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Interleukin-9 promotes mast cell progenitor proliferation and CCR2-dependent mast cell migration in allergic airway inflammation

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Abstract

Allergic asthma is a chronic lung disease characterized by airway hyperresponsiveness and cellular infiltration that is exacerbated by immunoglobulin E-dependent mast cell (MC) activation. Interleukin-9 (IL-9) promotes MC expansion during allergic inflammation but precisely how IL-9 expands tissue MCs and promotes MC function is unclear. In this report, using multiple models of allergic airway inflammation, we show that both mature MCs (mMCs) and MC progenitors (MCp) express IL-9R and respond to IL-9 during allergic inflammation. IL-9 acts on MCp in the bone marrow and lungs to enhance proliferative capacity. Furthermore, IL-9 in the lung stimulates the mobilization of CCR2+ mMC from the bone marrow and recruitment to the allergic lung. Mixed bone marrow chimeras demonstrate that these are intrinsic effects in the MCp and mMC populations. IL-9-producing T cells are both necessary and sufficient to increase MC numbers in the lung in the context of allergic inflammation. Importantly, T cell IL-9-mediated MC expansion is required for the development of antigen-induced and MC-dependent airway hyperreactivity. Collectively, these data demonstrate that T cell IL-9 induces lung MC expansion and migration by direct effects on the proliferation of MCp and the migration of mMC to mediate airway hyperreactivity.

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AUTHOR CONTRIBUTIONS

A.P. and M.H.K. provided substantial contributions to the conception of the work and writing the manuscript. A.P. Y.F., and M.H.K. designed experiments. All authors contributed to the acquisition, analysis, or interpretation of data for manuscript and drafting, revising, and critically reviewing the manuscript for important intellectual content. All authors approved the final version of the manuscript to be published.

DECLARATIONS OF COMPETING INTEREST

The authors declare that they have no competing interests.

APPENDIX A. SUPPLEMENTARY DATA

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INTRODUCTION

Allergic asthma is a chronic lung disease characterized by allergen-induced airway hyperresponsiveness (AHR), eosinophilic infiltration, and intermittent airway obstruction[1]. Airway inflammation and AHR are further exacerbated by immunoglobulin (Ig)E-dependent mast cell (MC) activation[2]. Upon allergen exposure, MCs degranulate to release pro-inflammatory mediators, such as histamine, proteases, leukotrienes, cytokines, and chemokines, that mediate immediate hypersensitivity and contribute to the pathological features of allergic asthma[3,4].

Among the cytokines that regulate MCs, interleukin 9 (IL-9) was initially identified as an MC growth factor[5,6]. IL-9 signals by binding to its IL-9 receptor (IL-9R) consisting of a unique α chain (IL-9Rα) and a common γ chain. IL-9R is expressed in a variety of cells including epithelial cells, fibroblasts, granulocytes, lymphocytes, macrophages, and MCs[7]. The role of IL-9 in asthma pathophysiology and disease control has gained considerable attention[8–10]. Increasing evidence for the crucial role of IL-9 in allergic asthma is derived from clinical studies in which patients exhibited significantly elevated IL-9 and clusters of differentiation (CD4+) T helper 9 (T_H9) cell numbers in the peripheral blood[11–15]. Consistent with these reports, elevated IL-9 expression strongly correlated with high serum IgE concentration in atopic patients, suggesting that IL-9 is associated with IgE-mediated allergic responses[16]. In asthma models, IL-9 is required for MC accumulation[17–19], and IL-9 from T cells is required for MC expansion during allergic airway inflammation[20]. Yet, although MCs have the highest expression of IL-9R in the allergic lung[8,9,12,21], exactly how IL-9 regulates MC expansion and function *in vivo* is not well described.

MCs are tissue-resident cells of hematopoietic origin that complete their differentiation in the peripheral microenvironment. Although both MCs and basophils express FcεRI and may derive from common precursors[22,23], mature cells can be distinguished by their divergent expression of c-Kit and CD49b (Dx5)[23,24]. In mice, as MC progenitors (MCp) leave the bone marrow, they can be identified in the blood by Lineage $(Lin)^-$ c-Kit^{hi} $ST2^+$ integrin β7 $(Itgβ7)^{hi}$ CD16/32 $(FcγRII/III)^{hi}$ with variable maturity defined by FcεRI expression[25]. This is consistent with multiple studies in mice utilizing expression of c-Kit, FcεRI, and Itgβ7 to distinguish MC maturation status[23,26]. Thus, while both MCp and mature MC (mMCs) express c-Kit and FcεRIα, there is a consensus that MC differentiation can be distinguished using flow cytometric analysis of Itgβ7, FcγRII/III, and side scatter (SSC) profile[26–29].

In mouse models of allergic airway disease, MCp in the bone marrow can migrate to the allergic lung in a CCR2/CCL2, CXCR2/ CXCL3, and α4β7/VCAM-1-dependent manner[30–33]. Moreover, studies in mice have identified that IL-9 contributes to MC accumulation in lung tissue[8,9,20,21,34]. However, it is unclear if IL-9-mediated MC expansion is selective and if IL-9 only acts on tissue-resident mMCs.

Here, we define the mechanism for IL-9-mediated MC expansion in the allergic lung. We show that IL-9R is expressed at multiple stages of MC development and that proliferation and migration of MCs following allergen challenge was dependent upon IL-9 signaling.

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Additionally, we identify IL-9-producing CD4+ T cells as controllers of MC-dependent AHR.

RESULTS

IL-9R is constitutively expressed in multiple stages of MC development

To investigate if IL-9 affects MC at different stages of development, we first defined the expression of IL-9R on MCp and mMC within the lungs of naïve mice, identified based on published gating strategies: MCp (Lin^- $CD45^+$ $c\text{-}kit^+$ $CD49b^-$ $ST2^+$ $Itg\beta7^+$ $CD16/32^{int}$ $Fc\varepsilon RI\alpha^+$ SSC^{lo}) and mMC (Lin^- $CD45^+$ $c\text{-}kit^+$ $CD49b^{-/int}$ $ST2^+$ $Itg\beta7^{-/lo}$ $CD16/32^+$ $Fc\varepsilon RI\alpha^+$ SSC^{hi}) (Fig. 1A)[27,28]. A previous report demonstrated that in response to allergen exposure, IL-9R is upregulated in MCs compared to naïve controls[8]. In line with this, IL-9R was highly expressed on MCp and mMC with the greatest expression on mMC (Figs. 1B and 1C). Studies also show that inhibiting IL-9 signaling leads to significantly reduced lung MC numbers, indicating that MCs are highly responsive to IL-9 during an allergic response[8,9,20]. To begin to determine how IL-9 impacts MC populations in the lung, we investigated the effects of IL-9 blockade on MCp and mMC numbers in a chronic house dust mite (HDM) allergen exposure model using control or IL-9 neutralizing antibody during the last 2 weeks of HDM treatment (Fig. 1D). Blockade of IL-9 led to diminished MC accumulation in the trachea, while also significantly reducing MCp and mMC numbers in the bone marrow and lungs (Figs. 1E–G). Together, these data demonstrate that IL-9 can act on both MCp and mMC during an allergic airway inflammatory response.

