Expression of the transcription factor Interferon Regulatory Factor 4 (IRF4) is required for the development of lung conventional dendritic cells type 2 (cDC2s) that elicit Th2 responses, yet how IRF4 functions in lung cDC2s throughout the acute and memory allergic response is not clear. Here, we use a novel mouse model that loses IRF4 expression after lung cDC2 development to 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. IRF4-deficient DCs demonstrate defects in their migration to the draining lymph node and in T cell priming. Finally, T cells primed by IRF4-competent DCs mediate potent memory responses independently of IRF4-expressing DCs, demonstrating that IRF4-expressing DCs are not necessary 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 tissue resident-memory T cell (T_{RM cell})-dependent memory responses.
IRF4 expression by lung dendritic cells drives acute but not T<sub>Rm</sub>-dependent memory Th2 responses

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Abstract
Expression of the transcription factor Interferon Regulatory Factor 4 (IRF4) is required for the development of lung conventional dendritic cells type 2 (cDC2s) that elicit Th2 responses, yet how IRF4 functions in lung cDC2s throughout the acute and memory allergic response is not clear. Here, we use a novel mouse model that loses IRF4 expression after lung cDC2 development to 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. IRF4-deficient DCs demonstrate defects in their migration to the draining lymph node and in T cell priming. Finally, T cells primed by IRF4-competent DCs mediate potent memory responses independently of IRF4-expressing DCs, demonstrating that IRF4-expressing DCs are not necessary 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 tissue resident-memory T cell (T<sub>Rm cell</sub>)-dependent memory responses.

Keywords: dendritic cells; asthma; Th2 responses
Introduction

Tissue-resident memory T cells (TRM cells) mediate inflammatory responses in various non-lymphoid tissues, but questions remain regarding the mechanisms underpinning the contribution of CD4 TRM cells to allergic airway disease. Like circulating memory T cells, TRM cells develop in response to initial antigen exposure at mucosal surfaces and persist beyond the contraction phase of the immune response. However, TRM cells maintain proximity to barrier tissues, poised to respond rapidly upon subsequent antigen exposure (1). TRM cells can contribute to a “first-line” response until additional cells are recruited from the circulation and are often sufficient to control invading pathogens (2). However, how CD4 TRM cells are activated within the lung milieu and the role of DCs in CD4 TRM cell activation is not known. In settings where T cell responses are pathogenic, such as in allergic asthma, these sentinel TRM cells may serve as central mediators of disease. Thus, investigating how TRM cells are generated, maintained, and restimulated is critical to understanding chronic allergic lung disease.

Many questions remain unanswered regarding how long-lived TRM cells are maintained and how they are reactivated during subsequent responses, particularly with regard to CD4 TRM cells. While the pool of CD8 TRM cells remains stable in the skin for up to 200 days after its establishment (3-6), lung CD8 TRM cells decline more rapidly and rely on replenishment from the circulating memory population or reintroduction of the inflammatory stimulus (6-9). Evidence regarding the duration of CD4 TRM cell maintenance is limited, but one study showed that maintenance of vaginal CD4 TRM cells against HSV2 was shorter than that of CD8 TRM cells (10). Two studies have demonstrated declining allergen-specific CD4 TRM cells in the lungs in the 70- to 90-day period following allergen challenge (11, 12). IL-2, IL-4, and IL-7 are implicated in the development and maintenance of lung CD4 TRM cells in type 2 immunity during this time (11-13), but the source of these cytokines and whether these signals are sufficient remains to be elucidated.
Apart from requiring antigen presentation from a specific DC subset, it is possible that \( T_{RM} \) cells are fully licensed to respond upon antigen presentation from any source, or that \( T_{RM} \) cells may be more reliant on innate or cytokine signals to prompt a response (14, 15). As in all mucosal tissues, numerous DC populations exist in the lungs. 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 Basic Leucine Zipper ATF-Like Transcription Factor 3 (BATF3) and Interferon Regulatory Factor 8 (IRF8), are proficient at cross-presentation as well as priming Th1 and specific Treg responses (16-19). Roles for cDC2, which are dependent upon Interferon Regulatory Factor 4 (IRF4) for development, include promoting Th2, Th17, and specific Treg responses (20-29). The Th2-promoting IRF4-expressing cDC2 subset is also dependent on the transcription factor Kruppel-like Factor 4 (KLF4) and expresses CD24, while the Th17-promoting cDC2 subset is dependent on Notch2 and lacks CD24 (ref. (24) and Fig. S1). When mice lack IRF4 entirely or lack IRF4 during the CD11c\(^{int}\) pre-cDC stage, they fail to develop cDC2 populations in the lungs, lung-draining lymph nodes, small intestine, mesenteric lymph nodes, and spleen (25, 27, 30, 31). Because of systemic failure of cDC2 development in the absence of IRF4, it has been challenging 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 bone marrow-derived DCs (BMDCs). To address this problem, we have developed a mouse model using a CD11c-Cre strain that does not express Cre until after the pre-DC stage, thereby only deleting IRF4 in mature DCs. Thus, these mice have normally-developed cDC2s, but the mature cDC2s no longer express IRF4 (20). This mouse model has enabled us to isolate IRF4-dependent DC functions during immune responses in vivo.

