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Interleukin-2-dependent allergen-specific tissue resident memory cells drive asthma

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Abstract

Exposure to inhaled allergens generates T helper 2 (Th2) $CD4^+$ T cells that contribute to episodes of inflammation associated with asthma. Little is known about allergen-specific Th2 memory cells and their contribution to airway inflammation. We generated reagents to understand how endogenous $CD4^+$ T cells specific for a house dust mite (HDM) allergen form and function. After allergen exposure, HDM-specific memory cells persisted as central memory cells in the lymphoid organs and tissue resident memory (Trm) cells in the lung. Experimental blockade of lymphocyte migration demonstrated that lung resident cells were sufficient to induce airway hyper-responsiveness, which depended upon $CD4^+$ T cells. Investigation into the differentiation of pathogenic Trm cells revealed that interleukin-2 (IL-2) signaling was required for residency and directed a program of tissue homing migrational cues. These studies thus identify IL-2-dependent resident Th2 memory cells as drivers of lung allergic responses.

Introduction

Atopic asthma affects between 50–80% of asthmatics and begins when children are exposed to common aeroallergens including pollen, animal dander, fungal spores or house dust mites

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Author Contributions: BH performed experiments, analyzed data and wrote manuscript. DA performed AHR experiments. JS performed and analyzed parabiosis experiments. KK, HS, GK, AK, EG performed experiments and helped with manuscript preparation. JM provided tetramer construct. DM designed parabiosis experiments and reviewed manuscript. WA designed AHR experiments and provided expertise in lung biology. MP designed experiments, analyzed data and wrote manuscript.

(HDM) (Locksley, 2010). The majority of atopic asthma cases are characterized by T helper 2 (Th2) cell-associated cellular processes (Wenzel, 2012). During the sensitization phase, allergen-specific $CD4^+$ Th2 cells expand, acquire the capacity to express "type 2" cytokines and up-regulate chemokine receptors and integrins associated with migration to various anatomical sites. The type 2 cytokines (IL-4, IL-5 and IL-13) orchestrate multiple events associated with asthma including eosinophil maturation and survival, airway hyper-responsiveness and B cell isotype switching to IgE (Holgate, 2012). After this period of expansion and differentiation, there is a protracted contraction phase in which approximately 90% of the expanded population dies and a small population of differentiated memory cells is retained. In both murine models of disease and in asthmatic patients, $CD4^+$ memory T cells are thought to be involved in recurrent episodes of inflammation (Lanzavecchia et al., 1983; Mojtabavi et al., 2002). Due to the difficulty in tracking small populations of $CD4^+$ Th2 cells that express allergen-specific TCRs, little is known about how endogenous Th2 memory cell differentiation, maintenance, or homing properties.

In humans and mice, there are circulating and non-circulating $CD8^+$ and $CD4^+$ memory T cells. Circulating memory T cells exist in two subsets: central memory (Tcm) and effector memory (Tem) T cells (Sallusto et al., 1999). Tcm cells express the chemokine receptor CCR7 and L-selectin, which direct recirculation through lymphoid tissues. $CCR7^-$ Tem cells express receptors needed for migration into nonlymphoid tissues and when stimulated with their relevant peptide-MHCII (pMHCII) ligand, rapidly produce cytokines. In models of Th1 memory, differentiation of $CXCR5^-$ Teff and Tem is promoted by signaling through the cytokine Interleukin-2 (IL-2), while differentiation of the $CXCR5^+$ T follicular helper cells (Tfh) and Tcm cells depends upon expression of the transcription factor BCL6 (Choi et al., 2011; Pepper et al., 2011). It is not known if these same mechanisms are involved in Th2 memory formation.

A third population of memory T cells has also been described that are non-circulating and are retained in the tissues, called tissue-resident memory (Trm) cells (Mueller et al., 2013). Studies primarily examining $CD8^+$ T cells have defined unique roles for Trm cells including the direct immediate control of local infection and the indirect modification of the tissue microenvironment to promote inflammation (Schenkel and Masopust, 2014). Although recent studies have begun to unravel the function of $CD4^+$ Trm cells in infection, less is known about how these cells contribute to immune pathology since antigen-specific memory cells that reside in nonlymphoid tissues are rare. Recent studies have overcome these issues by combining advances in MHC Class II tetramer generation with novel intravascular (i.v.) staining procedures, magnetic bead enrichment of rare cells and surgical techniques that allow residence to be experimentally defined (Anderson et al., 2014; Jiang et al., 2012; Moon et al., 2009).

In an effort to understand the differentiation of allergen-specific memory cells and determine their importance in the development of asthma, these technological advances were applied to a relevant murine model of asthma. We developed pMHCII tetramer reagents to interrogate the endogenous allergen-specific $CD4^+$ T cell response to HDM, focusing on the immunodominant Der p1 (Derp1) protein. Allergic sensitization with HDM in the absence of additional adjuvant led to the hallmark symptoms of airway inflammation

including eosinophilia, Immunoglobulin E production and airway hyper-responsiveness (AHR) associated with Th2 cytokine production (Gregory and Lloyd, 2011). These reagents were used in conjunction with i.v. staining and cell enrichment techniques to track small populations of allergen-specific $CD4^+$ T cells in the lymphoid organs and lungs of mice sensitized and challenged with HDM.

These studies demonstrated that two major types of allergen-specific memory Th2 cells formed and were maintained in response to HDM: a Tcm cell population in the lymphoid organs and a Trm cell population in the lungs. Furthermore, our studies demonstrated that lung resident cells were sufficient to induce asthmatic symptoms and memory T cells from the lymphoid organs were not required. Finally, these studies demonstrated that IL-2 signaling regulated a broad program of migrational cues and was critical for the development of pathological Th2 Trm cells in the lung. Thus, this report defines IL-2 dependent $CD4^+$ resident memory Th2 cells as promoters, and potential therapeutic targets, of allergic disease.

Results

$Derp1^+$ tetramer identifies an expanded population in the secondary lymphoid organs and lungs after allergic sensitization

These studies focused on a 30 kDa Group 1 cysteine protease found in HDM that breaks down epithelial tight junctions and enhances antigen presentation by dendritic cells (DCs) called Derp1 (Chua et al., 1988). Derp1 contains epitopes that are highly immunogenic in atopic individuals and certain strains of mice, including amino acids 110–131 (Hall et al., 2003; Hoyne et al., 1993; Yssel et al., 1992). Previously described algorithms were used to identify two distinct, but overlapping epitopes with affinity for the MHC class II allele $I\text{-}A^b$ (Fig. S1A) (Lee et al., 2012). We generated and tested two different $I\text{-}A^b$ tetramers containing epitopes 114–124 and 117–127 (Moon et al., 2007). The 117–127 epitope induced a more robust $CD4^+$ T cell response, and was therefore used for all subsequent experiments (data not shown).

Intranasal administration of an extract of HDM was used to determine if Derp1:$I\text{-}A^b$ -specific ($Derp1^+$) Th2 cells could be identified in a model of airway inflammation. Based upon previously published protocols, mice were sensitized with HDM intranasally on day 0 followed by five subsequent, smaller intranasal (i.n.) challenge doses of HDM on days 10–14 (Fig. S1B) (Kool et al., 2011). To differentiate between cells in the lung vasculature versus those in the lung parenchyma, i.v. staining with anti-Thy1.2 antibody was performed. The number and phenotype of $Derp1^+$ cells in the secondary lymphoid organs (SLO) or lungs of naive mice were compared to mice given either a single i.n. administration of HDM or the full allergic sensitization protocol. The SLO of naive mice contained mostly $CD44^{low}$ $Derp1^+$ cells (~80 cells per mouse) (Fig. 1A). Although a small number of $CD44^{low}$ cells could be found in the lung vasculature of some mice, no $Derp1^+$ cells were present in the lung tissues of naive mice, suggesting no prior exposure to HDM. A single i.n. administration of HDM induced an expanded population of ~3,800 $CD44^+$ $Derp1^+$ cells in the SLO and ~400 $Derp1^+$ cells were found in both the lung vasculature and lung tissue 6 days post-immunization (Fig. 1A). Five subsequent days of allergic challenge expanded the

Derp1$^+$ cells predominantly in the lungs, where nearly all were then found within the lung tissue and not in the vasculature (Fig. 1A). Immunofluorescent images from lungs showed that within three days of allergen challenge, expanded numbers of CD4$^+$ cells infiltrated the lung parenchyma surrounding the blood vessels (BVs) and in close proximity to the airways (AWs), highlighting the inflammation induced by this system (Fig. 1B). These results demonstrated that endogenous Derp1$^+$ CD4$^+$ T cells could be identified and tracked in the lymphoid organs and lungs in a relevant murine model of asthma.