IL-9-mediated MC expansion is tissue-specific

We next sought to test whether local IL-9 production in the lungs has systemic effects on other peripheral tissues. To analyze the ability of IL-9 to mediate effects in the airways, we treated wild-type (WT) mice intranasally with recombinant IL-9 (rIL-9) for 3 consecutive days. Compared to phosphate-buffered saline (PBS)-treated mice, IL-9-treated mice exhibited MC accumulation in the trachea, but not in the skin or tongue (Fig. **b** and Supplementary Fig. 1A). However, bone marrow and lung MCs exhibited expansion in response to intranasal IL-9 that was not observed in the blood, mediastinal lymph node (draining lymph node), spleen or peritoneal cavity (Figs. 2B–D and Supplementary Fig. 1B). In contrast, intranasal IL-9 was insufficient to expand lung neutrophils, eosinophils, or basophils; suggesting that these cell types do not independently respond to IL-9 in this assay (Figs. 2E–G). Therefore, expansion of MC populations in the bone marrow and lungs following IL-9 administration likely reflects a cell type-specific and tissue-specific effect of IL-9 in the airways.

We then tested whether our findings are relevant for human MCs by generating humanized NSG-SGM3 mice that develop human MC and intranasally treating these mice with recombinant human IL-9 for 3 days (Fig. 2H)[35]. Similar to our findings in murine MC, we demonstrate that intranasal IL-9 enhances MC numbers in the lungs and bone marrow (Figs. 2I and 2J). Together, these findings support a conserved function of IL-9 in human and mouse MC expansion in the lungs and bone marrow.

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IL-9 acts intrinsically on bone marrow MCs

To distinguish between direct and indirect effects of IL-9 on MCp and mMC, we utilized a mixed bone marrow chimera experiment in which C57BL/6 x Boy/J F1$^{CD45.1+\ CD45.2+}$ recipients were irradiated and reconstituted with bone marrow from donor WT$^{CD45.1+}$ and *Il9r*$^{-/-\ CD45.2+}$ (Fig. 2K). Prior to HDM treatment, chimeric mice were confirmed to express comparable frequencies of bone marrow reconstitution in the blood (Fig. 2L). After treatment with HDM, we observed greater frequencies of eosinophils, consistent with the induction of allergic airway inflammation (Fig. 2M). *Il9r*$^{-/-}$-derived cells were enriched in the bone marrow mMC population, while lung MC were primarily WT-derived cells (Fig. 2N). The preferential increase of Il9r$^{-/-}$ mMC in the bone marrow could arise from altered MCp expansion and differentiation or that IL-9 signaling had an impact on mMC mobilization from the bone marrow.

IL-9 enhances MCp proliferative capacity

To directly test the impact of IL-9 signaling on MCp expansion, we analyzed mixed bone marrow chimeras to assess the contribution of WT and *Il9r*$^{-/-}$ -derived cells on MCp expansion in the bone marrow and lungs. MCp in the lungs and bone marrow were primarily WT-derived cells, indicating that MCp is largely dependent upon IL-9 signaling for expansion (Fig. 3A). From these results, we speculated that the stimulatory effects of IL-9 on MC may impact stem cell factor-dependent proliferative capacity that was observed in a previous study[5]. To test this, we used bone marrow-derived MC cultures grown in IL-3 and stem cell factor (SCF) and stimulated these cells with IL-9 for 2 hours. IL-9 increased bone marrow-derived MCp but not mMC proliferation (Figs. 3B and 3C). A short 3-day treatment with intranasal IL-9 was sufficient to induce lung but not bone marrow MCp proliferation (Fig. 3D).

We then compared the ability of allergen challenge to alter proliferative capacity. After a 6-week HDM chronic challenge, bone marrow and lung MCp and mMC displayed greater Ki67 expression compared to PBS-treated controls (Fig. 3E). To examine whether IL-9 contributed to allergen-dependent MCp proliferation *in vivo*, we utilized IL-9 deficient mice (*Il9*$^{-/-}$) and treated the mice with HDM for 6 weeks. *Il9*$^{-/-}$ mice exhibited diminished MCp and mMC proliferation in the bone marrow (Figs. 3F and 3G). Likewise, IL-9 deficiency led to reduced lung MCp proliferation, whereas mMC displayed a trending decrease in proliferation as compared to PBS controls (Figs. 3H and 3I). This is consistent with mMC having a limited proliferative potential[36]. Together, these results indicate that IL-9 signaling enhances MCp proliferative capacity but that although IL-9 promotes the expansion of mMC in the lung, it does not promote the proliferation of lung mMC.

IL-9 promotes CCR2-dependent bone marrow MC recruitment to the allergic lung

Previous studies have shown that IL-9 promotes CCR2-mediated bone marrow monocyte migration to the lung and that MC can migrate in a CCR2/CCL2-dependent manner to the allergic lung in BALB/c mice[8,30]. Thus, we hypothesized that IL-9 may be involved in CCR2-mediated lung MC recruitment. Supporting our hypothesis, using the bone marrow chimera model in Fig. 2, we found that 10–20% of the MC in the allergic lung express CCR2 and that CCR2+ MCs were primarily WT-derived, suggesting that IL-9 impacts

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CCR2 migration (Fig. 4A). We then tested whether blockade of CCR2 signaling impacts MC migration in a chronic HDM allergen model (Fig. 4B). Consistent with previous reports, blockade of CCR2 led to diminished eosinophil frequencies in the lungs, demonstrating effective blockade of CCR2 (Fig. 4C)[37]. Notably, compared to vehicle dimethylsulfoxide (DMSO)-treated controls, blockade of CCR2 led to greater frequencies of mMC in the bone marrow while mMC numbers were profoundly diminished in the trachea and lungs, indicating that mMCs utilize CCR2 to emigrate from the bone marrow to the lungs (Figs. 4D–F). Next, we tested whether IL-9 directly impacts CCR2 expression on MC. Using bone marrow-derived MC (BMMC) cultures, stimulation with IL-9 enhanced CCR2 expression, while neutralizing IL-9 decreased CCR2 expression (Figs. 4G and 4H).