Whether there is a role for tissue DCs in maintaining or reactivating CD4 \( T_{RM} \) 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\textsubscript{RM} cells can mediate a response in the absence of DCs (32). However, another vaginal infection model in which CD8 T\textsubscript{RM} cells were generated using the prime and pull technique, demonstrated that mice without MHC-I-expressing CD301b(+) cDC2s in the vaginal lamina propria were much more susceptible to infection compared to mice possessing this DC subset (33). One study focusing on lung CD4 T\textsubscript{RM} cells demonstrated that the proportion of lung cDC2s increases during an allergic memory response, with more DCs expressing CD86 (34). 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\textsubscript{RM} cell responses is needed.

This study addresses fundamental questions regarding whether IRF4-expressing DCs regulate the development and recall response of type 2 T\textsubscript{RM} cells. Using our mice which have mature cDC2s that lack IRF4 expression, we demonstrate that mice with IRF4-deficient DCs during sensitization exhibit diminished T\textsubscript{RM} cell-dependent memory responses to allergen. In addition, we demonstrate that IRF4 controls DC expression of IL-10 and IL-33 as well as migration to the draining lymph node during allergic sensitization. After finding that IRF4-expressing DCs are necessary during sensitization, we investigated the ongoing role for IRF4-expressing DCs in maintaining T\textsubscript{RM} cells that were primed by IRF4-competent DCs in wild-type hosts. T cells primed 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 impaired CD4 T\textsubscript{RM} cell-dependent responses stem from earlier defects.
Results

IRF4-expressing dendritic cells regulate the CD4 $T_{RM}$ cell-restricted type 2 inflammatory memory response to HDM rechallenges

We previously reported that novel $Irf4^{fl/fl}$CD11cCre mice, in which IRF4 is depleted after DC maturation, do not develop robust type 2 effector responses in the lungs following house dust mite (HDM) sensitization and challenge (20). However, it remains unknown whether these defects persist throughout subsequent memory recall responses, or if the defective development of the type 2 response resolves over time. 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. 1A, ref. (12)), 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 (35). We confirmed that FTY720 treatment does not affect the lung $T_{RM}$ cell population but effectively depletes circulating T cells (Fig. S2A-D). Mice lacking IRF4 in DCs exhibit a severely mitigated type 2 inflammatory $T_{RM}$ cell-dependent 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. 1B). 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. 1C).

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 WT counterparts (Fig. 1D), yet they express less CD86 (Fig. 1E). None of the examined antigen-presenting cells (APCs) exhibit IRF4-dependent expression of CD80 (data not shown). 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,
Irf4fl/flCD11cCre mice display diminished effector responses to HDM (20) and have defective memory type 2 responses.