Exposure to HDM generates CD4$^+$ Th2 cells and T cell-dependent airway inflammation

Clinical symptoms including airway remodeling, mucus production, Th2 cytokine production, eosinophilia and airway hyper-responsiveness (AHR) were assessed to demonstrate atopic asthmatic disease (Wenzel, 2012). Analyses of sections of formalin fixed lung tissue from HDM sensitized or naïve mice stained with hematoxylin and eosin (H&E) or a Periodic-acid Schiff (PAS) stain were performed. In naïve mice, the lung parenchyma was largely devoid of inflammatory cells and there was little mucus production, while in the sensitized lung there was inflammatory leukocyte infiltration, airway remodeling and significant mucus production as measured by PAS (Fig. 2A).

Several different asthmatic “endotypes” have been described that are driven by various types of T helper (Th2, Th17, Th9) and innate lymphoid cell subsets (Agache et al., 2012). Although the HDM model of asthma has previously been described as a Th2-mediated disease, it was important to determine if the endogenous Derp1$^+$ cells that developed indeed expressed Th2 cytokines that contribute to airway inflammation (Hammad et al., 2009). To substantiate that Derp1$^+$ cells produced Th2 cytokines *in vivo*, IL-13 and IL-4 expression were assessed in HDM sensitized reporter mice (*IL13-eGFP* and *KN2* mice, respectively) (Mohrs et al., 2005; Neill et al., 2010). These mice provide a snapshot of cytokine production in allergen-specific cells directly *ex vivo*. In accordance with the development of type 2 immunity induced by helminth infection, three days after allergen sensitization, IL-4 was primarily expressed by Derp1$^+$ CXCR5$^+$ T follicular helper cells in the SLO, whereas very few Derp1$^+$ cells expressed IL-13 (Fig. 2B) (King and Mohrs, 2009; Liang et al., 2012; Zaretsky et al., 2009). Both IL-4 and IL-13 were produced in the lungs by Derp1$^+$ CD4$^+$ and total lung CD4$^+$ T cells (Fig. 2B and Fig. S2A). As the Derp1$^+$ cells are in lung tissue after allergic sensitization (Fig. 1A), expression of CD69 (associated with tissue residency in both CD8$^+$ and CD4$^+$ memory T cells) was also assessed (Schenkel and Masopust, 2014; Teijaro et al., 2011). Of interest, the majority of the cytokine producing cells expressed CD69 in the lung, suggestive of either recent activation, residency, or both (Fig. 2B).

To demonstrate that Th2 cytokine production was antigen-specific, single cell suspensions of lungs from HDM-sensitized mice were cultured *in vitro* in the presence or absence of the Derp1 peptide (117–127) and cytokine production was measured by ELISA. Measurements of IL-4, -13, -5,-17 or IFNγ demonstrated that only IL-4, -5, and -13 were significantly expressed in response to the Derp1 peptide, while expression of IFN-γ and IL-17 expression were not significantly increased in response to peptide (Fig. S2B). These data demonstrate that the Derp1$^+$ CD4$^+$ T cells in this system primarily produced Th2 cytokines in response to antigenic challenge.

The type 2 cytokine IL-5, produced by Derp1$^+$ lung cells, is critical for the recruitment and survival of eosinophils (Locksley, 2010). To test whether CD4$^+$ T cells are responsible for eosinophil recruitment in this model, WT mice or mice that lack CD4$^+$ T cells were sensitized and challenged with HDM and the percentage of eosinophils in the lung were quantified by flow cytometry. As shown in Fig. 2C, induction of airway inflammation with HDM leads to enhanced lung recruitment of Siglec-F$^+$SSC-A^{high} eosinophils (that are also CD3$^-$Class II$^-$CD11b$^+$, data not shown) and this recruitment is significantly reduced in MHC Class II deficient mice that lack CD4$^+$ T cells (Fig. 2C).

The most stringent hallmark of allergic airway inflammation, AHR, can be measured using assessment of methacholine resistance by Flowvent. Neither naive B6 nor naive MHC Class II deficient mice exhibited AHR in response to methacholine challenge compared to a PBS challenge baseline control (Fig. 2D). Sensitized B6 mice, but not sensitized MHC Class II KO mice, display a significant increase in methacholine resistance, demonstrating that airway inflammation depends upon the presence of CD4$^+$ T cells (Fig. 2D). These data together demonstrate that allergic sensitization with i.n. HDM leads to the development of allergic airway inflammation associated with type 2 immunity mediated by CD4$^+$ T cells.

Heterogeneous populations of Derp1$^+$ memory cells persist in the lymphoid tissues and lungs

Derp1$^+$ cells in the SLO and lung tissue were quantified at various time points to determine if endogenous allergen-specific Th2 cells form memory. Early in the primary response, Derp1$^+$ cells preferentially expanded in the SLO compared to the lungs (Fig. 3A). Allergen challenge however, lead to the expansion of Derp1$^+$ T cells in the lung parenchyma (and a concomitant decline in the spleen), such that 3 days after challenge, approximately equal numbers of Derp1$^+$ cells could be found in both sites (Fig. 3A). Total Thy1.2$^-$ CD4$^+$ cells in the lung reflected similar kinetics after HDM exposure (Fig. 3A). Following a short period of contraction in the SLO and a longer contraction in the lung, the numbers of Derp1$^+$ cells slowly declined in both sites as previously demonstrated for Th1 memory (Pepper et al., 2010). After contraction, antigen-experienced CD44$^+$ Derp1$^+$ cells were present at all timepoints examined. These data demonstrate that long-lived Derp1$^+$ memory populations persist in the SLO and lungs.

We next determined if Th2 cells undergo an early bifurcation in Teff and Tfh cells (and later Tem and Tcm, respectively) as previously described for Th1 cells (Pepper et al., 2011). Tfh cells can be identified by intermediate expression of CXCR5, while germinal center (GC) Tfh cells display higher expression of CXCR5 and down-regulate CCR7 (Crotty, 2014; Lee et al., 2015). Six days after a primary immunization and before a germinal center response started, approximately half of the Derp1$^+$ cells were CXCR5$^+$ Tfh (Fig. 3B). CXCR5$^+$ GC Tfh cells are present 3 days after allergen challenge and comprised the majority of the CCR7$^-$ cells in the lymphoid organs (Fig. 3B and data not shown). The vast majority of the Derp1$^+$ memory cells that remain in the SLO at late time-points (>100 days post-challenge) retain CCR7$^+$ and resemble Tcm cells. Unlike Th1 memory cells, there is no clear delineation of Tem and Tcm that correlates with CXCR5 expression, demonstrating that Th1 and Th2 CD4$^+$ T cells undergo different programs of memory cell differentiation.

The phenotype of the allergen-specific memory cells that formed in the lungs was also assessed. Most Derp1$^+$ cells in the lung parenchyma lacked CCR7 and resembled Tem or Trm cells (Fig. 3C). The expression of CD69 was also analyzed for 70$^+$ days after the primary sensitization, to determine if CD69 positivity correlated with lung retention. The percent of Derp1$^+$ cells that express CD69 in the lung increases over time such that approximately 85% of the cells are CCR7$^-$ CD69$^+$, therefore resembling Trm (Fig. 3C). Very few of the cells expressed CD103 as has been described for CD8$^+$ Trm (data not shown) (Schenkel and Masopust, 2014). Together, these data demonstrate that memory Th2 cells that persist in SLO predominantly resemble Tcm while those in the lung resemble CD4$^+$ Trm that have previously been described during infection (Teijaro et al., 2011).