To further explore how IL-9 affects CCR2-dependent MC migration, we utilized an *in vitro* migration assay of BMMC cultures to test chemoattraction to CCL2. We found that IL-9 or CCL2 alone did not promote MC migration; however, the combination of IL-9 and CCL2 resulted in significant MC migration (Fig. 4I). IL-9-induced MC migration was CCL2-specific, since IL-9 was unable to enhance MC migration toward CXCL1, CXCL2, CXCL3, CXCL8, or CXCL12 (Supplementary Fig. 2A). Moreover, IL-4 in combination with CCL2 was unable to promote MC migration, suggesting that not all type 2 cytokines impact this response (Supplementary Fig. 2B).

We next sought to test whether IL-9 induced CCR2-dependent MC migration *in vivo* using intranasal IL-9 treatment and intravenous treatment with the CCR2 inhibitor (Fig. 4J). Blockade of CCR2 in rIL-9-treated mice significantly increased mMC numbers in the bone marrow and reduced mMC accumulation in the lungs (Figs. 4K and 4L). We further demonstrate that blockade of CCR2 led to diminished MC protease 1 (MCPT1) and IL-6 expression in the bronchoalveolar lavage fluid (BALF) (Fig. 4M), suggesting that the accumulation of MCs in the lung greatly contributes to the amount of MC degranulation markers: MCPT1 and IL-6 levels in the BALF. Taken together, these results indicate that bone marrow MCs respond to IL-9, enhancing CCR2 expression to facilitate migration to the allergic lung.

IL-9 has also been shown to enhance interstitial macrophage expansion and function in allergic airway inflammation in an IL-9-dependent manner[8]. Since monocytes and macrophages are known to migrate in a CCL2/CCR2 axis in allergic airway inflammation[8,38], we wanted to confirm whether blockade of CCR2 impacted macrophage recruitment to the lungs. Indeed, blockade of CCR2 reduced macrophage frequencies in the lungs (Supplementary Fig. 3A). To test whether IL-9 responsive macrophages impact MC expansion, we depleted macrophages in WT mice using clodronate liposomes prior to intranasal IL-9 treatment (Supplementary Fig. 3B). We demonstrated that a single dose of clodronate was sufficient to reduce macrophage numbers in the lungs (Supplementary Fig. 3C). Importantly, there was a significant increase in MC numbers even when macrophages were depleted, although the absolute numbers were diminished compared to mice treated with control liposomes (Supplementary Fig. 3D). These findings, coupled with the bone marrow chimera results and *in vitro* assays showing that IL-9 promotes MCp proliferation and STAT5 activation (Figs. 3A–C and Supplementary Fig. 3E), suggest that IL-9 acts

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directly acts on MC to promote expansion in the allergic lung and that macrophages may contribute to MC expansion.

T cell-derived IL-9 promotes MC expansion in the allergic lung

CD4+ T helper 9 cells (T_H9) are an important source of IL-9 in mouse models of allergic airway inflammation[8,20,39,40]. MC and innate lymphoid cells have been demonstrated to contribute to the pool of IL-9 in allergic airway inflammation[17]. Thus, we first assessed the contribution of T cell IL-9 in comparison to MC and ILCs IL-9. Using our chronic allergen model, we demonstrate that T cells contribute the majority of IL-9 in the lung after chronic allergen exposure (Supplementary Fig. 4A). Next, to define the role of T_H9-derived IL-9 on MC expansion, we adoptively transferred *in vitro* polarized OTII-derived T_H9 or T_H2 cells into WT recipient mice followed by intranasal ovalbumin (OVA) challenge for 5 days to elicit allergic airway inflammation (Fig. 5A). Following allergen challenge, adoptively transferred T cells were present in the lungs but was also found in the bronchoalveolar lavage and draining lymph node (Supplementary Fig. 4B). We further demonstrate that adoptive T cell transfer successfully sensitized mice to ovalbumin by showing increased serum OVA-specific IgE in T_H2 and T_H9 adoptive transfer conditions. (Supplementary Fig. 4C). Adoptive T cell transfer and treatment with ovalbumin for 5 days induced allergic airway inflammation as evident by eosinophil infiltration in the lungs (Fig. 5B). As compared to T_H2 cells or PBS controls, recipients of T_H9 cells exhibited greater MC numbers evident within the trachea, bone marrow, and lungs (Figs. 5C–G). Furthermore, we assessed MCp proliferative capacity and showed that T_H9 cell transfer led to enhanced lung MCp proliferation, in contrast to T_H2 cells or PBS controls (Fig. 5H).

To determine if T_H9 cells are a sufficient source of IL-9 mediating MC accumulation in the allergic lung, we adoptively transferred WT-derived *in vitro* polarized T_H9 cells into $Il9^{-/-}$ mice (Fig. 5I). T_H9 adoptive transfer led to greater MC accumulation in the trachea compared to PBS controls (Fig. 5J), and increased numbers of MCp and mMC in the bone marrow and lungs following T_H9 cell transfer and allergen challenge (Figs. 5K and 5L).

To directly test the requirement for T cell IL-9, we used a recently generated mouse strain with a conditional deletion of *Il9* transgene in T cells ($Il9^{fl/fl}$ $CD4^{cre}$)[40]. Following the allergen challenge, mice with T cell-specific IL-9 deficiency had diminished MC accumulation in the trachea and bone marrow (Figs. 5M and 5N). $Il9^{fl/fl}$ $CD4^{cre}$ mice exhibited reduced lung MCp numbers and decreased Ki67 expression (Figs. 5O and 5P). Moreover, we observed a decrease in lung mMC numbers and lung mMC CCR2 expression (Figs. 5Q and 5R). Thus, T cell IL-9 is a central source of IL-9 mediating MCp proliferation and CCR2-dependent mMC migration in allergic airway inflammation.

MC-mediated airway hyperresponsiveness is dependent upon T cell IL-9

MCs are important effector cells mediating airway hyperresponsiveness in response to allergen[41,42]. Having demonstrated that IL-9 promotes mMC recruitment to the lungs, we next wanted to investigate whether mMCs are required for antigen-induced airway reactivity using adoptive transfer of WT MC, or PBS as a control, into MC-deficient mice. To test this, we sorted mMCs from HDM chronically treated mice and transferred

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cells intranasally to MC-deficient *Kit*$^{WSH/WSH}$ recipient mice. Mice were then treated with HDM for 2 weeks to allow for expansion in the allergic lung (Fig. 6A). Transfer of mMC into the airways of *Kit*$^{WSH/WSH}$ recipients allowed for successful reconstitution of MC in the lung tissue (Supplementary Fig. 5A). To directly test antigen-induced airway hyperresponsiveness and avoid any confounding effects of IL-9 on IgE production, HDM-challenged mice were passively sensitized to 2,4-dinitrophenol (DNP) and challenged with DNP-BSA intratracheally (Fig. 6A). We found that the increased airway response to antigen challenge required mMC transfer, and there was minimal to no response in mice receiving PBS (Fig. 6B). We measured known human MC degranulation markers taken one hour after antigen challenge[35,43]. Transfer of mMC led to greater MC degranulation marker expression of MCPT1, IL-6, and CCL2 as compared to PBS transfer controls (Supplementary Fig. 5B), supporting the use of these cytokines as indicators of MC activation. These results demonstrate that mMCs respond to antigen challenge and are sufficient to induce antigen-induced airway reactivity.

