared to their WT counterparts (Fig. 5-1B). These findings indicate that IRF4-expressing DCs are not required for overt measures of health during infection with *C. rodentium*. Interestingly, total *Irf4*$^{-/-}$ mice have been shown to lose weight and succumb to infection with *C.*

*rodentium* (Satpathy et al. 2013). These data suggest a role for the IRF4 in the response, but it is not possible to determine if IRF4-dependent cDCs play a role since *Irf4*$^{-/-}$ mice exhibit defects in other cells such as T cells and B cells. Among the IRF4-dependent cDC2s reside a KLF4-dependent fraction and a Notch2-dependent ESAM(+) fraction. One study found that Notch2-dependent cDC2s are required for survival and weight maintenance during *C. rodentium* infection, whereas neither KLF4-dependent cDC2s nor BATF3-dependent cDC1s are necessary (Satpathy et al. 2013). Thus, it is interesting that, in *Irf4*$^{fl/fl}$CD11cCre mice where this population is theoretically restored but unable to express IRF4, the mice are able to survive and do not lose weight in response to *C. rodentium*.

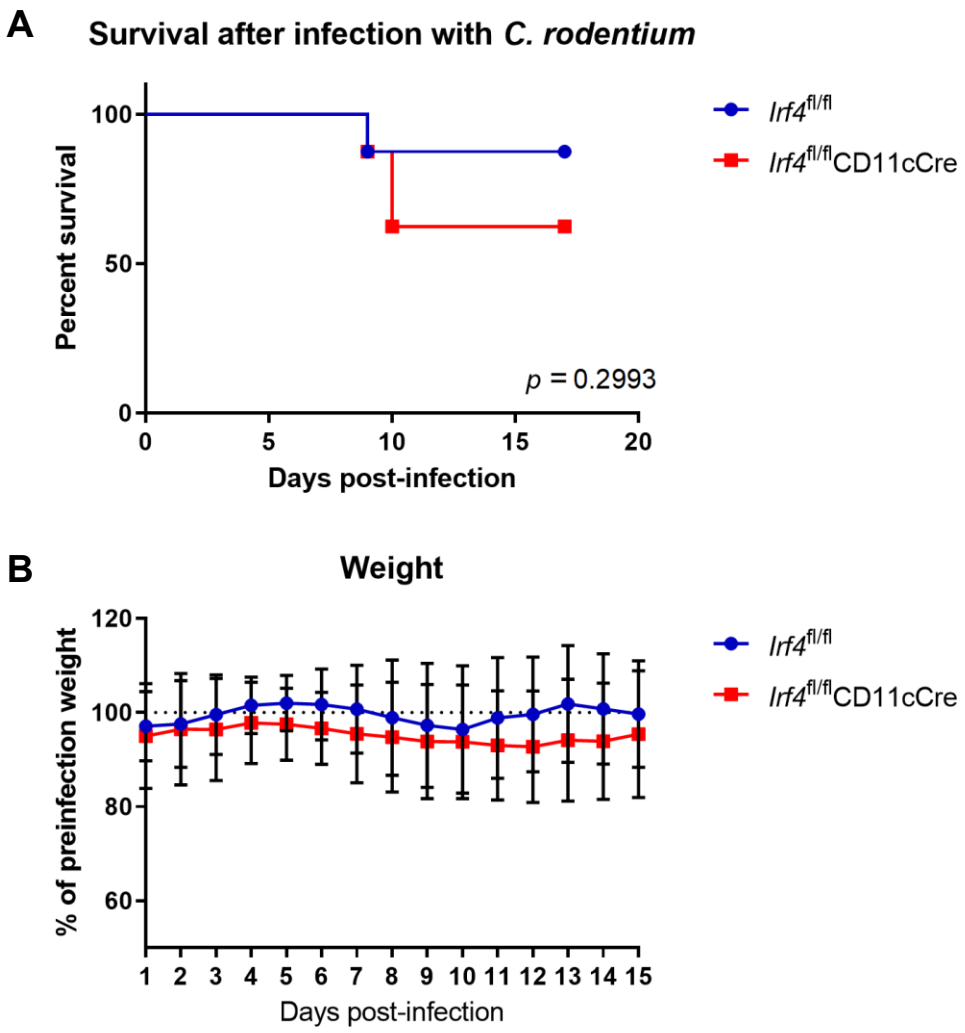


**Figure 5-1.** IRF4-expressing DCs are not required for survival or weight maintenance during infection with *C. rodentium*.

*Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice were infected o.g. with 2 x $10^9$ CFU of *C. rodentium*. (A) Survival was monitored and groups were compared using the Log-rank (Mantel-Cox) test. (B) Weight was monitored. Data represent the mean ± SD. The data represent one experiment with n = 8 mice per group on the day of infection.

*IRF4-expressing DCs are required for efficient clearance of* C. rodentium

During the course *C. rodentium* infection, feces were collected from the *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$CD11cCre mice every third day. *C. rodentium* was cultured from the fecal contents for quantification as a measure of the severity of infection over time. Mice with IRF4-expressing DCs were able to reduce their bacterial load more rapidly, with a significant difference between the groups emerging by day 15 (Fig. 5-2). It is unclear whether this is due to an inability of *Irf4*$^{fl/fl}$CD11cCre mice to clear the pathogen, or if instead the response is delayed. Conducting another time course experiment in which the mice are followed for a longer period of time would address this question.

While subsequent studies should examine additional outcomes such as histopathological score, this finding was an indication that IRF4-expressing DCs may impart an immune advantage apart from survival and weight maintenance, which merited further study.