Derp1$^+$ cells in lung are Trm cells

Although most resident T cell studies have focused on CD8$^+$ T cells in the context of viral infections, functional CD4$^+$ Trm that express CD69 have also been observed after influenza infection (Teijaro et al., 2011). To test the hypothesis that allergen-specific CD4$^+$ T cells could also form bona fide Trm, we performed parabiotic experiments on CD45.1$^+$ congenic mice that underwent allergic challenge and were subsequently unperturbed for 50 days while memory populations formed (Jiang et al., 2012) (Fig. 4A). Pairs of naive CD45.2 mice and allergen-challenged CD45.1 mice were surgically joined and 15 days later, the composition of CD45.1 and CD45.2 lymphocytes in the blood was assessed by flow cytometry (Fig. 4A). After i.v. staining, the two congenically marked CD4$^+$ T cell populations were evenly mixed in the blood of both the naive and sensitized parabionts, demonstrating that vascular CD4$^+$ T cells were recirculating (Fig. 4A). To assess if the memory cells that form in the SLO or lungs of the immunized animal were resident or transient, Derp1$^+$ cells were enriched from the organs of the sensitized or naive parabionts and stained for congenic markers. In the spleen and lymph nodes of both animals, CD45.1$^+$CD44$^+$ Derp1$^+$ cells could be found in roughly equal percentages (Fig. 4B). While there was a significant population of Derp1$^+$ cells in the lungs of the sensitized parabiont, very few CD45.1$^+$ Derp1$^+$ could be found in the lungs of the naive parabiont (Fig. 4B). Furthermore, while the cells found in the lungs of the sensitized parabiont predominantly expressed CD69 and were in the lung tissue, the Derp1$^+$ cells found in the lung of the naive parabiont were CD69$^-$ and in the vasculature (Fig. 4C). These data demonstrate that the vast majority of CD69$^+$ memory cells found in the immunized lung were resident.

Cells resident in the lung are sufficient to induce AHR

The previous experiments showed that aerosol HDM sensitization induced a long-lived pulmonary CD4$^+$ Th2 Trm population. We therefore tested the hypothesis that Th2 Trm do not require circulating memory cells to drive airway inflammation after allergic challenge. Treatment with the sphingosine-1-phosphate receptor 1 (SIPR1) agonist Fingolimod (FTY720), was used to assess if the cells within the lung parenchyma are sufficient to induce airway inflammation. FTY720 blocks lymphocyte egress from lymphoid organs and does not reposition Trm cells to secondary lymphoid organs (Matloubian et al., 2004; Schenkel and Masopust, 2014). Mice were therefore sensitized with HDM extract and left unperturbed for 25–35 days to form Tcm and Trm memory populations prior to FTY720 treatment. Mice were then challenged with allergen for five days in the presence or absence

of FTY720 (for nine days total) (Fig. S3). Blood was sampled to monitor circulating lymphocytes before, during and after challenge in animals treated with FTY720. Both CD45.2$^+$ leukocytes (black circles) and CD3$^+$CD4$^+$ T cells (white circles) rapidly decline by approximately 98% in the blood of FTY720 treated animals and at no time were more than 5 total CD4$^+$ T cells/μl (+/− 2 cells) found in the blood during FTY720 treatment (Fig. 5A).

Once assured that cells were no longer trafficking, AHR was assessed by methacholine resistance by Flowvent and the quantity and phenotype of Derp1$^+$ cells was measured. Control naive mice that were either treated or untreated did not demonstrate airway resistance compared to a baseline PBS challenge (Fig. 5B, data not shown). HDM-challenged mice that were treated with FTY720 exhibited at least as much AHR to untreated HDM-challenged mice that had circulating memory cells (Fig. 5B). Furthermore, there were no significant differences in the number and phenotype of Derp1$^+$ cells found in either the lymphoid organs or the lungs of treated or untreated mice, which contained phenotypically similar populations of CD69$^+$ Trm (Fig. 5C, 5D, data not shown). These experiments therefore demonstrated that cells from the secondary lymphoid organs were not required for AHR and that resident lung cells that contained the CD69$^+$ CCR7$^-$ Derp1$^+$ cells were sufficient to cause AHR after methacholine challenge.

CD25-mediated IL-2 signaling is required for lung residency

IL-2 signaling, which depends upon binding of IL-2 to the IL-2 receptor α chain (CD25), has been shown to be important for CD4$^+$ Teff cell differentiation as well as Th2 differentiation (Cote-Sierra et al., 2004; Pepper et al., 2011). Furthermore, it is not known how IL-2 modulates Trm formation. To determine how IL-2 may be involved in the differentiation of Th2 Trm cells, mixed bone marrow chimeras were generated with CD25 sufficient and deficient bone marrow such that the allergen-specific response could develop in the presence of a normal T regulatory compartment (also IL-2 dependent) (Malek et al., 2002). Congenically marked wild type (WT) and CD25 deficient (CD25KO) bone marrow were mixed and transferred into congenically mismatched, irradiated hosts and after reconstitution was assessed, airway inflammation was induced with HDM. Mice were sacrificed at various timepoints after sensitization or challenge, i.v. stained with anti-Thy1.2, and tetramer-specific cells were quantified in the lymphoid tissues or lungs as described above. Six days after a primary immunization, WT and CD25 KO cells CD44$^+$ Derp1$^+$ had expanded in similar ratios in the SLO with a slight advantage for WT cells, suggesting a less profound role for IL-2 on CD4$^+$ T cell proliferation *in vivo* than seen in *in vitro* experiments as previously described (Fig. 6A) (Khoruts et al., 1998). The ratio of total tetramer positive WT and CD25KO cells in the SLO remained constant at all timepoints examined (Fig. 6A). The lungs however revealed a very different profile, as significantly greater numbers of WT than CD25KO Derp1$^+$ cells were found in the lungs as early as six days after sensitization and this ratio increased dramatically after allergen challenge and was maintained at 27 days after allergen challenge (Fig. 6A). These data demonstrate that after HDM sensitization, allergen-specific cells that lack CD25 do not take residency in the lungs early and are not retained as Trm.

We next investigated how IL-2 signaling could be regulating the early residency of allergen-specific CD4+ T cells in the lung. To accomplish this, the expression of known molecules associated with lymphoid retention, egress and migration early after primary HDM sensitization were examined. One possibility was that in the absence of IL-2 signaling, allergen-specific cells displayed higher expression of the B cell follicle homing receptor, CXCR5 as previously seen in the absence of IL-2 in a Th1 response, and were therefore in the B cell area (Pepper et al., 2010). An alternative possibility was that IL-2 regulated adhesion molecule expression (CD62L) and cells were unable to leave the lymphoid tissue. A third possibility is that IL-2 regulated lymphoid egress through the expression of CD69, which can regulate SIPR1 (Mackay et al., 2015; Shinoda et al., 2012; Shiow et al., 2006). The expression of CXCR5, CD69 and CD62L were therefore examined on Derp1:I-A^b-specific WT or CD25KO cells in the lymphoid organs in mixed bone marrow chimeras six days after HDM sensitization (Lee et al., 2015; Skon et al., 2013). While there were no significant differences in the ratios of $CXCR5^-$ to $CXCR5^+$ $Derp1^+$ cells in the lymphoid organs in the absence of CD25, there was however a small, but significant decrease in the percent of the $CXCR5^-$ cells that were $CD62L^-$ in the absence of IL-2 signaling compared to WT cells in the spleens/lymph nodes (Fig. 6B). Similar CD69 expression (primarily on Tfh cells as previously described (Lee et al., 2015)) was observed on WT and CD25KO $Derp1^+$ $CD4^+$ T cells in the SLO however expression was significantly higher on WT cells than the few CD25KO cells in the lungs of the same animals, suggesting that it may have a role in retaining cells in this compartment (Fig. 6B and S5) (Mackay et al., 2015). These data suggest that lymphoid and perhaps tissue retention, but not follicular homing or CD69-regulated egress, was affected by IL-2 signaling.