To determine whether T cell IL-9 contributed to antigen-specific airway hyperresponsiveness, we performed airway testing using a similar passive sensitization and challenge model described in Fig. 6A. Treatment with an α-IL-9 blocking antibody, during the last 2 weeks of allergen treatments, protects against antigen-induced airway hyperresponsiveness compared to isotype controls (Fig. 6C). Moreover, adoptive transfer of T_H9 cells and OVA allergen treatment led to a consistent increase in airway resistance in response to intratracheal antigen challenge (Fig. 6D). Importantly, MC degranulation was evident following T_H9 cell transfer with high expression of serum MC protease 1 (MCPT1) (Fig. 6E).

Using mouse models of T_H9 deficiency (*Sfpi1*$^{fl/fl}$ *CD4*^{cre} and *Il9*$^{fl/fl}$ *CD4*^{cre}), we further examined the contribution of T cell-derived IL-9 on antigen-induced airway hyperresponsiveness[44]. Despite the decrease in airway resistance in *Sfpi1*$^{fl/fl}$ *CD4*^{cre} mice upon antigen challenge, the overall response to antigen was insufficient to induce a robust protective response (Fig. 6F). Therefore, we utilized mice deficient in T cell-specific IL-9 (*Il9*$^{fl/fl}$*CD4*^{cre}) and demonstrate that *Il9*$^{fl/fl}$ *CD4*^{cre} mice are protected against antigen-induced airway resistance (Fig. 6G). Moreover, deficiency in T cell IL-9 led to decreased *Mcpt1* transcripts in the lungs and MCPT1 levels in the bronchoalveolar lavage fluid, indicating diminished MC degranulation (Fig. 6H and Supplementary Fig. 5C). We further tested expression of additional cytokines expressed in MC including *Il2*, transforming growth factor-beta (*Tgfb1*), *Vegf*, and *Fgf2*[9,45]. We observed that T cell-specific IL-9 deficiency did not alter *Il2* or expression of profibrotic mediators, *Tgfb1, Vegf,* and *Fgf2* transcripts (Supplementary Fig. 5C). These data suggest that our antigen challenge may be too short of a duration to observe MC induction of these cytokines. Collectively, these findings demonstrate that T cell IL-9 expansion of lung mMC regulates antigen-induced allergic airway responses.

Discussion

This study defines a mechanism for IL-9-mediated MC accumulation in the allergic lung that includes a combination of IL-9-dependent MCp proliferation in the lungs and CCR2-

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dependent recruitment of MCs to the lungs[4,20]. Our mixed bone marrow chimera model revealed that IL-9 responsive MCp were preferentially expanded in the bone marrow and the lungs, suggesting that MCp responds to IL-9 signaling. Our data also indicate that IL-9 enhances mMC CCR2 expression and contributes to MC migration from the bone marrow to the allergic lung. We further demonstrate that MCs are required to generate antigen-induced airway reactivity and that these responses are dependent upon IL-9. We also show that T cell IL-9 is an important source of IL-9 for MC expansion in the lung and allergen-induced airway reactivity.

mMCs are a highly IL-9 responsive cell type in allergic asthma[9,20]. However, knowledge about the impact of IL-9 signaling on MCp and how MCs are sustained within the allergic lung is limited. This study demonstrated that IL-9R is constitutively expressed in multiple stages of MC development albeit at varying intensities depending on the maturation stage. We show that mMCs express the greatest amount of IL-9R and that mMC can respond to IL-9 in the bone marrow and lungs. This is consistent with studies demonstrating that IL-9 signaling enhances MC growth in the bone marrow and that MCs are key IL-9 responders in the lungs[5,9]. In comparison, MCp expresses an intermediate level of IL-9R compared to mMC, thereby suggesting that IL-9 signaling can be regulated by controlling its IL-9R expression during the multiple stages of MC development. Thus, IL-9R expression is linked to the MC differentiation stage with distinct functional outcomes.

MCs can migrate toward the allergic lung environment in a variety of ways: α4β7 expressed on human MCp binds VCAM-1 expressed on epithelial cells, CCR3-expressing MCp migrates toward CCL5 and CCL11, and the CCR2/CCL2 axis[4,30,46]. However, the role of IL-9 on MC migration in allergic airway inflammation is not well defined. Our data demonstrate that IL-9 upregulates CCR2 expression on MC to promote MC migration from the bone marrow to the allergic lung. While *in vitro*-cultured bone marrow MCs are a valuable tool to study mucosal MC, these culture conditions have several caveats in their interpretation, particularly, on how the culture conditions differ from the MC phenotypes found in the allergic microenvironment. Still, we observed parallel effects on MC directly analyzed *ex vivo* from allergen-challenged mice. Despite reports that MCp migrates from the bone marrow, we were unable to identify CCR2-dependent MCp migration in mice treated with a CCR2 inhibitor (data not shown). Thus, if the IL-9/CCR2 axis does promote MCp migration to the allergic lung, an alternative pathway may function in its absence. These data indicate that the deficit in MC expansion observed in the CCR2 inhibitor-treated mice does not result from insufficient proliferation of lung-resident MC, and likely reflects a primary contribution of migratory MC. Further investigation on IL-9-dependent MC migration *in vivo* will define MC migration and localization within the lungs that can impact allergic responses.

IL-9 is a pleiotropic cytokine, and its intrinsic and extrinsic effects on MC responses remain to be defined. Our data support an intrinsic effect of IL-9 during multiple stages of MC development. Our mixed bone marrow chimera model revealed that IL-9R-expressing WT-derived mMC were preferentially recruited to the allergic lung, while IL-9R-deficient mMC primarily remained in the bone marrow, suggesting an effect of IL-9 on MC migration that allows a selective advantage to emigrate from the bone marrow to the lungs. We have

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also found that less than 10% of the bone marrow MCp were *Il9r*$^{-/-}$-derived, indicating that IL-9 signaling on MCp is essential for MCp expansion. However, these results do not exclude the possibility that IL-9 acts on other cells in the allergic lung to contribute to MC expansion. One possibility is that IL-9 acts on CD11c+ macrophages or dendritic cells to promote an inflammatory response that can then promote MC responses. In support of this, Fu *et al*. demonstrate that macrophages express IL-9R at a comparable level to MC and that IL-9 expands the CD11c+ interstitial macrophages in the allergic lung[8]. Moreover, Dahlin *et al* show that CD11c+ cell depletion severely inhibited allergen-induced MC recruitment to the lungs[47]. These cells could be sources of, or inducers of, CCR2 ligands required for mMC recruitment. Notably, macrophage depletion diminished but did not eliminate IL-9-induced MC expansion in the allergic lung, suggesting that macrophages can contribute to MC expansion but are not required. While it is not clear how macrophages contribute to these responses, further experiments will define these circuits in the lung.