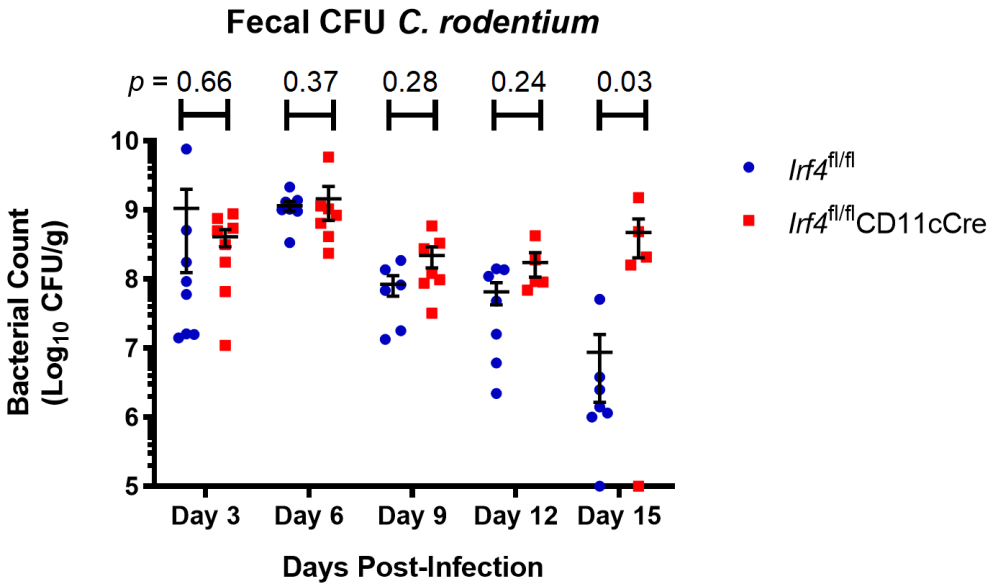


**Figure 5-2.** IRF4-expressing DCs are required for efficient clearance of *C. rodentium.*

*Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice were infected o.g. with 2 x $10^9$ CFU of *C. rodentium*. Feces were collected on the days indicated and CFU were enumerated. Data represent the mean ± SEM, and groups were compared using the Kolmogorov-Smirnov test. The data represent one experiment with n = 8 mice per group on the day of infection.

*Development of a Th22 response, but not a Th17 response, to* C. rodentium *depends on IRF4-expressing DCs*

To begin to address why the IRF4-deficient mice were failing to clear the bacteria at the same rate, we evaluated the T cell response to infection with *C. rodentium*, and whether this response is dependent upon IRF4-expressing DCs. To address this, we harvested the mesenteric lymph nodes (mLNs) on day 17 post-infection and evaluated IL-17 and IL-22 production by the CD4(+) T cells. The number of overall CD4(+) T cells found in the mLN was equal even in the absence of IRF4-expressing DCs, as was the percent of the CD4(+) T cells that were CD44(high), indicating previous activation (Fig. 5-3A). Thus, the overall ability to generate activated T cells is intact when IRF4-expressing DCs are absent in this model.

Examination of the specific type of T cell responses generated revealed no dependence on IRF4-expressing DCs for the generation of IL-17-expressing T cells (Fig. 5-3B). However, the percentage and number of IL-22-expressing CD4(+) T cells were reduced in half in the mice having IRF4-deficient DCs (Fig. 5-3C). For comparison, IL-5-production by CD4(+) T cells was not different between *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$CD11cCre mice (data not shown). These data suggest that IRF4-expressing DCs are important for mounting Th22 responses to intestinal *C. rodentium* infection. Protective effects of IL-22 include acting on intestinal epithelium to upregulate claudin-2, which drives diarrhea and pathogen clearance (Tsai et al. 2017). As there is an early and a late wave of IL-22 production, induced by ILCs and T cells respectively, and since our data indicate that no difference in pathogen clearance emerges until the later phase, this suggests a specific role for IRF4-expressing DCs on the Th22 cells in particular, and not in an interaction involving ILCs (Basu et al. 2012).

Interestingly, it has been shown that the Notch2-dependent DCs are a requisite source of IL-23 during infection with *C. rodentium*, and IL-23 is important for the induction of IL-22 and Reg3γ (Satpathy et al. 2013). Thus, it is possible that IRF4 regulates expression of IL-23 in the Notch2-dependent cDC2 subset, but this must be investigated further. Indeed, the specific mechanisms by which IRF4-expressing DCs mediate pathogen clearance as well as which DC subset within the cDC2 compartment mediates protection remains to be addressed.

In future experiments, it will be important to determine whether there are any defects in intestinal DCs or T cells prior to infection, as this has not yet been evaluated. In a study where T cells from uninfected PBS-treated control mice are compared to those from infected mice, it will be possible to compare baseline DC and T cell populations, to confirm the T helper responses induced by *C. rodentium*, and which of these responses are dependent upon IRF4 expression in mature intestinal DCs. CD4(+) T cells in the colon, where the infection is occurring, should also be examined, in addition to histopathologic evaluation of extent of disease.

Here, we present early evidence that mature IRF4-expressing DCs are involved in the Th22 response to *C. rodentium* infection. While this was not associated with a significant survival advantage, *Irf4*$^{fl/fl}$CD11cCre mice were slower to clear the pathogen compared to *Irf4*$^{fl/fl}$ mice, as measured in the fecal contents. Additional experiments are necessary to further determine whether these findings are indeed causal and to specify the mechanism by which these effects occur.