An additional, and somewhat underappreciated function of IL-2 that could impact $CD4^+$ T cell lung residency is its role in regulating migration (Zheng et al., 2007). We therefore investigated the possibility that chemokine receptors that could direct lung migration (including CCR4, CXCR3, CCR6, CCR8) were regulated by IL-2 signaling (Islam and Luster, 2012). Again, WT:CD25KO mixed bone chimeras were generated, and chemokine expression was assessed on $Derp1^+$ in the SLO and blood within six days of HDM sensitization to catch the earliest migrating cells (Fig. 6C). WT $Derp1^+$ cells expressed significantly higher levels of CCR4 (in the lymphoid organs and blood) and CXCR3 (in the lymphoid organs) compared to CD25KO Derp1:I-A^{b+}-specific cells (Fig. 6C and S4). There was heterogeneous expression of CCR4 and CXCR3 by the $Derp1^+$ cells in the SLO and blood, including a $CXCR3^+CCR4^+$ population that has been previously described in airway inflammation (Sekiya et al., 2000) (Fig. S4). Of interest, CCR6 expression was not found to be significantly different between WT and CD25KO $Derp1^+$ cells and the genetic ablation of CCR8 in a mixed bone marrow chimeric setting (as no commercial antibody is available) had no effect on $Derp1^+$ lung residency (data not shown). These data demonstrate that IL-2 regulates a broad migrational program of lymphocyte retention and migration.

As IL-2 has also been shown to regulate IL-4 production and Th2 differentiation, which could possibly impact Trm formation, we also examined production of IL-4 by CD25-deficient cells in a mixed bone marrow chimeric setting (Cote-Sierra et al., 2004), (Zhu et al., 2003). To accomplish this, KN2 mice were bred to CD25 KO mice and KN2/+ CD25KO: KN2/+ WT mixed bone marrow chimeras were generated and sensitized. Six

days after primary immunization no significant differences in IL-4 production were observed between WT and CD25KO Derp1$^+$ cells in the SLO (Fig. S6). While this demonstrates that cells in the lymphoid organs that produce IL-4 (primarily Tfh cells) do not exhibit a defect in IL4 production in the absence of IL-2 signaling, it does not rule out that Th2 eff/Trm cells (non-Tfh) would not have a defect in cytokine production in the absence of IL-2 signaling, but this was difficult to assess with so few CD25KO cells in the lungs.

Th2 cells undergo an early effector or Tfh fate choice

The preceding studies demonstrated that the generation of Th2 lung resident cells occurred early and required IL-2 signaling. Furthermore, the decrease in CD62L$^-$ CXCR5$^-$ cells suggested that the Trm precursor population is a component of the early CXCR5$^-$ Teff pool. Since early CD4$^+$ Teff cell differentiation can be promoted by STAT5 (downstream of IL-2 signaling), and inhibited by BCL6, we hypothesized that in the absence of BCL6, more Derp1$^+$ tissue resident cells should form (Choi et al., 2011; Pepper et al., 2011). Mixed bone marrow chimeras were generated with CD45.1$^+$ bone marrow from WT mice and CD45.2$^+$ BCL6 deficient bone marrow to test this hypothesis. After allergic sensitization, a greater proportion of WT Derp1$^+$ cells were present in the spleen and lymph nodes of immunized mice three days after HDM challenge (Fig. 7A). In the lungs however, this ratio was inverted such that there were far fewer WT than BCL6KO Derp1$^+$ cells (Fig. 7A). Since interactions with B cells are required to maintain BCL6 expression in CD4$^+$ T cells, we hypothesized that B6.129S2-*Ighm*^{tm1Cgn}/J mice that lack mature B cells (μMT) mice would phenocopy the BCL6KO cells in the mixed bone marrow chimeric setting. If true, this would allow further analysis of the asthmatic response in a non-chimeric setting. As expected, Derp1$^+$ Tfh cells were absent in spleen and lymph nodes of μMT mice while allergen-specific CD69$^+$ tissue resident cells were enhanced in the lungs, confirming the above findings with BCL6-deficient cells and creating a system where asthmatic symptoms could be compared to a WT control (Fig. 7B–E). WT and μMT mice were therefore sensitized and AHR was assessed after methacholine challenge by Flowvent. Both sensitized C57BL/6 and μMT mice had increased AHR compared to naïve B6 or μMT mice (Fig. 7F). These findings therefore demonstrate that entry into the lung by pathogenic Derp1$^+$ cells is inhibited by BCL6 and interactions with B cells, therefore highlighting the fact that B cells potentially have pleiotropic effects in the development of asthma.

Discussion

Allergen-specific Th2 memory cells are important regulators of airway inflammation associated with atopic asthma in both mice and humans (Cohn et al., 2004). Little is known however about how memory Th2 responses form or persist *in vivo* (Paul and Zhu, 2010). These studies used tetramer enrichment and i.v. staining techniques to identify small populations of endogenous Th2 cells responding to inhaled HDM directly *ex vivo* in the secondary lymphoid organs and lungs. Administration of i.n. HDM results in mucosal sensitization within the lungs without adjuvant, as sensitization is thought to occur in humans. Within six days of HDM administration, Derp1$^+$ Th2 cells expand and differentiate in both lymphoid organs and lungs, but further antigenic stimulation is required for the preferential accumulation of allergen-specific cells in the lung tissue. Functional analysis of

the Derp1$^+$ specific effector cells confirmed that as seen in other model systems, IL-4 is predominantly produced by Tfh cells in the SLO (King and Mohrs, 2009; Vijayanand et al., 2012; Zaretsky et al., 2009), while IL-5 and -13, are expressed by Derp1$^+$ in the lung tissue. Interestingly, the vast majority of lung cells that express cytokine 3 days after sensitization, also express the C-type lectin CD69.

Previous studies demonstrated that allergen-specific Th2 memory cells can be generated after *in vitro* differentiation or by using TCR transgenic T cells responding to model antigens (Endo et al., 2014; Mojtabavi et al., 2002; Salek-Ardakani et al., 2003). The present studies demonstrate that endogenous allergen-specific Th2 memory cells form and persist for at least 100 days in both lymphoid organs and lungs. The Derp1$^+$ memory populations are heterogeneous: CCR7$^+$ Tcm form in lymphoid tissues while CCR7$^-$ Trm form in lungs. This is a different pattern from Th1 memory cells that develop in response to acute bacterial or viral infections, where both Tem and Tcm are found in the lymphoid organs. Additionally, unlike Th1 cells, there is no clear delineation of Tem and Tcm that correlates with CXCR5 expression in the lymphoid organs, demonstrating that Th1 and Th2 CD4$^+$ T cells undergo different programs of memory cell differentiation. Since CCR7$^+$ Tcm have greater proliferative capacity and plasticity than CCR7$^-$ Tem, this finding raises interesting questions about allergen-specific Tcm and their ability to propagate the allergic response with continuous exposure to allergen.

These studies also demonstrate that allergen-specific Th2 cells that persist in lungs are CD4$^+$ Trm cells. CD4$^+$ Trm have been shown to be important mediators of protection in response to infection with Sendai (Hogan, 2001), influenza virus (Teijaro et al., 2011) or HSV-2 (Iijima and Iwasaki, 2014; Shin and Iwasaki, 2012). CD4$^+$ Trm have also recently been shown to enhance protection to *Leishmania* in the skin (Glennie et al., 2015). This appears to be tissue and/or pathogen-specific however, since epicutaneous infection with HSV-1, does not promote CD4$^+$ T cell residency (Gebhardt, 2011, Nature). Using parabiotic mice, we show that CD69$^+$ allergen-specific cells are resident and are not recovered in the lungs of naive parabionts. Furthermore, treatment of memory mice with FTY720, which isolated Trm cells in the lung, demonstrated that allergen-responsive cells in the lung are sufficient to mediate AHR. These findings are supported by clinical studies in which a lung transplant from mildly asthmatic donor patients into non-asthmatic recipients can transfer airway disease, while transplant of a non-asthmatic lung into an asthmatic patient can ameliorate disease (Corris and Dark, 1993).