Lungs of mice with IRF4-deficient DCs contain fewer Der p 1-specific Th2RM cells during the memory phase

We hypothesized that the reduced memory response to HDM in Irf4fl/flCD11cCre mice was due to a defect in the underlying CD4 T_{RM} cell pool that develops in response to sensitization and challenge. Without rechallenging the mice, we harvested the lungs 4-5 weeks after acute sensitization and challenge of Irf4fl/fl or Irf4fl/flCD11cCre mice to HDM or PBS vehicle control (Fig. 2A). 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 anti-CD45 antibody minutes prior to sacrifice (36). CD4 T_{RM} cells were identified as CD3(+) CD4(+) CD44(high) CD62L(low) cells that expressed CD69 and CD11a, but that did not display intravascular CD45 staining (Fig. S2B-D). While no significant difference in the total number of CD4 T_{RM}, T central memory (T_{CM}), or T effector memory (T_{EM}), cells was evident in the absence of IRF4-expressing DCs (Fig. 2B, S3A-C), fewer CD4 T_{RM} cells in Irf4fl/flCD11cCre mice expressed the IL-33 receptor (ST2), which is a marker of Th2 cells and a subset of Tregs (Fig. 2B). We used tetramers to identify antigen-specific cells, using the tetramer on two fluorochromes to increase confidence in the specificity that the cells are recognizing the intended epitope as opposed to the fluorochrome itself (37). We found that Irf4fl/flCD11cCre mice had fewer GATA3(+) ST2(+) Der p 1-specific T_{RM} cells and Foxp3(+) Der p 1-specific T_{RM} cells (Fig. 2C), suggesting that decreased antigen-specific CD4 T_{RM} cells may limit memory Th2 and Treg responses.

To determine the stage at which Th2 defects begin, we sensitized Irf4fl/fl or Irf4fl/flCD11cCre mice to HDM and evaluated the T cells in the lung seven days later (Fig. 3A). This revealed a reduced proportion of lung parenchymal T effector cells and reduced expression
of CD69 after HDM sensitization (Fig. 3B), demonstrating a defect in the recruitment of activated T cells to the lungs after sensitization. We examined the expression of T cell lineage-specifying transcription factors in the lung-draining lymph nodes (LdLNs) (Fig. 3C) and found that the number of conventional Th2 cells was reduced in \(Irf4^{fl/fl}\)CD11cCre mice by four days post-sensitization (Fig. 3D). Further, the number of Der p 1-MHC-II tetramer-positive CD4 T cells was also reduced at this time point, demonstrating a diminished antigen-specific response during priming (Fig. 3E). These data indicate that IRF4 is acting in DCs during the earliest stages of HDM sensitization to initiate type 2 lung responses.

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

We hypothesized that IRF4 regulates particular DC processes required to initiate Th2 responses during sensitization in vivo. A first step in initiating the immune response is the phagocytosis of allergens by DCs. To assess allergen phagocytosis, 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. 4A). There was no difference in the overall number of any DC subset (Fig. 4A, Fig. S4A). The CD24(-) cDC2 subset, which is also IRF4-dependent, had a slight but statistically significant reduction in the proportion that were HDM-positive (Fig. S4B), but no reduction in cell number (Fig. S4C) or HDM MFI (Fig. S4D). Thus, while there is a small difference in allergen uptake in the IRF4-deficient lung cDC2s, 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 (30, 31). Thus, we found an expected reduction in the number of migratory CD24(+) cDC2s in the LdLNs of \(Irf4^{fl/fl}\)CD11cCre mice (Fig. 4B). While the proportion of CD24(+) cDC2s that were HDM-positive was equal in the LdLNs between \(Irf4^{fl/fl}\) or \(Irf4^{fl/fl}\)CD11cCre mice, there was a reduction in the number of HDM+
DCs in the $Irf4^{fl/fl}$CD11cCre mice (Fig. 4B). No consistent defects were seen for other APC subsets lacking IRF4 in the LdLN (Fig. S4E-H). Thus, as was previously described for skin DCs (30, 31), the migration of IRF4-deficient lung CD24(+) cDC2s to the lymph node is impaired. 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 BMDCs, we previously showed that IRF4-deficient DCs display a reduced capacity to promote Th2 differentiation in vitro (20). 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 mixed with the surrogate reagent DQ Red BSA, which becomes fluorescent upon proteolytic cleavage. IRF4-deficient CD24(+) cDC2s were capable of processing antigens both in the lungs (Fig. 4C) and in the LdLN (Fig. 4D). 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, pooled 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 T-cell receptor (TCR) transgenic mice (Fig. 5A). We found that ex vivo IRF4-deficient CD24(+) cDC2s were less effective at inducing T cell proliferation (Fig. 5B). Even when OVA peptide is added to the culture, the IRF4-deficient DCs were still slightly deficient in stimulating OTII proliferation (Fig. 5C). 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 ($p = 0.0002$), suggesting that CD24(-) cDC2s are not well-suited to T cell priming in response to HDM (Fig. S5).
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. Our previous in vitro work demonstrated that IRF4-deficient DCs express reduced IL-33 and IL-10 (20). To determine whether IRF4 regulates IL-33 and IL-10 expression in vivo, we sensitized \textit{Irf4}^{fl/fl} and \textit{Irf4}^{fl/fl} \textit{CD11cCre} mice to HDM, sorted lung cDC2s 18 hours later, and evaluated IL-33 and IL-10 expression by qPCR. \textit{Il33} and \textit{Il10} expression by lung cDC2s, as assessed by qPCR, is dependent on IRF4 during in vivo HDM sensitization (Fig. 5D). This suggests that IRF4 controls a pro-Th2 program involving these factors, culminating in Th2 polarization.