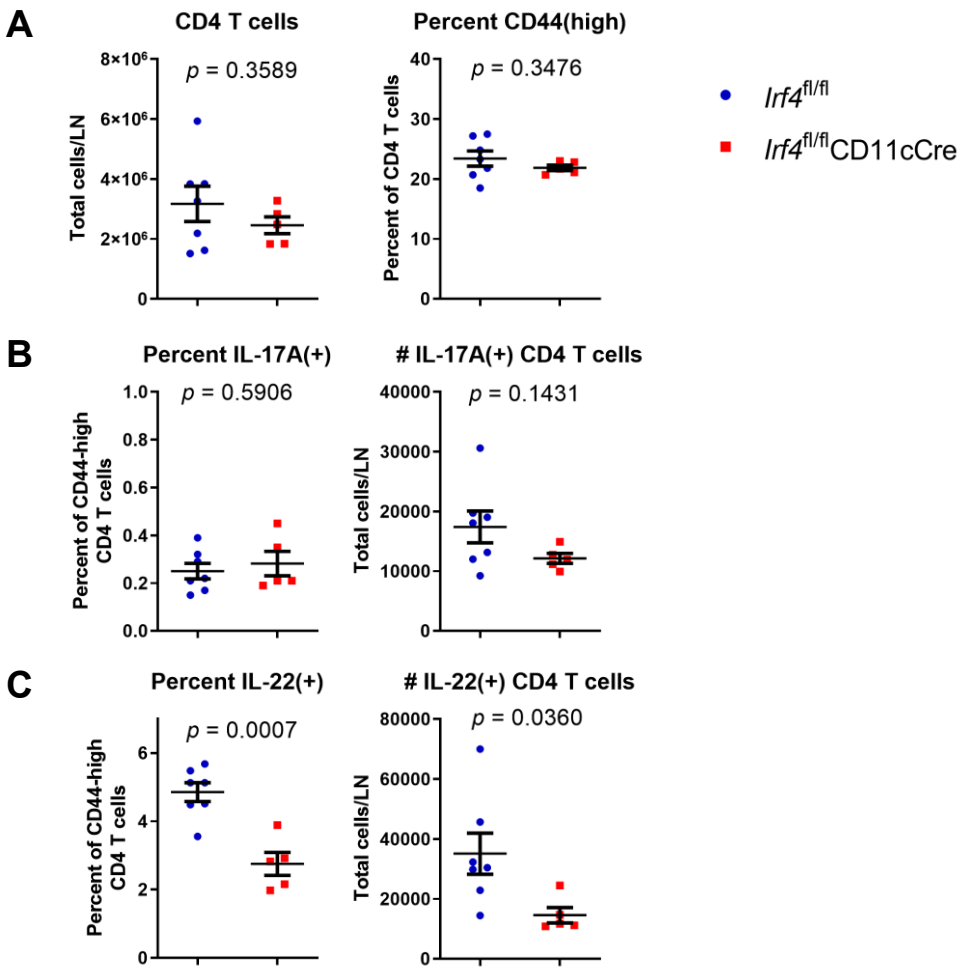


**Figure 5-3.** Development of a Th22 response, but not a Th17 response, to *C. rodentium* depends on IRF4-expressing DCs.

*Irf4*$^{fl/fl}$ or *Irf4*$^{fl/fl}$CD11cCre mice were infected o.g. with 2 x $10^9$ CFU of *C. rodentium*. Mesenteric lymph nodes were collected from the surviving mice on day 17 post-infection, cells were stimulated with PMA and ionomycin, and T cells were analyzed by FACS. (A) Total CD4(+) T cells and percent expressing CD44 are plotted. (B) Percentage of CD44(+) cells expressing IL-17 and the number of IL-17 expressing CD4(+) T cells in the LN. (C) Percentage of CD44(+) cells expressing IL-22 and the number of IL-22-expressing CD4(+) T cells in the LN. Data represent the mean ± SEM, and groups were compared using unpaired t tests. The data represent one experiment with n = 8 mice per group on the day of infection.

# Chapter VI: Discussion

In this work, I have detailed mechanisms by which antigen-presenting cells (APCs) contribute to the development of inflammation at mucosal sites in vivo. In particular, I have established that the Th2-associated cytokine IL-33 upregulates chemokines in the lungs, including monocyte chemoattractants which recruit Ly6C(+) monocytes to the lung interstitium upon the formation of OVA-ICs (Chapter 3). Further, I found that IRF4 has a role in mature DCs during allergic sensitization by promoting DC migration to the lung-draining lymph nodes and supporting the expression of pro-Th2 signals such as IL-33, IL-10, and OX40L for the education of naïve T cells. However, these DCs do not perform ongoing roles during memory responses (Chapter 4). Finally, I demonstrated early evidence that intestinal IRF4-expressing DCs are required for Th22 immunity during infection with *C. rodentium* (Chapter 5). Taken together, these data support the notion that mucosal APCs are crucial mediators between innate and adaptive immune responses and bring novel insight to the mechanisms by which they promote inflammatory responses to a variety of stimuli.

IL-33 is a multifaceted cytokine implicated in type 2 immunity. IL-33 is canonically released from epithelial cells in mice but can be produced by numerous cell types (Martin and Martin 2016). The release of IL-33 activates ST2-expressing cells such as mast cells, DCs, macrophages, ILC2s, and memory lymphocytes to promote type 2 immunity (Martin and Martin 2016). Intracellular IL-33 localizes to the nucleus where it binds chromatin, but there is controversy regarding whether its nuclear activity is involved in chromatin organization and transcriptional regulation (Carriere et al. 2007), or if its ability to bind DNA does not actually regulate transcription (Travers et al. 2018).