Th1 memory formation was previously shown to depend upon competition between IL-2 signaling and BCL6 (Pepper et al., 2011). It was unknown if this framework applied to Th2 memory or Trm cells. Using mixed bone marrow chimeras, these studies demonstrated that although formation of Th2 memory in the SLO was largely normal, CD25 was required for lung Th2 Trm differentiation while BCL6 was not. In support of this finding, a previous study revealed that Treg deficient scurfy (Sf) mice that lack Foxp3 display severe skin, liver and lung inflammation while IL-2 deficient mice that lack both Tregs and IL-2 signaling by effectors do not display inflammation in the skin and lungs (Zheng et al., 2007). This study demonstrates that allergen-specific CD4$^+$ T cells express a broad program of migrational cues regulated by IL-2. Of interest, expression of CXCR3, demonstrated in these studies to

be diminished on cells lacking IL-2 signaling, was previously shown to be important for $CD4^+$ effector T cell migration to the lung during parainfluenza virus infection (Kohlmeier et al., 2009). While specific programs downstream of IL-2 signaling *in vivo* remain unclear, one possibility is IL-2-mediated regulation of the transcription factor KLF2. KLF2 contributes to the regulation of lymphocyte migration out of the lymphoid tissues through the control of CCR7 and SIPR1 (Schober, 1999). Consistent with this hypothesis, inhibition of the PI(3)K-AKT pathway ameliorates cytokine-induced down-regulation of KLF2 and SIPR1, suggesting that this may be an important downstream mediator of IL-2 in these studies (Skon, 2013). Our data demonstrating a partial loss of $CD62L^-CCR7^-$ Teff cells six days after a primary immunization further supports this argument. This data raises important questions about whether treatment for specific cancers with recombinant IL-2 or drugs like Daclizumab that inhibit IL-2 signaling by binding CD25 are directing $CD4^+$ T cell trafficking and residency, more than function.

Understanding what retains $CD4^+$ allergen-specific Trm in the lungs and how these signals are regulated will be essential for developing new therapies and vaccines. Expression of CD69, shown to be required for both $CD8^+$ Trm residency in the skin (Mackay et al., 2015) as well as $CD4^+$ memory cell residency in the bone marrow (Shinoda et al., 2012) may be involved in tissue retention as the proportion of $CD69^+$ cells in the lung increases with time and $CD69^+$ cells were absent in naive parabiont lungs. We are currently assessing the importance of this program and dissecting the signals upstream of CD69 that may be involved.

Experimental Procedures

Mice

Male and female C57BL/6, B6.SJL-$Ptprc^a$ $Pepc^b$/BoyJ ($CD45.1^+$), B6(Cg)-$Rag2^{tm1.1Cgn}$/J, B6.129S4-$Il2ra^{tm1Dw}$/J (CD25KO), B6.129S2-$Ighm^{tm1Cgn}$/J (μMT)-$Rag2^{tm1.1Cgn}$/J and B6.129S2-$Ciita^{tm1Ccum}$/J (Class II KO) mice were purchased and maintained/bred under specific pathogen free conditions at the University of Washington. C57BL/6 human CD2 IL-4 reporter mice (KN2) and *4get* mice were provided by Dr. Mohrs (Trudeau Institute). Mice were maintained on homozygous backgrounds and bred to generate KN2/*4get* mice. KN2/+CD25KO mice were made by breeding CD25 heterozygote mice to KN2 mice. Mice were screened for KN2 and CD25 by PCR. C57BL/6 IL-13 eGFP reporter mice were provided by Drs. McKenzie (MRC) and Holtzman (Washington University). BCL6 heterozygote mice were obtained from Dr. Dent (Indiana University). CCR8 KO mice were obtained from Dr. Lira (Icahn School of Medicine). All experiments were performed in accordance with the University of Washington Institutional Care and Use Committee guidelines.

Bone Marrow Chimeras

Mixed bone marrow chimeras were generated as described previously (Pepper et al., 2011). Briefly, bone marrow cells were depleted of T and NK cells, and wild-type cells and BCL6 deficient or CD25 deficient cells were mixed in equal portions. Recipient mice were lethally

irradiated (1000 rads) and injected with 5×10^6 total bone marrow cells and given antibiotic treated water for 6 weeks.

Immunizations

Whole house dust mite antigen (HDM) (Greer, NC) is resuspended in PBS. Mice are anaesthetized with ketamine/xylazine and given the equivalent of 23 μg of Derp1 protein i.n. for the primary immunization. HDM challenge occurs on day 10–14 with the equivalent of 5.75 μg of Derp1. For FTY720 (Enzo Life Science, Farmingdale, NY) experiments, treated mice were given 25 μg of FTY720 for the indicated days i.p (Suppl. Fig. 3).

Tetramer production

Biotinylated I-A^b molecules containing the covalently attached Derp1 114–124 (SNYCQIYPPNV) or Derp1 117–127 (CQIYPPNVNKI) epitope (nonamer core plus two N-terminal flanking amino acids) were made as previously described (Moon et al., 2007). Monomers were tetramerized with streptavidin-PE or streptavidin-APC (Prozyme).

Isolating single cell suspensions of lung cells and secondary lymphoid organs

Mice were injected i.v. with 1 μg of anti-Thy1.2-PE-Cy7, APC-eFluor 780, or Thy1.2 BUV395 (53-2.1) for ~3 minutes. After CO_2 asphyxiation, the SLO (cervical, mediastinal, axillary, brachial, pancreatic, renal, mesenteric, inguinal, lumbar, and popliteal Lns) were pooled and mashed through Nitex mesh (Amazon.com). Lungs were diced with scissors and forced through a 70–100μm filter with a 3 cc syringe. Lungs were placed in HEPES buffer (10 μM HEPES, 5mM KCl, 1.8mM $CaCl_2$, 150 mM NaCl, 1mM $MgCl_2$) containing Liberase (70 μg/ml) (Roche #05401127001) and aminoguanidine (10mM) (Sigma) and tissue was dissociated on the gentleMACS Dissociator (Miltenyi Biotec). Lungs were incubated for 30 minutes at 37°C, then again dissociated. Lung cell suspensions were poured over a 70 μm mesh and washed with DMEM with 10% fetal calf serum to inhibit liberase function.