\textit{IRF4 expression in DCs is not required for CD4 T_{RM} cell maintenance or recall responses}

Having demonstrated early defects in the immune response when DCs lack IRF4, the question remained 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 priming and differentiation by sensitizing and challenging WT CD45.1 mice with HDM, isolating CD4 T cells from the inflamed lungs, and then adoptively transferring these lung T cells into either \textit{Irf4}^{fl/fl} or \textit{Irf4}^{fl/fl} \textit{CD11cCre} mice (Fig. 6A). Recruitment of the transferred cells was assisted by “pulling” with intratracheal instillation of rIL-33, which induces lung expression of chemokines (38). The mice were then rested for 4-5 weeks to allow for contraction of the adoptively transferred effector CD4 T cells into T_{RM}. Polyclonal and tetramer-positive donor T_{RM} cells were present to an equal extent in the lungs of both \textit{Irf4}^{fl/fl} or \textit{Irf4}^{fl/fl} \textit{CD11cCre} mice (Fig. 6B), demonstrating that T cells primed by IRF4-expressing DCs were capable of taking up residence and persisting for many weeks without the continued presence of IRF4-expressing DCs. We found a modest reduction in allergen uptake and CD86 expression by CD24(+) cDC2s in this adoptive transfer model (Fig.
S6A-C), similar to in the aforementioned experiments, validating our use of this T cell adoptive transfer to investigate IRF4-dependent cDC2 defects.

To determine the role of IRF4-expressing DCs during recall responses, we rechallenged the mice with HDM during simultaneous FTY720 treatment to restrict the memory response to the lung T_{RM} cells (Fig. 6C). Upon challenge, Irf4^{fl/fl}CD11cCre mice displayed allergic responses equal in magnitude to those mounted by Irf4^{fl/fl} littermates, as they were equally capable of recruiting eosinophils and CD4 T cells to the airways (Fig. 6D). There were equal numbers of the donor CD4 T cells in the BAL, suggesting that their memory response was similar whether the lung DCs were IRF4-deficient or IRF4-sufficient. Taken together, our data demonstrate that IRF4-expressing DCs are necessary for the development of the effector Th2 response but not for reactivating resting T_{RM} cells.
Discussion