Previous studies from our lab have focused on demonstrating the circumstances in which APCs are a source of IL-33. In Chapter 4 of this thesis I focus instead on the effector functions of IL-33 during type 2 inflammatory responses by investigating the role of IL-33 in recruiting inflammatory cells to sites of allergen exposure. An early study suggested that IL-33 acts directly as a chemokine in vitro for the recruitment of human Th2 cells expressing ST2 (Komai-Koma et al. 2007). In vivo experiments in the same study demonstrated that footpad injection of IL-33 recruits adoptively transferred ST2(+) Th2 cells. However, there was also some degree of recruitment of $ST2^{-/-}$ cells, suggesting that IL-33 may also recruit inflammatory cells by an indirect mechanism such as via induction of chemokines. This hypothesis has since been supported by studies conducted in neuronal tissues, in which stress or damage to either retinal cells or to the spinal column resulted in IL-33-dependent chemokine production for the recruitment of monocytes and other inflammatory cells (Gadani et al. 2015, Xi et al. 2016). In Chapter 3, I demonstrated an analogous phenomenon in the lungs, in which administration of IL-33 to the airways induces monocyte chemoattractants and monocyte migration to the lung interstitium. The chemokines induced also had the capability to recruit other cells involved in

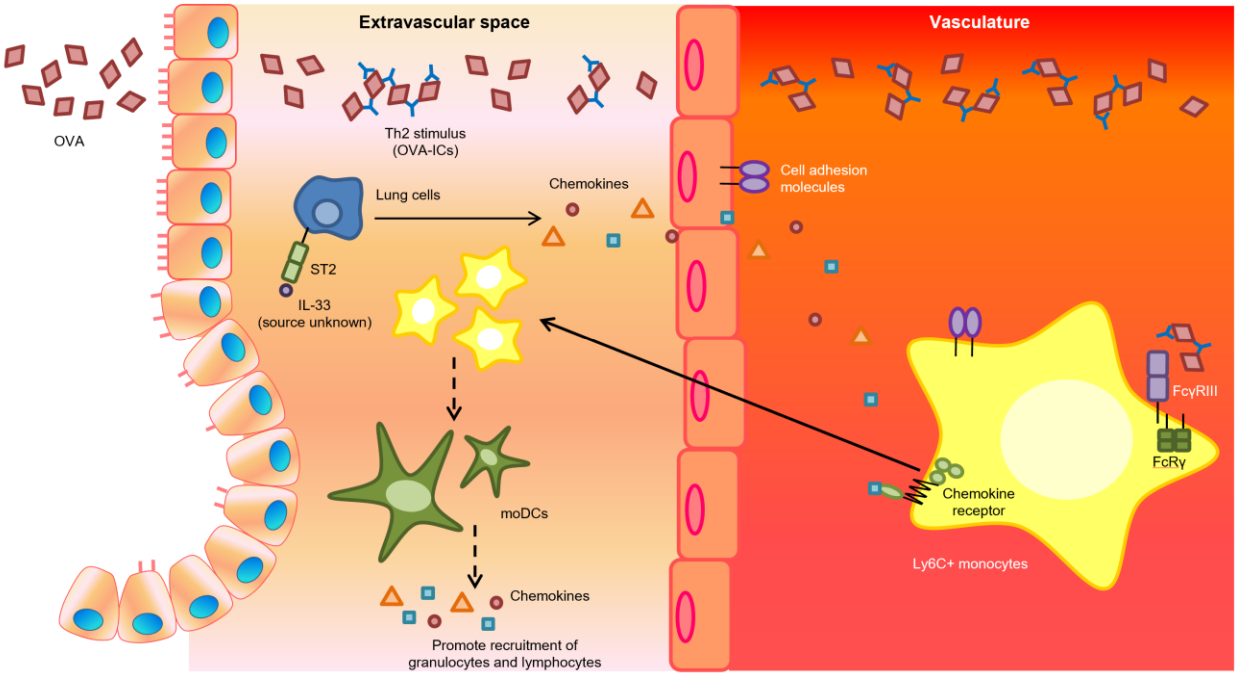


**Figure 6-1.** IL-33 drives monocyte recruitment to the lung extravascular space through chemokine upregulation.

αOVA antibodies in the vasculature and interstitium form OVA-IC when mice are given OVA intratracheally. OVA-IC formation in the lungs leads to FcγRIII signaling and promotes uptake of allergen by Ly6C(+) monocytes, and also leads to the production of IL-33 in the lungs. IL-33 induces the expression of monocyte chemoattractants, among other chemokines, which recruit monocytes to the lung extravascular space as quickly as three hours following challenge. These tissue monocytes may then mediate certain aspects of the allergic response, such as tissue eosinophilia, following allergen challenge.

type 2 inflammation, such as eosinophils (eotaxins) and Th2 cells (CCL17, CCL22). As OVA-ICs induce IL-33 upon their formation, this supports a model by which IL-33 indirectly promotes inflammatory responses in addition to its direct effects on ST2-expressing cells (Fig. 6-1).

Recent studies have enumerated various modification processes that act upon IL-33 in the extracellular compartment to render it inactive. For example, IL-33 may experience oxidation (Cohen et al. 2015), binding by the soluble receptor sST2 (Hayakawa et al. 2007), or additionally proteolytic cleavage by caspase-1 (Cayrol and Girard 2009), mast cell chymase (Waern et al. 2013), or neutrophil proteinase 3 (Bae et al. 2012). These findings support an emerging hypothesis that the activity of IL-33 must occur primarily at short distances, in which case induction of chemokines with effects of longer duration may be a critical role for IL-33 to promote inflammation.