Additional procedures are detailed in Supplemental Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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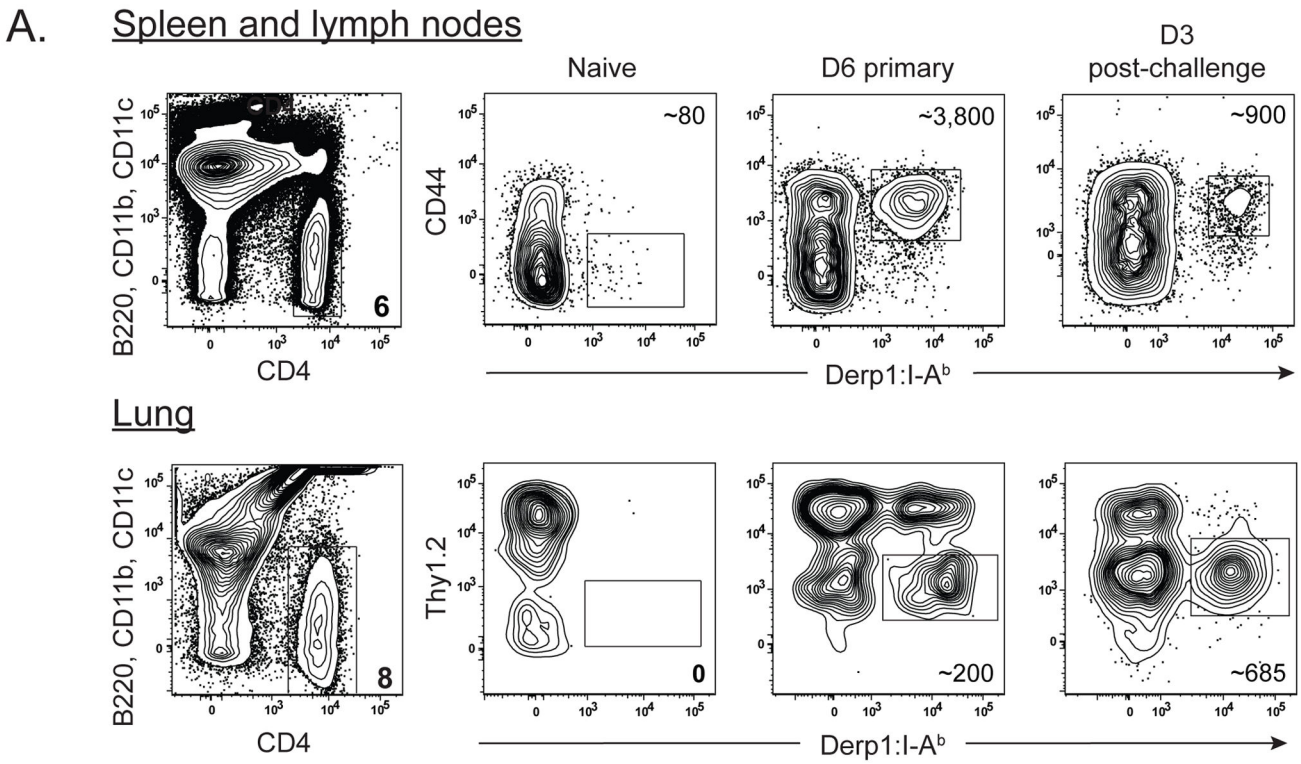

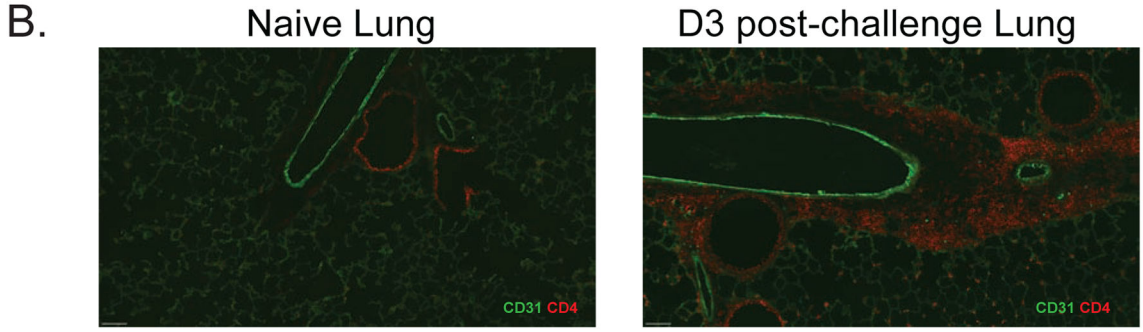

Figure 1. Detection of Derp1^{+} CD4^{+} T cells

A) Gating scheme to identify naïve or antigen-experienced Derp1+ CD4^{+} T cells in the SLO (top row) and lung (Thy1.2^{-}) (bottom row). B) Representative immunoflourescent sections of lungs from naïve (left panel) or day 3 post-challenge (right panel) mice showing the presence of CD4^{+} T cells only in the lungs of challenged mice (AW = airway and BV = blood vessel). These data were from one of two independent experiments from a total of 3 naïve and 3 challenged mice. See also Fig. S1.

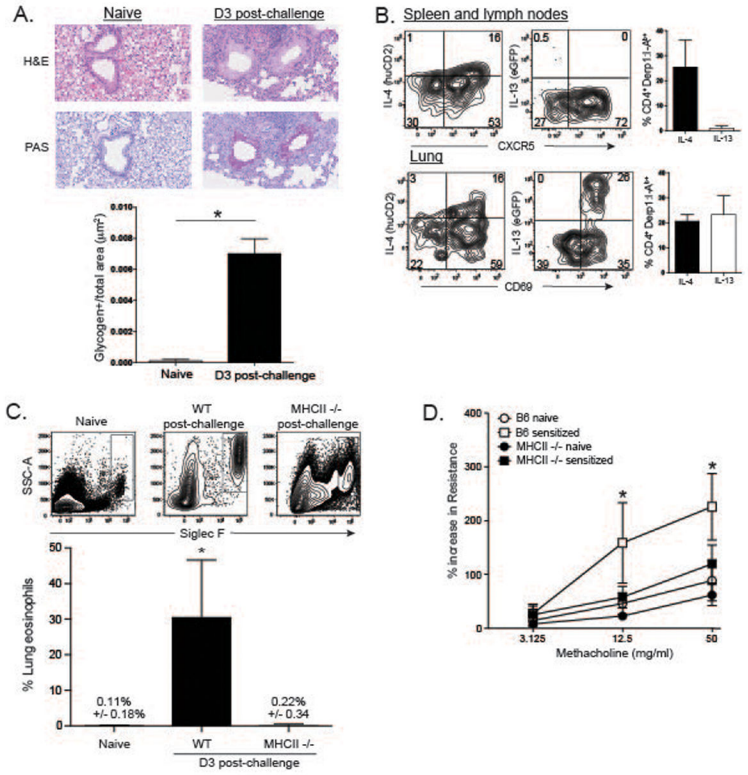

Figure 2. Derp1^{+} specific cells produce Th2 type cytokines and cause inflammation

A) H&E and PAS stains of naïve or day 3 post challenge lungs. Top row shows the cellular infiltrate in sensitized lungs (right) compared to lungs from naïve mice (left). The PAS sections show glycogen (purple, right panel) production in lungs described above. Bar graph depicts the average (+/− S.D.) amount of glycogen in lungs of naïve or day 3 post challenge mice. The data shown are from 10 sections from two independent experiments with a total of 3 mice in each group and (*) ($p<0.05$) indicates statistical significance. B) Representative huCD2 (IL-4, left column) and IL-13 (middle column) plots from KN2 (IL-4) and IL-13eGFP reporter mice, respectively. Data are gated on CD4^{+} Derp1^{+} events acquired 3 days after challenge and were measured in 2–3 separate experiments. Bar graphs show the average percentage (+/− S.D.) of IL-4 and IL-13 in the SLO (top row) or lung parenchyma (bottom row). Data are the combined average from two independent experiments (n=5 for IL-4 expression, n=4 for IL-13 expression). C) Contour plots showing SSC^{+} Siglec F^{+} eosinophils from naïve WT or immunized WT or MHCII−/− mice (3 days post challenge). Graph shows the percentage of gated live cells from lungs that were eosinophils (CD3^{-} Class II^{-} CD11b^{+} Siglec F^{+} SSC^{+}). Data were collected from 2–9 separate experiments (naïve mice: n=4, WT challenged mice n=17, Class II KO challenged mice n=4). (*) indicates significantly different ($p<0.05$) compared to naïve and Class II KO mice. D) Graph shows the increase in airway resistance versus PBS control from naïve or immunized B6 and Class II KO mice. The data shown were collected from two experiments and the average +/− S.D. is graphed (naïve B6 n=4, immunized B6 n=4, naïve MHC class II KO n=3, immunized MHC Class II KO n=4) and (*) ($p<0.05$) indicates statistically different compared to all other groups. See also Fig. S2.