In this study, we find that mature IRF4-expressing CD24(+) cDC2s play numerous crucial roles in the priming and differentiation of naïve T cells in response to HDM in vivo. IRF4-deficient CD24(+) cDC2s display minor defects in their ability to phagocytose inhaled allergens, no defect in antigen processing, and 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 and IL-33 during sensitization. Intriguingly, we find that mature DCs need not express IRF4 during the homing of Th2_{EM} cells to the lungs, the development of Th2_{EM} cells into Th2_{RM} cells, or the subsequent persistence of Th2_{RM} cells in the lungs. Finally, 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 entire CD11c(+) compartment causes the absence of lung, intestine, lymph nodes, and spleen CD24(+) cDC2s (25, 27, 30, 31, 39), we find that IRF4-deficient CD24(+) cDC2s are present to a normal extent in the lungs and spleen of our mice (Fig. 4A and ref. (20)). The CD11c-Cre mice used in this study excise IRF4 only after the pre-cDC stage, thereby producing mature IRF4-deficient cDC2 cells (20). While other models have not been useful for studying IRF4-deficient lung DCs in vivo, dermal DCs could still develop independently of IRF4. In fact, one study found increased numbers of dermal CD11b(+) DCs due to IRF4-dependent deficiency in CCR7 and reduced drainage to the skin-draining LNs (30). In our mouse model, lymph node homing of migratory CD24(+) cDC2s was severely limited in the context of IRF4-deficiency (Fig. 4B). However, this did not appear to cause an increase in the number of these DCs in the lungs (Fig. 4A).
IRF4-deficient BMDCs have inferior T cell priming capabilities due to defects in antigen processing and presentation pathway genes such as CtsS (cathepsin S), H2-Oa, H2-DMb2, Ciita, and Cst3 (25). In our study, IRF4-deficient lung DCs could process exogenous antigen and even had higher levels of antigen processing as measured by BSA-DQ fluorescence (Fig. 4C). 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 (40). 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 suggest particular roles for IL-10 and IL-33 in Th2 differentiation. A recent report has corroborated the IRF4-dependent production of IL-10 by lung DCs (41). While other cells may produce 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 (42). 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 (43). 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 (44, 45) or is inactivated by oxidation (46). 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 (20, 25, 26, 41). HDM sensitization and challenge generate a pool of CD4 T_{RM} cells, but less potently induce CD8 T_{RM} cells (34). We find that HDM sensitization promotes allergen-specific CD4 T_{RM} cells with both Th2 and Treg subsets, but that the absence of IRF4-expressing DCs results in a more significant loss of the Th2 subset. This loss of Th2 cells may result from diminished downstream mediators of IRF4 such as IL-33 and IL-10.

We have demonstrated that IRF4 expression by DCs is not necessary for the maintenance and recall response of Th2 cells that IRF4-expressing DCs previously primed. In particular,
adoptively transferred T\(_{EM}\) cells from mice sensitized and challenged to HDM were capable of homing to the lungs and persisting as T\(_{RM}\) cells in the absence of allergen or IRF4-expressing DCs. 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 could proliferate in response to an LCMV peptide in the female reproductive tract when CD11c(+) cells were depleted (32). MHC-II-expressing epithelial cells have also been shown to present antigen to lung CD4 T\(_{RM}\) cells (47). Together with our data that IRF4-expressing DCs are expendable for recall responses, these studies suggest that other non-classical APCs can present antigen and stimulate appropriately-educated T\(_{RM}\) cells. Alternatively, T\(_{RM}\) cells could be licensed to conduct an allergic recall response by the presence of allergen-triggered cytokines. The latter possibility is consistent with previous investigations pointing to tissue-derived signals such as Thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 as important for licensing lung Th2 cells (48), particularly the requirement and sufficiency of IL-33 signaling in memory Th2 cells for IL-5-mediated eosinophilic responses (48). 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. For instance, nearly all microbe-specific clonal effector populations give rise to their own pool of long-lived memory cells (49). 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.

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 impaired T\(_{RM}\) cell-dependent memory responses when DCs lack IRF4 stem from defects in earlier T cell education.
**Methods**

**Mice.** C57Bl/6 mice (WT) were purchased from Harlan Laboratories (Indianapolis, IN). B6 CD45.1 (B6.SJL-Ptprc<sup>Peap<sup>c</sup>/BoyJ, stock #002014, (50-52)) and conditional Irf4 mutant (Irf4<sup>fl/fl</sup>; B6.129S1-Irf4<sup>tm1Rdf</sup>/J, stock #009380, (53)) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Irf4<sup>fl/fl</sup> mice were bred to CD11c-Cre-GFP transgenic mice (CD11cCre; C57BL/6J-Tg(Itgax-cre,-EGFP)4097Ach/J, stock #007567, (54)), which were developed and kindly provided by Dr. Alexander Chervonsky (University of Chicago, Chicago, IL). 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.

**HDM-induced mouse model of allergic airway disease.** 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 37°C, 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 biotinylated or PE-conjugated...
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 rechallenge 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) was used in murine experiments.

**Tetramer production.** As previously described, I-A^b^ containing the Der p 1 peptide 117-127 (CQIYPNNVKI) were biotinylated and tetramerized with streptavidin-PE or streptavidin-APC (Prozyme) (55, 56). Tetramers were produced in Boston, MA and shipped to Chicago, IL.