The best approach I tested for the recruitment of Th2 effector cells to the lungs was intravenous injection of the cells followed by intratracheal instillation of rIL-33. In Chapter 3, I showed that intratracheal instillation of rIL-33 induces CCL17 and CCL22 production, two chemokines that mediate signaling through CCR4, which is implicated in both Th2 responses and lung homing (Morimoto et al. 2005, Mikhak, Strassner, and Luster 2013). As CCR4 is expressed by Th2 cells in the setting of allergy to HDM (Cates et al. 2004), it is interesting that rIL-33 recruits these cells more strongly than rCCL17 and rCCL22. This raised the possibility that the endogenous induction of these chemokines by rIL-33 is more potent than exogenous instillation of chemokines into the airways. It has been shown that glycosaminoglycan (GAG) binding is essential for the in vivo activity of certain chemokines, via a mechanism that promotes chemokine oligomerization (Proudfoot et al. 2003). CCL17 is capable of dimerization (Wang et al. 2013), and thus its activity may benefit from GAG binding, however whether CCL22

dimerizes is unclear. While monomeric chemokines have high affinity for their receptors and dimerization is not required for binding, it is thought that during in vivo conditions involving fluid flow, monomeric chemokines may otherwise be washed away and diluted, preventing a chemokine gradient from forming and impeding their chemotactic effects. In contrast, when chemokines are bound to the endothelium on GAGs, gradients can be formed by their presentation or by their concentration in chemokine clouds (Graham, Handel, and Proudfoot 2019). Thus, these processes may constitute important effects of IL-33 apart from those that are the direct result of ST2 signaling in target cells. There may also be synergy between the chemokines induced by IL-33 to organize a chemokine milieu, in that chemotaxis driven by a particular chemokine receptor can be enhanced by the presence of seemingly unrelated chemokines or cytokines (Sebastiani et al. 2005, Suzukawa et al. 2008). Thus, cells may migrate based on integrating complex signals that are not simply additive. Investigating how networks of chemokine synergy operate may be important for understanding cell migration or for therapeutic targeting.

As the "prime and pull" strategy potently attracts CXCR3-expressing Th1 cells to chemokine-treated tissue (Shin and Iwasaki 2012), it seems that CD8(+) T cell- and Th1 cell-attracting chemokines CXCL9 and CXCL10 do not need to be presented on GAGs to function. This is important to consider when implementing prime and pull strategies in other contexts, whether experimentally or therapeutically. For example, in the context of asthma or allergy immunotherapy it may be desirable to pull Tregs to the relevant mucosal site, whereas promoting immune responses to helminths may require recruitment of Th2 cells. Efficient pulling requires an understanding of which chemokine receptors are expressed by the intended leukocyte

population as well as an understanding of how those specific chemokines function in a physiologic context.

Following our studies, it remains unclear which lung cells produce chemokines in response to IL-33. Directly responding cells may include ILC2s, mast cells, eosinophils, or basophils. For example, one study found that human basophils stimulated in vitro with IL-33 were capable of producing MCP-1, MIP-1α and MIP-1, among other cytokines (Smithgall et al. 2008). To understand which cells respond to IL-33 by producing chemokines, it will be necessary to sort-purify many populations of lung cells from IL-33-treated or vehicle-treated lungs and examine chemokine production by these various cells. Alternatively, this matter could be addressed by single cell RNAseq.

Previous studies from our lab demonstrated that two type 2 immune stimuli, OVA-ICs and the common allergen HDM, activate APCs by signaling through two different FcRγ-associated receptors that upregulate IRF4 among other factors such as IL-33 and IL-10 (Williams et al. 2013, Tjota et al. 2014). Further, both stimuli promote IL-33-dependent type 2 inflammatory responses in mice. Importantly, we found that IRF4 regulates DC expression of IL-33 and IL-10 during responses to these stimuli. A prominent role for IRF4 in DC development had been well-appreciated, as deletion of IRF4 during the pre-cDC stage results in the absence of cDC2s in mice (Bajana et al. 2016), but the upregulation of IRF4 in response to stimulation with allergen suggested that IRF4 continues to play an active role in mature DCs during allergic responses. In fact, deletion of IRF4 after the pre-cDC stage demonstrates that the mere presence of cDC2s is not sufficient for type 2 inflammation; mature cDC2s must be able to express IRF4 to orchestrate type 2 allergic lung responses (Williams et al. 2013). However, prior to my

studies, the IRF4-dependent processes performed by lung DCs during the in vivo response to HDM were not known.

Our strain of *Irf4*$^{fl/fl}$CD11cCre mice may be referred to as "late-deleting," as IRF4 is not deleted until after the pre-cDC stage (Williams et al. 2013). The strain more commonly used by other investigators can be called "early-deleting," since IRF4 is deleted once CD11c expression begins in the pre-cDC stage (Ainsua-Enrich et al. 2019). The discrepancy in early- versus late-deleting mice is due to differences in the CD11cCre mouse strains; the early-deleting strain was based on a bacterial artificial chromosome (BAC) (Caton, Smith-Raska, and Reizis 2007), while the late-deleting strain possesses a transgene expressing Cre after CD11c is highly upregulated during development. One study compared these two CD11cCre strains crossed to *Irf8*$^{fl/fl}$ mice to demonstrate that IRF8 is required in both the development of cDC1s and for their continued survival (Sichien et al. 2016). Direct comparison of these two CD11cCre strains crossed to *Irf4*$^{fl/fl}$ mice has the potential to provide new insight for tissue-specific requirements of IRF4 in DC development. In particular, it is unclear why early-deleting mice do not have lung or spleen cDC2s (Bajana et al. 2016), whereas skin DC populations remain intact (Bajana et al. 2012). In contrast, later deletion of IRF4 restores normal lung and spleen DC populations (Williams et al. 2013). This suggests that skin DCs could be less reliant on IRF4 for development, but what special role there may be for IRF4 in DCs developing in other tissues is unclear. A comparison of late-deleting versus early-deleting mouse models may give insight to special requirements for the development of lung cDC2s. One study using early-deleting mice demonstrates that the lung CD24(+) cDC2 subset is ablated in these mice (Bajana et al. 2016). In that study, there appears to be a compensatory increase in CD24(-) cDC2s, which others suggest should also be IRF4-dependent (Tussiwand et al. 2015). However, the authors fail to explicitly account for moDCs,

which would fall into that gate and may account for the residual appearance of IRF4-independent cDC2s.