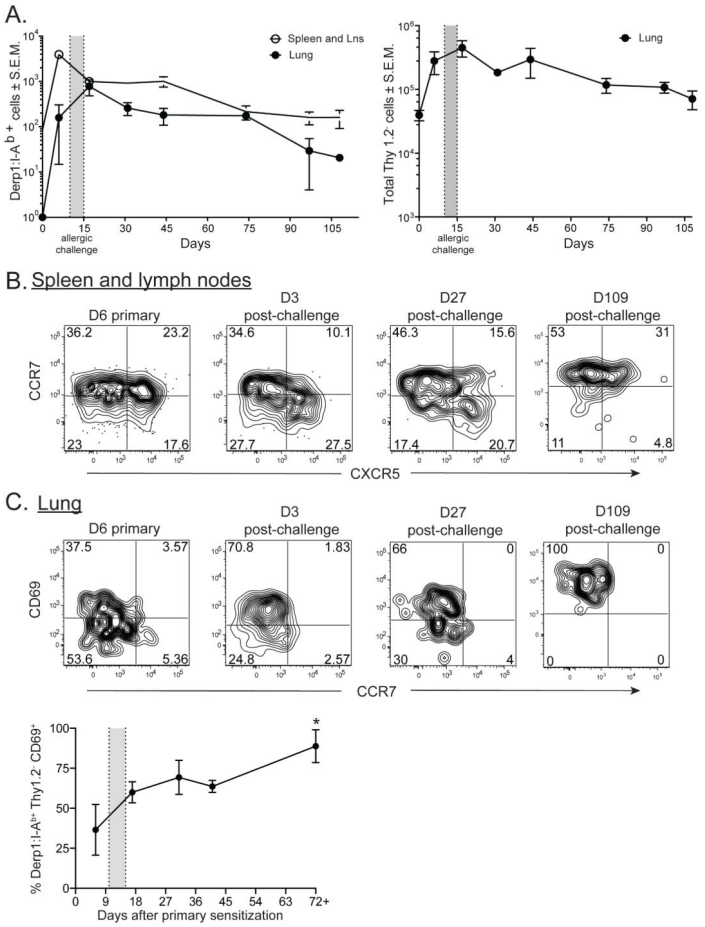

Figure 3. Derp1^{+} memory cells are primarily CCR7^{+} in the spleen and lymph nodes but CD69^{+} CCR7^{-} in the lung

A) A timecourse of the number of CD4^{+} Derp1^{+} cells in the SLO (left, white circles), Thy1.2^{-} Derp1^{+} cells in the lung (left, black circles), and total Thy1.2^{-} CD4^{+} in lungs (right). Data for the graph were compiled from two experiments with 2–5 mice per timepoint. B) Representative contour plots of CCR7 and CXCR5 expression from CD4^{+} Derp1^{+} cells from the SLO. C) Representative contour plots of CCR7 and CD69 expression from Thy1.2^{-} CD4^{+} Derp1^{+} cells from lungs. The graph shows the average percentage +/− S.D. of CD4^{+} Thy1.2^{-} Derp1^{+} cells that are CD69^{+} at indicated timepoints in lungs. The grey column shows the time of allergic challenge. The represented data were collected from two experiments with 2–5 mice per timepoint. (*) indicates significantly higher percentage of CD69 compared to all other timepoints ($p<0.05$).

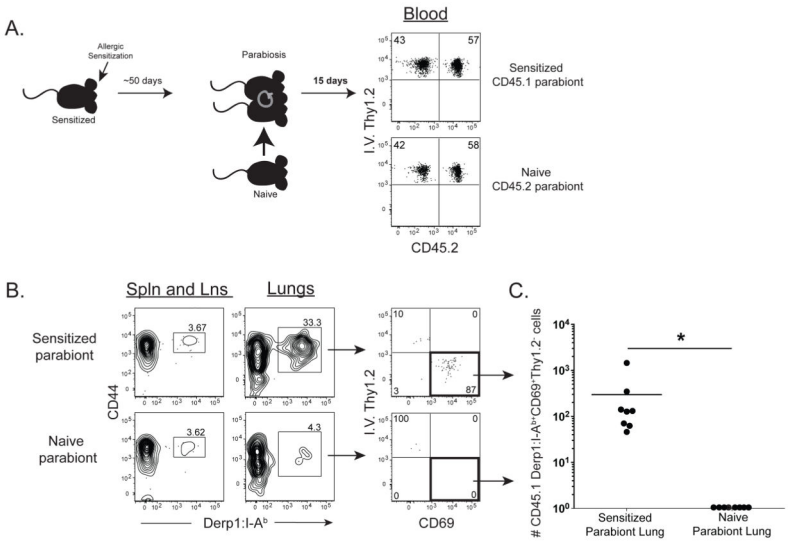

Figure 4. HDM immunization induces CD4$^+$ CD69$^+$ Derp1$^+$ Trm in the lung

A) Cartoon summary of the parabiosis protocol. Dot plots demonstrate that i.v. anti-Thy1.2 antibody labeled all blood T cells and that both mice in the parabiont pair had roughly equal amounts of cells from naïve and immunized parabionts. B) Representative contour plots showing CD4$^+$ Derp1$^+$ cells from the SLO and lungs from immunized and naïve parabionts. The representative dot plots in the third column show that the resident CD4$^+$ CD44$^+$ Derp1$^+$ are CD69$^+$ and only found in the immunized parabiont. C) The graph indicates the number of CD69$^+$ Derp1$^+$ cells per mouse (n=8) found in lungs of naïve and immunized parabionts. The experiment was performed twice with 4 mice in each group and the (*) indicates that the two groups are significantly different ($p<0.05$).

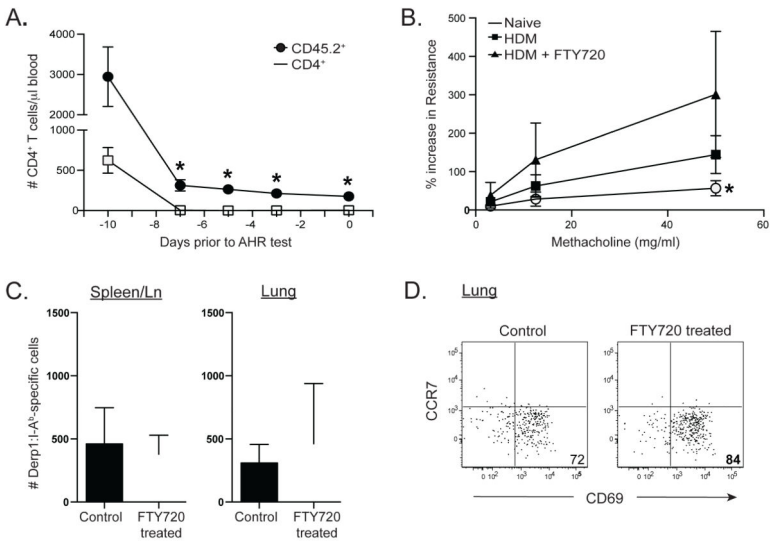

Figure 5. Resident lung cells are sufficient to induce AHR

A) Graph demonstrates the number of $CD45.2^+$ (black circle) and $CD4^+$ (white squares) in the blood before and during FTY720 administration. Each time point shows the average number +/− S.D. from 3 mice of the indicated cell population per µl/blood from one of two experiments. (*) significantly less $CD45.2^+$ and $CD4^+$ cells during FTY720 treatment compared to pre-treatment ($p<0.05$) B) Graph shows the increase in airway resistance to increasing doses of methacholine versus PBS. Each point on the graph is the average +/− S.D. airway resistance from 5 mice from two independent experiments. (*) indicates significantly greater airway resistance versus naïve B6 mice ($p<0.05$). C) The bar graphs indicate average +/− S.D number of $CD4^+$ $Derp1^+$ cells from 5 mice in SLO or lungs from control (black bars) or FTY720 treated (white bars) mice. Data were compiled from two experiments. D) Representative dot blot from two experiments showing CCR7 and CD69 expression on $Thy1.2^-$ $CD4^+$ $Derp1^+$ cells from lungs of control or FTY720 treated mice. See also Fig. S3.