We utilize a novel method of testing MC function *in vivo* using antigen-induced airway testing. Our model allows us to directly test MC activation *in vivo* by passively sensitizing mice to a heterologous antigen and challenging intratracheally with antigen. We demonstrate that these responses are MC-dependent, as we do not observe antigen-induced airway reactivity in MC-deficient mouse models (*Kit*$^{WSH/WSH}$). Because intravenous MC adoptive transfer is insufficient to reconstitute MC in the lung[48], we utilized intranasal transfer of HDM-sensitized MC followed by treatment with HDM for 2 weeks. We demonstrate that mMC intranasal transfer can reconstitute MC populations in the respiratory tract. We further demonstrate that intranasal transfer of MC was sufficient to mediate antigen-induced airway reactivity. Our findings support that IL-9 mediates MC expansion in the lower airways: trachea and allergic lung, whether IL-9 promotes MC expansion in the upper airways requires further investigation.

The source of IL-9 in these studies was also determined. Consistent with previous results in OVA-Alum allergen models, compared to T_H2 cells, we demonstrate that T_H9 cells have an independent role in promoting airway cellular infiltration of MC and MC-mediated airway hyperreactivity in a chronic 6-week HDM allergen model[20,49,50]. While T_H2 cells elicited increased eosinophil infiltration compared to PBS controls, T_H2 cell transfer was insufficient to significantly increase MC numbers in the trachea and lungs to similar levels as T_H9 cells, and ultimately, unable to promote antigen-induced airway hyperresponsiveness. Despite the reduced MC expansion by neutralizing T_H2 cytokines, IL-4 and IL-13 play important roles in maintaining IgE antibody production, and thus may indirectly contribute to mast survival[51]. These assays define an independent function for the IL-9/MC circuit that is distinct from the ability of T_H2-dependent inflammation to increase methacholine-induced airway hyperreactivity[52]. Previous studies have utilized mice with a T cell-specific PU.1 deletion (*Sfpi1*$^{fl/fl}$ *CD4*^{cre}) as a model for T_H9 deficiency; however, these mice are not without limitations. Although PU.1-deficiency in T cells impairs T cell IL-9 production, *Sfpi1* transcripts are also expressed in T_H1, T_H2, and T_H17 cells, suggesting that it may also regulate these T cell lineages[20,44]. In this report, we utilize a newly generated T cell-specific IL-9 deficient mouse strain (*Il9*$^{fl/fl}$ *CD4*^{cre}) that does not impact ILC2 or MC IL-9 production but eliminates T cell IL-9[40]. Given that T cells, ILC2, and MC can contribute to IL-9 levels in the cytokine milieu[53,54], that *Il9*$^{fl/fl}$ *CD4*^{cre} mice still exhibited reduced MC

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expansion, suggests that ILC2 and MC IL-9 are insufficient and that T cells are a critical source of IL-9 promoting MC expansion in allergic airway inflammation.

The clinical implications of using anti-IL-9 antibody treatments have been demonstrated in mouse models and human clinical trials[55–57]. Thus far, anti-IL-9 treatment efficacy is variable; however, for individuals that benefit from this treatment, blocking IL-9 can improve asthma exacerbation rates in subjects with mild asthma[55]. We observed a similar greater effect of IL-9 blockade on intermittent exposures in mouse models[40]. Here, we further demonstrated that intravenous administration of anti-IL-9 during the last 2 weeks of allergen challenge, protected against allergen-mediated airway hyperreactivity. Data presented here and in other preclinical studies provide evidence that an IL-9/ MC pathway regulates airway inflammation and airway reactivity[58]. Therapies such as c-Kit kinase inhibition (Imatinib) have also been shown to reduce allergic airway hyperresponsiveness by decreasing MC numbers and tryptase expression[59]. Importantly, MCs offer protective roles in other diseases[60]. Therefore, complete ablation of MC using Imatinib raises important concerns in the design of therapeutics that have the potential to modulate MC biology. Thus, a greater understanding of which patients will benefit from targeting the IL-9/MC pathway will provide more personalized asthma therapies.

MATERIALS AND METHODS

Mice

All mice were on C57BL/6 background. WT mice (C57BL/6, Strain #002014), OTII mice (Strain #004194), and *Kit*$^{WSH/WSH}$ (C57BL/6, Strain #005051) were purchased from the Jackson Laboratory. *Il9r*$^{-/-}$ mice (C57BL/6) were a gift from Dr. Jean-Christophe Renauld[61]. *Il9*$^{-/-}$ mice (C57BL/6) were provided by Drs. Andrew McKenzie[62], Alexander Kirsch, and Sophie Paczesny. *Il9*$^{fl/fl}$ mice were generated by Jackson Laboratories using Cas9-mediated targeting as characterized in Ulrich & Kharwadkar *et al*[40]. Experiments were performed using 6–16-week-old female and male mice with no observed differences between sexes. All mice were maintained in SPF animal facilities (ambient temperature 70–72° F, humidity 50%, light/dark cycle 12/12 hour). All experiments were performed with the approval of the Indiana University Institutional Animal Care and Use Committee.

Humanized mouse model

Triple transgenic [NOD.Cg-Prkdc scid Il2rg tm1wjl Tg (CMV-IL 3, CSF2,KITLG)1Eav./ MloySzJ] (NSG-SGM3) mice were purchased from the Jackson Laboratory (Strain #013062) and bred in house by the Indiana University Melvin and Bren Simon Comprehensive Cancer Center *In Vivo* Therapeutics Core. Mice were maintained under pathogen-free conditions in ventilated cages and were given ad libitum irradiated Teklad Uniprim Medicated Diet (Harlan Laboratories, Indianapolis, IN, TD 06596) and autoclaved, acidified water. Before transplant, cryopreserved human cord blood CD34+ cells (STEMCELL Technologies, Vancouver, Canada) were thawed and cultured for 4 hours at (5×10^5) cells/ml in StemSpan serum-free media (STEMCELL Technologies, Vancouver, Canada) supplemented with stem cell factor (SCF) at 100 ng/ml (PeproTech, Cranbury, NJ). Cells were then counted in try-pan blue and were 95 to 98% viable. Adult female

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NSG-SGM3 mice (6 to 8 weeks old) were sublethally irradiated (100 cGy) and injected intravenously 4 hours later with 2×10^4 human CD34+ cells.