Early-deleting mice had been used to examine roles for IRF4 in skin DCs, with the major finding that DCs present in the skin but unable to express IRF4 were deficient in CCR7 and unable to migrate to the skin-draining lymph nodes (Bajana et al. 2012). Similarly, we found that lung DCs unable to express IRF4 had reduced CCR7 expression and impaired migration to the lung-draining lymph nodes. We suspected that there were additional IRF4-dependent processes contributing to Th2 skewing, as we had previously published that IRF4-deficient BMDCs have defects in promoting Th2 skewing in vitro, in conditions where they were equal in number to their IRF4-expressing counterparts and where long-scale migration was not necessary (Williams et al. 2013). Indeed, ex vivo lung CD24(+) cDC2s were less potent at priming naïve T cells and expressed less OX40L, IL-33, and IL-10 (Chapter 4, Fig. 6-2). The previous understanding of the role for IRF4 in DCs, apart from in migration, had been limited to studies of IRF4-deficient BMDCs. In these BMDCs, antigen processing and presentation appears to be impaired without IRF4 (Vander Lugt et al. 2014). However, my in vivo studies do not replicate these findings. I find that antigen processing is intact (Fig. 4-7), and antigen presentation is also intact as $T_{FH}$ responses are unimpaired (Williams et al. 2013). Further analysis of the RNAseq dataset will allow for a more in-depth evaluation of the genes associated with these pathways. The discrepancy between my finding and others may be due to effects specific to the lung tissue, artifacts of BMDC cultures, or responses dependent upon the stimulus.

Astonishingly, we found that IRF4-expressing DCs were dispensable during the memory response in a setting where T cells were previously educated by IRF4-expressing DCs during sensitization and challenge. This begins to challenge the notion MHC-II-expressing APCs are

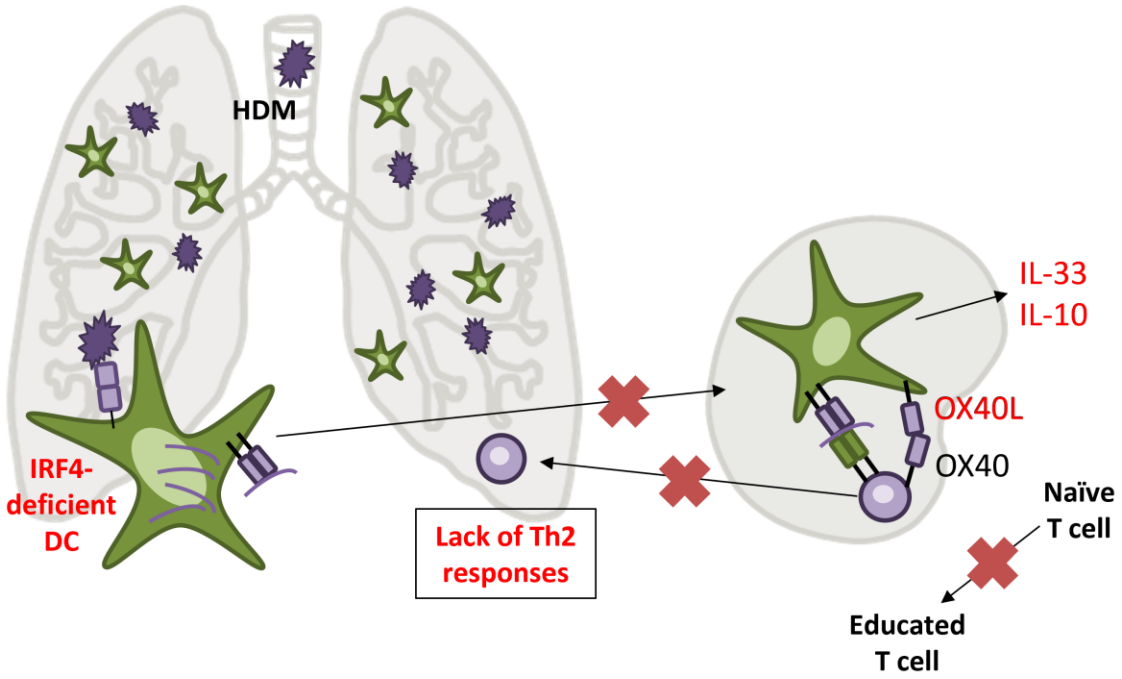


**Figure 6-2.** IRF4 controls a Th2-promoting program in CD24(+) cDC2s which includes the regulation of migration as well as the expression of IL-33, IL-10, and OX40L.

HDM is an inhaled allergen phagocytosed, processed, and presented primarily by lung CD24(+) cDC2s in a manner largely independent of IRF4. However, IRF4 controls the migration of CD24(+) cDC2s to the lung-draining lymph nodes as well as their production of IL-33, IL-10, and OX40L, all of which are implicated in Th2 differentiation.

actively required for the maintenance of memory cells (Schuijs, Hammad, and Lambrecht 2019) and raises the question of how memory cells are activated during recall responses. For example, it is possible that once IRF4-expressing DCs generate full-fledged $T_{RM}$ cells, antigen presentation from any source may be sufficient for their reactivation. Alternatively, $T_{RM}$ cells may perhaps be activated by cytokines. As I observed that antigen-specific $T_{RM}$ cells express ST2, it is possible that memory Th2 cells can be activated by IL-33 in a manner independent of antigen-specificity.