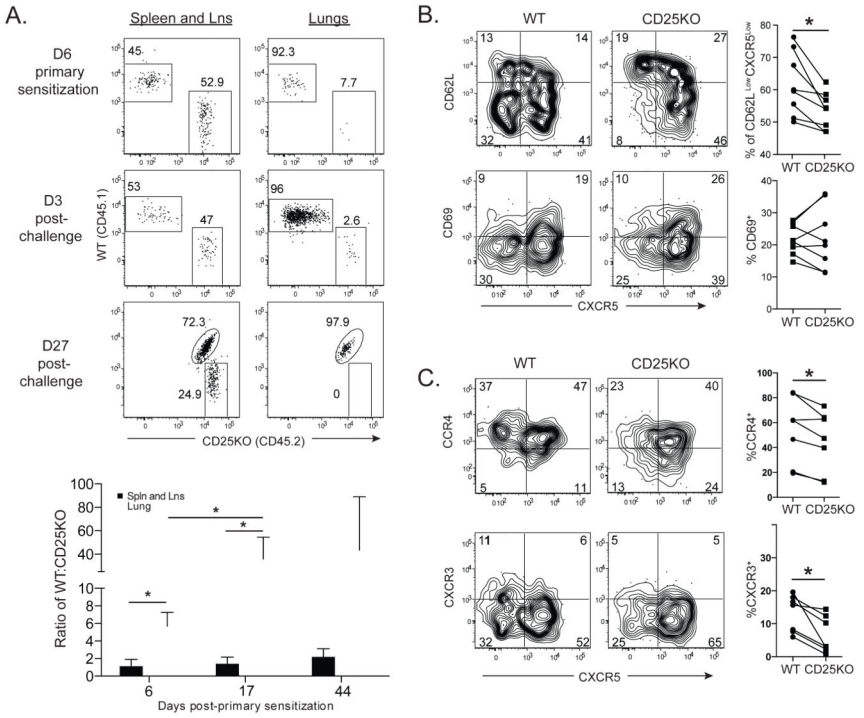

Figure 6. Trm accumulation in the lungs is dependent on CD25

A) Representative dot plots from WT:CD25KO mixed bone marrow chimeric mice 6 days after various timepoints showing the percent of $CD4^+$ $CD44^+$ $Derp1^+$ originating from WT versus CD25KO mice. Bar graphs depict the ratio of the percentage WT/CD25KO $CD4^+$ $CD44^+$ $Derp1^+$ cells from 6 days post primary sensitization (n=4 from 3 separate experiments), day 3 post challenge (n=5 from 3 independent experiments), and 27 days post challenge (n=2 from 1 experiment). (*) indicates a significant difference between the indicated groups ($p<0.05$). B) Representative contour plots from WT:CD25KO mixed bone chimeras six days after primary sensitization showing CD62L or CD69 levels by CXCR5 expression on $CD4^+$ $Derp1^+$ in the SLO. Plots show percentage of $Derp1^+$ cells that are $CXCR5^-$ $CD62L^-$ (top) or the total percentage of $CD69^+$ cells (bottom) from SLO of individual WT:CD25KO chimeric mice. Data are compiled from 7–8 mice from four independent experiments. (*) indicates a significant difference between the WT and CD25KO cells ($p<0.05$). C) CCR4 and CXCR3 expression were compared in $CD4^+$ $Derp1^+$ cells from WT and CD25KO cells in WT:CD25KO mixed BM chimeras. Contour plots depict the indicated chemokine receptor expression 6 days after primary sensitization from $CD4^+$ $Derp1^+$ WT or CD25KO cells. Both CCR4 and CXCR3 expression (*) ($p<0.05$) are significantly greater in WT $Derp1^+$ cells versus CD25KO $Derp1^+$ T cells. Data were compiled from 4 independent experiments with a total of 7 mice. See also Fig. S4–S6.

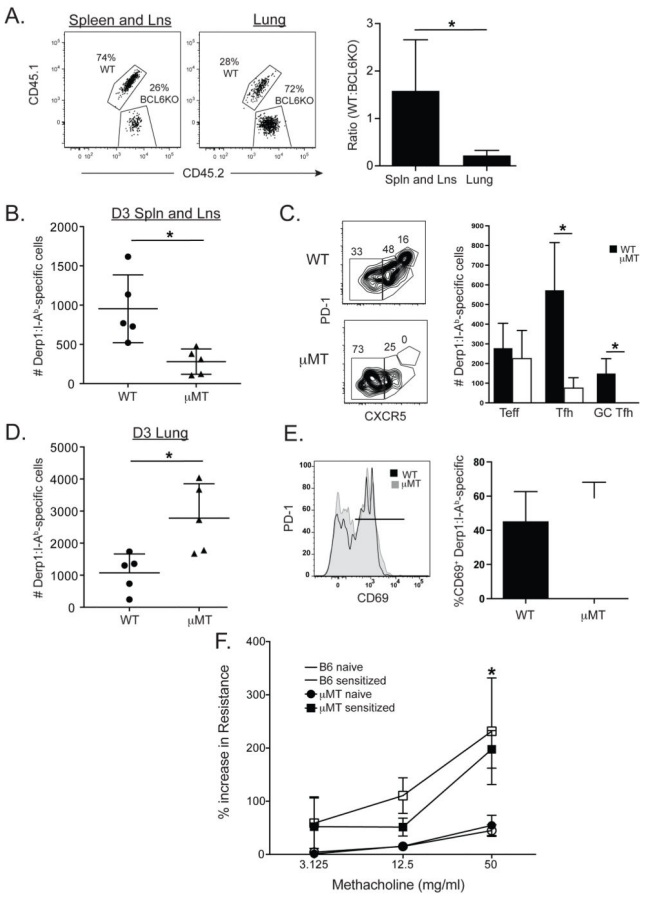

Figure 7. Trm accumulation in the lungs is inhibited by BCL6 and B cells

A) Dot plots showing $CD4^+$ $CD44^+$ $Derp1^+$ from WT:BCL6KO mixed bone marrow chimeras 3 days after challenge in the SLO (left dot plot) and lung (right dot plot). Bar graphs represent data from two separate experiments using four mice total showing the ratio of $CD44^+$ $Derp1^+$ of WT/BCL6KO origin in the SLO and lungs. (*) indicates the ratios are significantly different in the SLO versus the lungs ($p<0.05$). B) Graphs depict total numbers of $CD4^+$ $Derp1^+$ in the SLO and D) lungs 3 days after challenge. Each dot represents an individual mouse from a total of three separate experiments. (*) indicates that number of $Derp1^+$ cells are significantly different between the two groups ($p<0.05$). C) Representative contour plots of PD1 and CXCR5 expression on $CD4^+$ $Derp1^+$ cells in the SLO of WT and µMT mice three days after challenge. Numbers in the contour plots indicate the percentage of cells in each gate shown. Bar graph shows the total number of $CD4^+$ $Derp1^+$ being : Teff ($CXCR5^-$), Tfh ($CXCR5^{int}$), and GC Tfh ($PD1^+$ $CXCR5^+$). Data were compiled from five mice in three separate experiments and (*) indicate statistical differences between WT and µMT mice ($p<0.05$). E) Histogram showing CD69 expression on $Thy1.2^-$ $CD4^+$ $Derp1^+$ cells in the lung 3 days after challenge between WT (black line) and µMT mice (shaded grey). The bar graph depicts the percent $CD69^+$ from $Thy1.2^-$ $CD4^+$ $Derp1^+$ cells in the lungs from five mice each in three independent experiments. F) Graph shows the increase in airway resistance to increasing doses of methacholine versus PBS. Each point on the graph

is the average +/− S.D. airway resistance from 3–6 mice from 2–3 separate experiments. (*) indicates significantly greater airway resistance versus naïve B6 or μMT mice ($p<0.05$).