Induction of allergic airway inflammation Acute adoptive cell transfer model

Naïve CD4+ T cells isolated from OTII mice, mice that express ovalbumin (OVA)-specific T cell receptors, were cultured *ex vivo* to polarize toward a T_H2 (IL-4) or T_H9 (IL-4 + TGFβ) phenotype. We assessed cytokine production by stimulating cells with PMA (50ng/mL) and ionomycin (1mg/mL). After 3 hours, monensin (2μM) was added to stimulated cells for another 3 hours. Cells were fixed, permeabilized, and stained for intracellular cytokines: IL-4, IL-9, and IL-13. 5×10^5 *in vitro* polarized T_H2 or T_H9 cells were adoptively transferred intravenously to recipient mice. Twenty-four hours after transfer, mice were intranasally treated with 100 μg OVA (Sigma, St. Louis, MO, Catalog #A5503) in 25 μl for 5 days.

Chronic HDM challenge model

House dust mite (HDM; Greer Laboratories, Lenoir, NC, Catalog #XPB91D3A2.5) was diluted with PBS. Mice were challenged intranasally with 25 μg HDM three times a week for 6 weeks. When specified, mice were treated intravenously with CCR2 inhibitor (2 mg/kg, R&D systems, Minneapolis, MN, Catalog #RS 504393) or DMSO twice a week during the last 4 weeks of HDM treatment. For some experiments, mice were treated intravenously with antibodies every other day for the last 2 weeks of HDM treatment: α-IL-9 (150μg/mouse, BioXcell, Lebanon, NH, Catalog #9C1), α-IL-13 (25 μg/ mouse, R&D, Catalog #MAB413–500), and α-IL-4 (50 μg/mouse, BioXcell, Catalog #BP0045); or its isotype control antibody: IgG2A isotype (BioXcell, Catalog #BE0085), IgG2A (R&D, Catalog #MAB006), and IgG1 isotype (BioXcell, Catalog #BP0088), respectively.

Macrophage depletion

Mice were intravenously injected with 200 μL of Clodronate liposomes (Liposoma; The Netherlands, SKU #C-010) 48 hours prior to intranasal IL-9 treatment.

Tissue harvest and processing

Mice were euthanized and lungs were lavaged with cold PBS. BAL fluid was collected, and cells were centrifuged at 1500 g for 5 minutes at 4°C for further surface staining. Lung tissue was digested in collagenase A (1 mg/ml, Roche, Indianapolis, IN, Catalog #10103586001) in DMEM (Dulbecco's Modified Eagle Medium) (5 ml/lung, Gibco, Detroit, MI, Catalog #11965084) for 45 minutes at 37°C in rotation. After digestion, the lungs were filtered through a sieve (Bellco Glass, Vineland, NJ, SKU #1985–85000) to obtain single-cell suspensions. Cells were pelleted and red blood cells were lysed using ACK (Ammonium-Chloride-Potassium) Lysis for 5 minutes. Blood was collected for FACS analysis, and the remaining blood samples were used for plasma. Femur-derived bone marrow was flushed with cold PBS, pelleted, filtered, and proceeded to ACK lysis. Single-cell suspensions were used for flow cytometry and RNA isolation using TRIzol reagent (Invitrogen, Waltham, MA, Catalog #15596026) or the RNeasy Plus Micro Kit (QIAGEN, Germantown, MD, Catalog #74004).

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Flow cytometry

Single-cell suspensions were stained with a fixable viability dye (eBioscience, San Diego, CA, Catalog #65-0865-18) and antibodies for surface markers for 30 minutes at 4°C. After fixation with IC Fixation (eBioscience, Cat #00-8222-49) for 10 minutes dark at room temperature, cells were permeabilized with permeabilization buffer (eBioscience, Catalog #00-8333-56) for 30 minutes at 4°C and stained for cytokines for 1 hour at 4°C. For transcription factor staining, after surface staining, cells were fixed with Fixation & Permeabilization Buffer (eBioscience, Catalog # 00-5521-00) for 2 hours or overnight at 4°C, and then permeabilized with permeabilization buffer. See Table 1 for information about flow reagents.

Mixed bone marrow chimera

F1/CD45.1^{+}CD45.2^{+} mice were irradiated at 1000 rds. One day after the irradiation, 8 million donor bone marrow cells [4 million from Boy/J mice (CD45.1+) and 4 million from *Il9r*$^{-/-}$ mice (CD45.2+)] were intravenously injected into the recipient mice. After donor cell injection, mice were rested for 12 weeks, and bone marrow reconstitution was assessed via blood. Expression of CD45.1^{+} and CD45.2^{+} cells was assessed via flow cytometry.

Bone marrow MC cultures and cell migration assay

Bone marrow MC were cultured in IL-3 (Biolegend, Cat#575504, 20 ng/ml) and stem cell factor (Biolegend, Cat#573902, 60 ng/ ml) in complete MC Roswell Park Memorial Institute (RPMI) 1640 Medium containing: FBS, L-Glutamine, sodium pyruvate, Pen/Strep, and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesul fonic acid). Cells were allowed to culture for 2–5 weeks with non-adherent MC transferred into a new culture dish every few days with the addition of fresh media. Cells (5×10^5 in 100 μl serum-free RPMI containing 1% BSA) were added to the upper chamber of the transwell insert (Millipore Sigma, Carlsbad, CA, Catalog #CLS3421). The lower chamber contained 400 μl complete MC media with chemoattractants: IL-4 (40 ng/ml, Biolegend, San Diego, CA, Catalog #:574302), IL-9 (40 ng/ml, Biolegend, Catalog #:556004), CCL2 (100 ng/ml, R&D, Catalog#479-JE/CF), CXCL1 (100 ng/ml, Peprotech, Cranbury, NJ, Catalog #250-11), CXCL2 (100 ng/ml, R&D, Catalog #452-M2), CXCL8 (100 ng/ml, R&D, Catalog #208–1L), CXCL12 (100 ng/ml, R&D, Catalog #MAB310). The plates were incubated at 37°C for 3 hours, and migrated cells were counted and stained for flow cytometry.

MC adoptive transfer

WT mice were treated with HDM three times a week for 6 weeks. Lungs were processed into single-cell suspension as described above. mMC were sorted using the gating strategy described in Fig. 1. 10^3 mMC were transferred intranasally to *Kit*$^{WSH/WSH}$ mice and were subsequently intranasally challenged with 25 μl of HDM (1 μg/μl) for 2 weeks. After the last HDM dose, mice were passively sensitized to α-DNP-IgE (3 μg/mouse) and challenged with DNP-BSA as described above.