A role for IL-33-mediated enhancement of the Th2 effector response is evident *during* antigen recognition. For example, in vitro-generated murine Th2 cells restimulated with αCD3 and αCD28 display enhanced production of Th2 cytokines when IL-33 is also added to the culture, but these in vitro experiments did not address the role of IL-33 on memory Th2 cells (Schmitz et al. 2005). Similarly, when human Th2 cells are restimulated with αCD3 and αCD28, IL-33 enhances production of Th2 cytokines (Pecaric-Petkovic et al. 2009).

There is less evidence regarding whether IL-33 alone can provoke memory Th2 responses. One in vitro study demonstrated IL-33-dependent enhancement of Th2 cytokines in response to HDM by human HDM-specific Th2 cells. Interestingly, IL-33 had a lesser effect without TCR stimulation (Smithgall et al. 2008). In an in vivo model of helminth infection in which ST2(+) $T_{RM}$ cells persist after clearance of the pathogen, $T_{RM}$ cells produced IL-5 and IL-13 following ex vivo stimulation with IL-33 in combination with IL-7. IL-33 alone displayed similar results, but without reaching significance (Steinfelder et al. 2017). A future experiment to address whether cytokines are sufficient to reactivate $T_{RM}$ cells recognizing HDM would be to conduct memory adoptive transfer experiments as in Chapter 4, but to administer rIL-33 to the lungs instead of performing recall challenges with HDM. Then tetramer(+) T cells would be evaluated for their ability to respond to stimulation with rIL-33. In such an experiment,

attributing inflammation to $T_{RM}$ cell activation would be fraught, due to the contribution of ILC2s. To address this, it would be necessary to perform the same experiment in $ST2^{-/-}$ hosts after having adoptively transferred WT $T_{EM}$, so that the WT $T_{RM}$ cells are the only cells able to respond to IL-33.

While there appears to be an influential role for IL-33 in amplifying Th2 responses, we also demonstrate an interesting interaction with IL-10. Other studies have also proposed a role for IL-10 in Th2 responses. For example, Peyer's patch DCs secrete IL-10 which is necessary for differentiation of Th2 cells (Iwasaki and Kelsall 1999). In another study, in which CD103(+) DCs were found to be the important drivers of Th2 responses among DCs, IL-10-deficient CD103(+) DCs were less capable of promoting Th2 differentiation (Nakano et al. 2012). This indicates that IL-10 is an important signal for developing Th2 cells. Indeed, previous studies from our lab have indicated that IL-10 and IL-33 from DCs are both essential for Th2 differentiation (Bandukwala et al. 2007, Williams et al. 2013). IRF4 and its binding partner PU.1 co-bind several regions in the *Il33* and *Il10* loci, and accordingly, IRF4-deficient BMDCs fail to upregulate IL-33 and IL-10 in response to HDM stimulation (Williams et al. 2013). Reintroduction of IL-33 and IL-10 to cultures in which IRF4-deficient BMDCs were used to differentiate OTII cells restored Th2 differentiation (Williams et al. 2013). In this setting, IL-33 and IL-10 were allowed to act on both the DCs and the T cells during their interaction. In my experiments, a corresponding add-back was performed in vivo, but as the cytokines were delivered intratracheally during sensitization, these cytokines were only allowed to exert their effects during a brief window of time and in a manner spatially restricted to the lungs. Thus, one potential caveat with this add-back experiment is that IL-33 and IL-10 were used to supplement DC activation in addition to HDM, but that these cytokines did not have the opportunity to

directly affect T cells in the lymph nodes. In fact, previous reports indicate that at least IL-33 has autocrine, activating effects on DCs which can promote Th2 responses (Besnard et al. 2011, Su et al. 2013). These effects may explain my finding that IL-33 and IL-10 cotreatment restored capacity for priming T cells that can produce the Th2 cytokines IL-4 and IL-5, but interestingly not IL-13. In addition, my study revealed that recruitment of CD4(+) T cells and eosinophils to the airways was not restored by add-back of IL-33 and IL-10, but it is unclear whether this was related to lack of restoration of IL-13, or due to other unmeasured factors that influence homing of these cells to the lung. It will be difficult to evaluate how IL-33 and IL-10 are mediating Th2 skewing and whether these effects occur directly on T cells or DCs. Ex vivo culture of lung DCs or T cells with these cytokines may reveal some phenotypic changes, but these may not accurately or entirely reflect what is going on in vivo. However, in vivo investigation of this question is complex. One approach is to examine LdLN DCs during sensitization in *Il1rl1*$^{fl/fl}$CD11cCre, *Il10ra*$^{fl/fl}$CD11cCre, or *Il1rl1*$^{fl/fl}$*Il10ra*$^{fl/fl}$CD11cCre mice to understand the role of these cytokine signals on the DCs. Similarly, evaluating T cell education during sensitization in *Il1rl1*$^{fl/fl}$LckCre, *Il10ra*$^{fl/fl}$LckCre, or *Il1rl1*$^{fl/fl}$*Il10ra*$^{fl/fl}$LckCre mice may reveal the role of IL-33, IL-10, or both cytokines in combination directly on T cells.

Because DCs perform numerous crucial roles in initiating T cell responses, and as we have demonstrated that IRF4 regulates multiple aspects, it is unlikely that any single IRF4-dependent factor is responsible for driving the allergic response. Instead, IRF4 orchestrates a Th2-promoting program, likely with multiple redundancies, which culminate in the allergic response. For example, experiments performed in our laboratory in which mice lacked DC-derived IL-33 showed that DC-derived IL-33 was not necessary for type 2 inflammation during the effector response to HDM, nor for T cell expression of IL-4, IL-5, or IL-13 (data not shown).