**T cell enrichment for adoptive transfer.** CD45.1 mice were sensitized and challenged as described above. On day 13, single-cell suspensions of lung cells were 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 $1 \times 10^6$ cells per mouse. Recipient mice were promptly intratracheally instilled with 100 ng recombinant mouse IL-33.

**T cell enrichment, labeling, and coculture.** 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.** For staining of dendritic cells, $1 \times 10^6$ 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, $5 \times 10^5$ 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 Supplementary Table 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 Aurora (Cytek). 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 (Fig. S1). 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(-) (57). 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 (58), which have also previously been defined by Ly6C, CD64 (FcγRI), and MAR-1 (FcεR1α) (22). 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 (24). We conducted subsequent analyses for each of the following DC populations: moDCs, CD103(+) cDCs, CD24(+) cDC2s, and CD24(-) cDC2s.

qPCR. RNA was isolated from sorted cells using a Quick-RNA Microprop 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'-CTGCGTCTGTTGAGCTATGA-3', reverse 5'-CACCTGGTCTTGCTCTTGGT-3'.

Statistics. 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.

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

**Author contributions**

DFC and AIS designed the research studies. DFC, TEV, MKH, EW, CL Howard, EPD, PAK, and CL Hrusch conducted experiments and acquired data. DFC, TEV, MKH, EW, CL Howard, DEK, and EPD analyzed data. JJM provided reagents. DFC, TEV, MKH, MRC, and AIS wrote the manuscript.

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References


**Figure 1:** IRF4-expressing dendritic cells regulate the T\textsubscript{RM} cell-restricted type 2 inflammatory memory response to HDM rechallenges.

(A) Schematic of experimental protocol for T\textsubscript{RM} cell-restricted memory response to HDM. (B) Total cellularity, eosinophils, CD4 T cells, and CD8 T cells in the airways (top) or lungs (bottom), n = 20. (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, n = 20. Scale bar represents 100 µm. (D) Number of CD24(+) cDC2s and CD24(-) cDC2s in the lungs, n = 63. (E) For lung CD24(+) cDC2s and CD24(-) cDC2s, MFI of CD86 normalized to the mean of the PBS-treated Irf4\textsuperscript{fl/fl} group, with representative flow plots (left), n = 40. Data are representative of (B, C) or combined from (D, E) 3 independent experiments with n ≥ 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). Also see Figures S2 and S4.
Figure 2

A

B

C

D
**Figure 2:** Lungs of mice with IRF4-deficient DCs contain fewer Der p 1-specific Th2\textsubscript{RM} cells during the memory phase.

(A) Schematic of experimental protocol for resting memory lung analysis. (B) Number of lung T\textsubscript{RM} cells and the proportion expressing ST2, n = 20. (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\textsubscript{RM} cells expressing GATA3 or Foxp3, n = 36. (B) Data are representative of 3 independent experiments with n ≥ 4 mice per group in each experiment. Analysis by ordinary one-way ANOVA with Tukey’s multiple comparisons test. (C) Three independent experiments (n ≥ 5 per group) were statistically significant. Shown are two of these experiments combined. Kruskal-Wallis test (with Dunn’s multiple comparisons test) were performed in GraphPad Prism. (A-C) Bar represents the mean ± SEM (*, p < 0.05; **, p < 0.01; ns, not significant). Also see Figure S3 and S4.
Figure 3

A

B

C

D

E
Figure 3: Mice with IRF4-deficient DCs are unable to adequately recruit T_{EM} cells to the lungs and produce Th2 cells or tetramer(+) T cells in the lung draining lymph nodes (LdLN) after HDM sensitization.

(A) Schematic of experimental protocol for analysis initiation of Th2 responses during sensitization phase in the lung. (B) Proportion of T effector cells out of lung extravascular CD4 T cells and CD69 expression by extravascular lung T cells, n = 7. (C) Schematic of experimental protocol for analysis initiation of Th2 responses during sensitization phase in the LdLN. (D) GATA3 and RORγt expression by LdLN T_{conv} cells on day 4 after HDM sensitization, n = 18. (E) Number of tetramer(+) LdLN CD4 T cells, n = 18. Data are representative of 2 independent experiments with n ≥ 3 mice per group, and statistics (B: unpaired t test with Welch’s correction; D, E: 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).
Figure 4
**Figure 4:** IRF4 regulates allergen phagocytosis and migration of CD24(+) cDC2s to the LdLNsduring 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, n = 11 (A) or LdLN, n = 10 as one LdLN could not be harvested (B). (C, D) Flow cytometry plots depict fluorescence of processed DQ Red BSA in CD24(+) cDC2s, proportion DQ(+), and MFI of DQ in either the lungs, n = 10 (C) or LdLN, n = 9 (D). Data are representative of 2 independent experiments with n ≥ 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). *Also see Figure S5.*
Figure 5