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Airway testing

Following the last allergen dose, mice were passively sensitized intravenously with 3 μg of anti-DNP IgE (Sigma-Aldrich, Burlington, MA, Catalog #D8406) and challenged intratracheally the day after with 20 μl of DNP-BSA (25 mg/ml, Life Tech Corporation, Waltham, MA, Catalog #A23018). Readings were obtained at baseline and after exposure to the allergen challenge. Data were collected for 5 minutes after 3 minutes of inhalation, and average values were expressed as airway resistance (RI). The airway response was measured for up to 60 minutes. Airway resistance was measured using a ventilator (Elan Series Mouse RC Site; Buxco Electronics, Wilmington, NC) and BioSystem XA software (Buxco Electronics, Wilmington, NC).

ELISA

MC protease-1 (MCPT1) (Invitrogen, Waltham, MA), CCL2 (Invitrogen), IL-6 (Biolegend, San Diego, CA), and Ovalbumin-specific IgE (Biolegend, San Diego, CA) enzyme-linked immunosorbent assay (ELISA) were performed according to the manufacturer's instruction. Briefly, a 96 well-plate was coated with coating antibody overnight at 4°C. After washing three times, 300 μl ELISA blocking buffer was added to the plate and incubated at room temperature for 2 hours. After washing three times, 100 μl standards and samples were added to the plate at different dilutions (neat or 1:2) and incubated at room temperature for 2 hours. After washing three times, 100 μl diluted detection antibody was added to the plate and incubated at room temperature for 1 hour. After washing the plate three times, 100 μl of diluted Avidin-HRP solution was added to the plate and incubated at room temperature for 30 minutes in the dark. After washing three times, 100 μl substrate was added to the plate. Plates were read at absorbance 450 nm and 570 nm.

Histology

Trachea and lung samples were preserved in 10% buffered formalin for 24 hours at room temperature and then transferred into 70% ethanol. Samples were then embedded in paraffin, sectioned, and stained with toluidine blue or chloroacetate esterase. MC numbers were quantified at 4x and 40x magnification.

Statistics and data analysis

All statistics were done using Prism software version 7 (Graph-Pad, San Diego, CA, USA). Flow cytometry data were collected using a Nxt Attune flow cytometer (Life Technologies, Waltham, MA, USA) and were analyzed using FlowJo version 10 (Tree Star FlowJo, BD Bioscience, San Diego, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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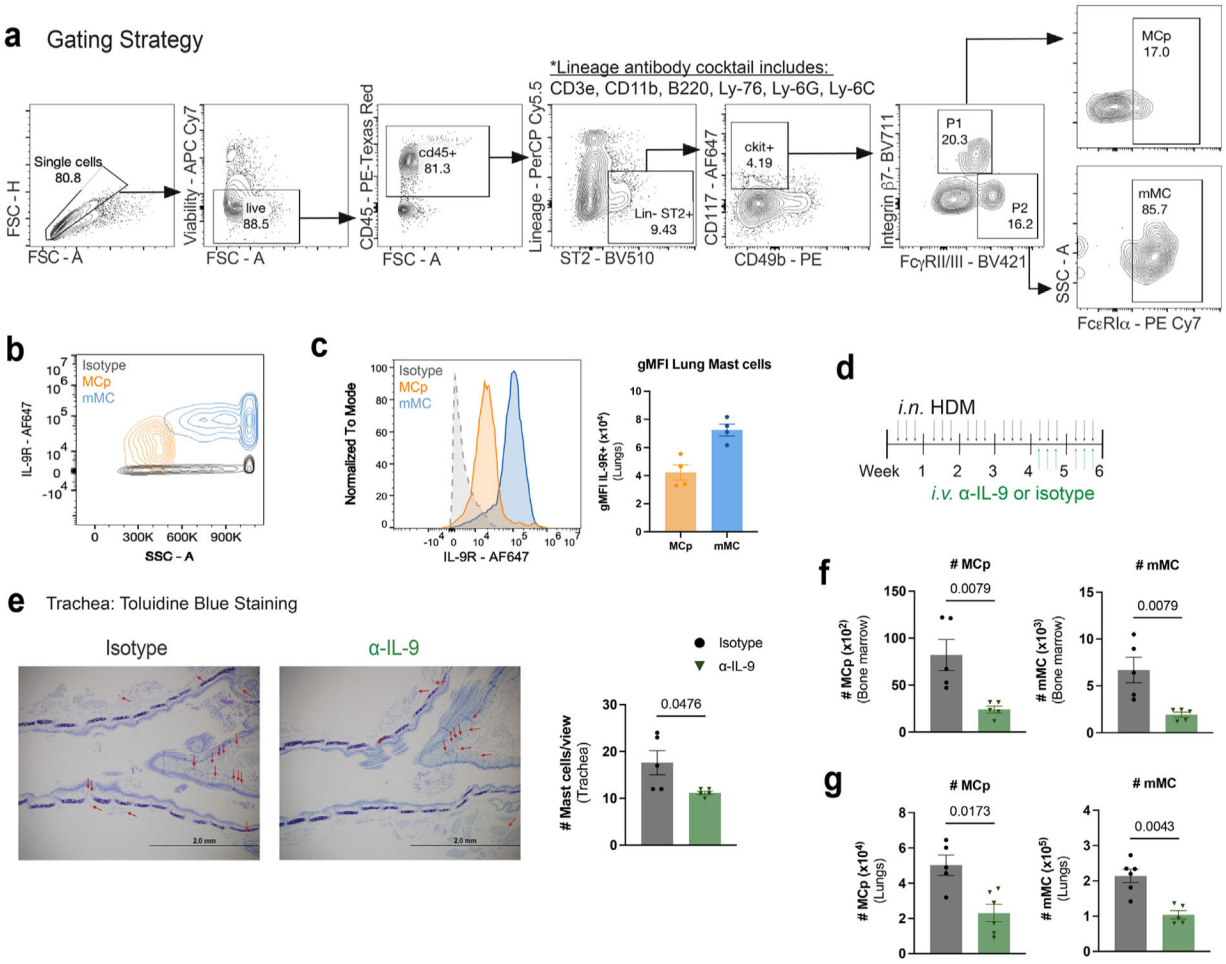

Fig. 1.