This was despite the fact that the CD24(+) cDC2s reporting IL-33 promoter usage from a GFP IRES expressed reduced OX40L. This suggests that IL-33 from other cellular sources is sufficient for Th2 induction.

One major question in the field pertains to the extent to which there are pre-existing DC subsets specialized for the specification of particular T helper responses, in contrast to the possibility that DCs retain a greater degree of multipotency whereby they integrate multiple signals and use that information to direct various responses. Initially, the latter was the prevailing hypothesis (Vroman, van den Blink, and Kool 2015). While this is a complex question that is difficult to address, several of our findings support the emerging fixed-subset hypothesis, which has gained traction as more DC subsets have been reliably defined. For example, I show that CD24(-) cDC2s are inferior at priming naïve T cells when stimulated with a Th2 stimulus, whereas CD24(+) cDC2s are adept at this task. As it has been suggested that the CD24(-) cDC2s promote Th17 responses (Tussiwand et al. 2015), using a Th17 stimulus in the lungs may reveal that the CD24(-) cDC2 subset is potent at priming naïve T cells in that context. Also, of the APC subsets studied in the LdLN, the greatest proportion of CD24(+) cDC2s contained HDM, suggesting that these cells are most adept at obtaining and delivering this Th2 stimulus to the LdLN. Additional analyses to address this question could make use of our RNAseq dataset described in Chapter 4, in which we sequenced transcripts from both CD24(+) cDC2s and CD24(-) cDC2s derived from mice recently sensitized to HDM or PBS control. In this experiment, HDM caused greater changes in gene expression in the CD24(+) cDC2 subset compared to the CD24(-) cDC2 subset (data not shown). This indicates that the CD24(-) cDC2 subset is ill-equipped to detect and respond to HDM, but further analysis is required to define these differences.

Strong support for distinct, pre-existing DC subsets specialized for the education of particular T helper responses will depend on clear surface markers to define these populations, and those DCs would depend upon one or more specific master transcription factors driving specialized programs. For example, each subset might express a distinct collection of C-type lectins or other PRRs for their activation. While these data are emerging, there remain contradictions in the literature regarding the division of labor among lung DCs. For example, KLF4-dependent lung CD24(+) cDC2s are essential for responses to HDM, which is considered primarily a type 2 stimulus (Tussiwand et al. 2015). Because KLF4-dependent DCs were not essential for *C. rodentium* responses in the same study, and Notch2-dependent DCs were, it was suggested that the Notch2-dependent subset of intestinal DCs is critical for Th17 responses. Confusingly, another study concluded that the CD24(+) cDC2 subset controlled Th17 responses to *Aspergillus fumigatus* in the lungs (Schlitzer et al. 2013). However, this study used early-deleting *Irf4*$^{fl/fl}$CD11cCre mice and failed to track whether the CD24(-) cDC2 population was also absent from the lungs of these mice. Furthermore, there is controversy regarding whether *C. rodentium* is truly a Th17 stimulus, as Th22 responses are suggested to be more protective during the response. Indeed, in Chapter 5, we presented early evidence that IRF4-expressing DCs are necessary for Th22 responses to *C. rodentium* but not T cell production of IL-17. Thus, answering the question of whether DC subsets are specialized for the promotion of specific T helper responses requires a detailed understanding of which DC subsets are affected in particular tissues in the mouse model at hand, as well as a careful evaluation of which T helper subsets are elicited in response to the stimulus.

To this end, questions remain regarding the study conducted in Chapter 5. For example, addition of uninfected control groups of *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$ CD11cCre mice will allow for the

evaluation of steady state differences dependent on IRF4-expressing DCs, as well as the particular T cell responses induced by *C. rodentium* infection in *Irf4*$^{fl/fl}$ mice. Special care will be required to confirm that a normal DC compartment is present among gut DCs in *Irf4*$^{fl/fl}$CD11cCre mice. While our studies focusing on the lungs demonstrated that defects in lung DCs present in early-deleting *Irf4*$^{fl/fl}$CD11cCre mice are corrected in our late-deleting *Irf4*$^{fl/fl}$CD11cCre mice, we have not characterized whether intestinal DC populations are normal in these late-deleting *Irf4*$^{fl/fl}$CD11cCre mice. However, our mice do not experience increased mortality when infected with *C. rodentium*, suggesting that DC defects in our model are less dramatic. Even so, it is likely that there is a defect in DC migration from the gut to the mLNs, as we have found to be the case for lung DCs, but this must be confirmed in future studies. Overall, these studies implicate IRF4-expressing DCs as playing essential roles in responses to various insults across tissues.

## Conclusion

Tissue APCs are a heterogeneous population of cells that are potently capable of mediating diverse T helper responses. In settings such as allergy that invoke type 2 inflammatory responses, APCs convert biological information regarding the allergic insult and contextual signals such as IL-33 to initiate an adaptive immune response centered around the promotion of Th2 cells. In this thesis, I find an important role for IL-33 in directing allergic responses by promoting the expression of chemokines associated with type 2 inflammatory responses. Further, I show in vivo for the first time that IRF4 is required in mature lung DCs for processes integral to allergic sensitization, such as migration and expression of pro-Th2 factors. However essential IRF4-expressing lung DCs are during the development of allergic responses, I demonstrate that

they are dispensable during the memory response, lending new insight to how activation of memory T cells differs from activation of effector T cells. Finally, I suggest that IRF4-expressing DCs are also necessary for Th22-dependent clearance of intestinal pathogens. In total, these studies contribute novel mechanistic understanding regarding the recruitment, activation, and function of APCs during responses to pulmonary and intestinal insults, which may one day be harnessed for therapeutic purposes.

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