A. Inflammasome or inflammasome CD11cCre
-1 0 4
Sort lung & LdLN DC subsets
L. HDM + OVA
Harvest

B. T cells cultured with CD24+ cDC2s
In vivo HDM + OVA
-1 0 4
Sort lung & LdLN DC subsets

Counts

C. T cells cultured with CD24+ cDC2s
OVAp added in vitro
-1 0 4
Sort lung & LdLN DC subsets

Counts

D. Lung cDC2s

Counts

**

### Figure Legends

**A.** Schematic representation of inflammasome or inflammasome CD11cCre intervention. 

**B.** Analysis of T cells cultured with CD24+ cDC2s after in vivo HDM + OVA treatment.

**C.** Evaluation of T cells cultured with CD24+ cDC2s following OVAp addition in vitro.

**D.** Assessment of lung cDC2s expression of i33 and i10 genes.
**Figure 5:** 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) Number of OTII cells after culture, % undivided, division index, proliferation index, and CFSE dilution histograms for (B) in vivo HDM+OVA sensitized CD24(+) cDC2s, n = 20, or (C) with OVA\textsubscript{323-339} peptide added, n = 20. (D) IL-33 and IL-10 expression by qPCR of sorted lung cDC2s after in vivo HDM sensitization, n = 6. (B-C) Data are representative of 2 independent experiments with n ≥ 4 wells per group, and statistics (unpaired t test with Welch’s correction) were performed in GraphPad Prism. (D) Data represent 1 experiment with n = 3 mice per group, and statistics (unpaired t test) were performed in GraphPad Prism. (A-D) Bar represents the mean ± SEM (*, p < 0.05; **, p < 0.01; ****, p < 0.0001; ns, not significant). Also see Figure S6.
Figure 6

A

Ly5.1

Day 0 7 8 9 10 13

HDM sensitization HDM challenges

Adoptive transfer of enriched lung CD4 T cells

~4 wks

lif6 or
lif6CD11cCre

Receive T cells & IL-33 pull to lungs

Harvest

B

Donor-derived T\textsubscript{RM}

Donor-tetramer\(^{+}\) T\textsubscript{RM}

Donor ST2\(^{+}\) T\textsubscript{RM}

C

Ly5.1

Day 0 7 8 9 10 13

HDM sensitization HDM challenges

Adoptive transfer of enriched lung CD4 T cells

HDM challenges

~4 wks

lif6 or
lif6CD11cCre

Receive T cells & IL-33 pull to lungs

FTY720 i.p.

Harvest

D

Total BAL cells

Eosinophils

CD4 T cells

Donor CD4 T cells

Normalized number

Normalized number

Normalized number

Normalized number

ns

ns

ns

ns
**Figure 6:** IRF4 expression in DCs is not required for CD4 T<sub>RM</sub> cell maintenance or recall responses.

(A) Ly5.1 mice were sensitized and challenged with HDM. Lung T<sub>EM</sub> cells were adoptively transferred to Irf4<sup>fl/fl</sup> or Irf4<sup>fl/fl</sup>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<sub>RM</sub> cells, donor-derived tetramer(+) T<sub>RM</sub> cells, and ST2(+) donor-derived T<sub>RM</sub> cells. Data represent 1 experiment with n ≥ 4 mice per group and total n = 21, 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, and donor CD4 T cells in the airways normalized to the mean of the Irf4<sup>fl/fl</sup> group receiving donor cells. Data represent 4 combined experiments with n ≥ 4 mice per group and total n = 52, and statistics (Mann-Whitney test) were performed in GraphPad Prism. Bar represents the mean ± SEM (*, p < 0.05; ns: not significant). Also see Figure S7.