MCp and mMCs are IL-9 responders in the allergic lung. (A) Flow cytometry MC gating strategy using representative flow cytometry plots. P1 and P2 are further gated on FcεRIα+ cells. Mast cell progenitors (MCp) and mature mast cells (mMC). (B–C) Naïve WT lung MC were analyzed using flow cytometry for IL-9 receptor expression: B, representative flow cytometry contour plot of IL-9R expression on MC. (C) Histogram for IL-9R and geometric mean fluorescence intensity (gMFI) for each population was compared to isotype control (n = 3–4). (D–G) HDM-treated WT mice were treated with α-IL-9 or isotype control during the last 2 weeks of HDM treatment. (D) Schematic of experimental design. (E) MC numbers and frequencies were assessed in the trachea via toluidine blue staining; F–G: flow cytometry analysis of bone marrow (F) and lung (G) MC (n = 5–6) Data are representative of two independent experiments with similar results. Error bars indicate ± standard error of mean. Mann-Whitney U test was used for comparisons to generate p values in (C) and (E–G). APC = Allophycocyanin; CD=clusters of differentiation; FSC = forward scatter; HDM = house dust mite; IL = interleukin; MC = mast cell; MCp = MC progenitors; mMC = mature MC.

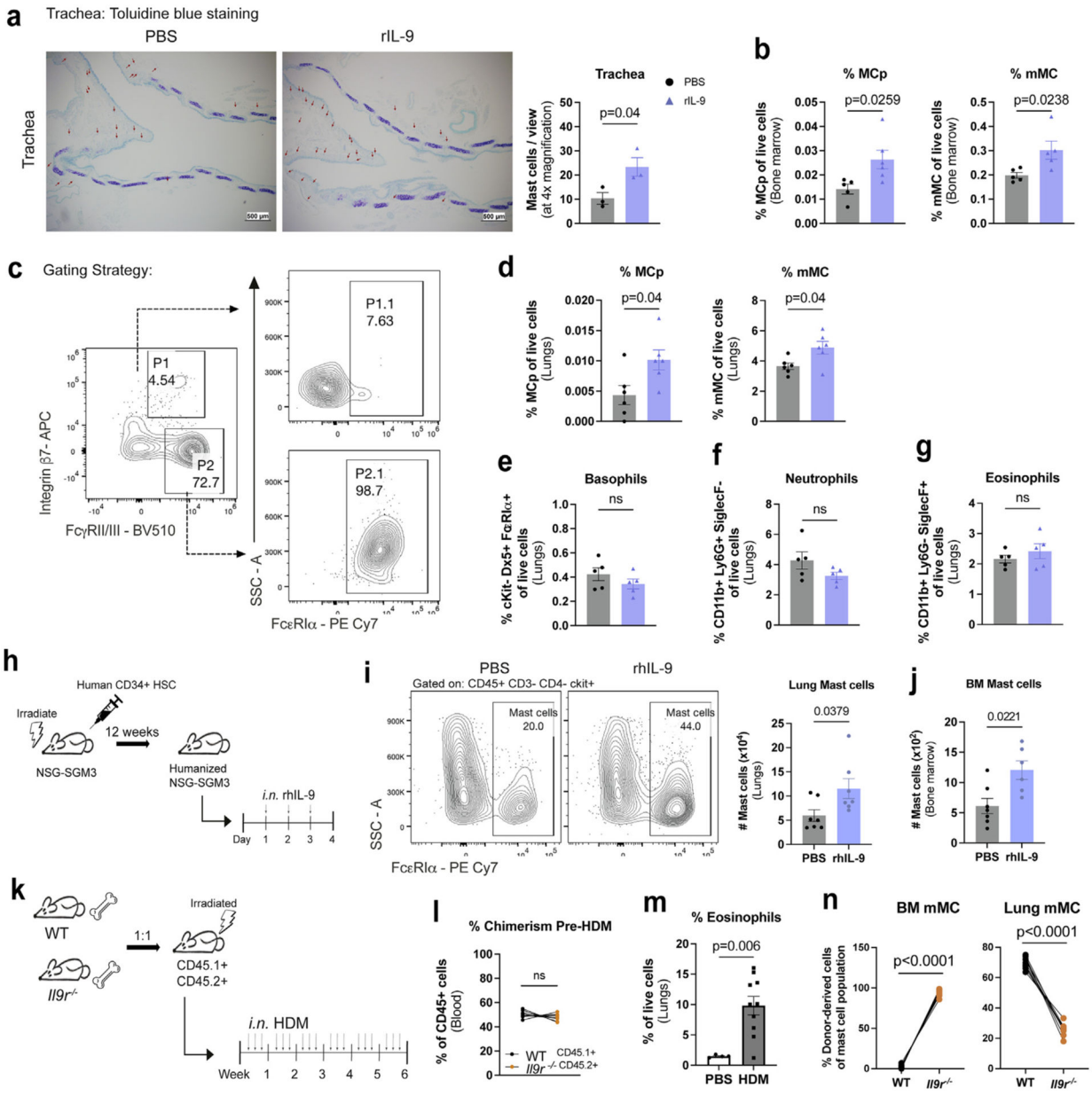

Fig. 2.

IL-9 acts intrinsically on bone marrow MC. (A–G) WT mice were treated intranasally with recombinant mouse IL-9 for 3 days: A, tracheal MC numbers were analyzed using toluidine blue staining. (B) MC frequencies in femur-derived bone marrow; C, flow cytometry gating strategy for lung MC frequencies in (D). (E–G) Flow cytometry analysis of Basophils (cKit- Dx5+ FcεRIα+), Neutrophils (CD11b+ Ly6G+ SiglecF-), and Eosinophils (CD11b+ Ly6G- SiglecF+). (n = 3–5) Data are representative of three independent experiments with similar results. (H–J) NSG mice were irradiated and reconstituted with human CD34+ cord blood cells. Following successful reconstitution of human CD45+ cells, mice were treated intranasally with recombinant human IL-9 for 3 days. (H) schematic of experimental design. (I) Flow cytometry analysis of lung MC. (J) Flow cytometry analysis of bone marrow MC.

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(n = 7–8). (K-N) WT and *Il9r*$^{-/-}$ bone marrow cells were transferred to lethally irradiated Boy/J x C57BL/6J F1 mice: (K) schematic of experimental design. (L) chimerism after 3 months was analyzed prior to HDM treatment using flow cytometry; M, frequency of lung eosinophils.(N) Bone marrow and lung MC were assessed for CD45.1 or CD45.2 expression by flow cytometry (PBS: n = 5; HDM: n = 10). Each data point represents an individual mouse. Data are representative of two independent experiments with similar results. Error bars indicate ± standard error of mean. Statistical significance was determined by analysis of variance, followed by Tukey's multiple comparison test (N) or Mann-Whitney U test (A–B, D–G, I–J, and L–M). APC =; CD = clusters of differentiation; gMFI = geometric mean fluorescence intensity; HDM = house dust mite; IL = interleukin; MC = mast cell; MCp = MC progenitors; mMC = mature MC; ns = not significant; NSG = NOD-SCID, gamma chain-deficient mouse; PBS = phosphate buffered saline; PE= R-phycoerythrin; rh = recombinant human; SSC = side scatter; WT, wild type.

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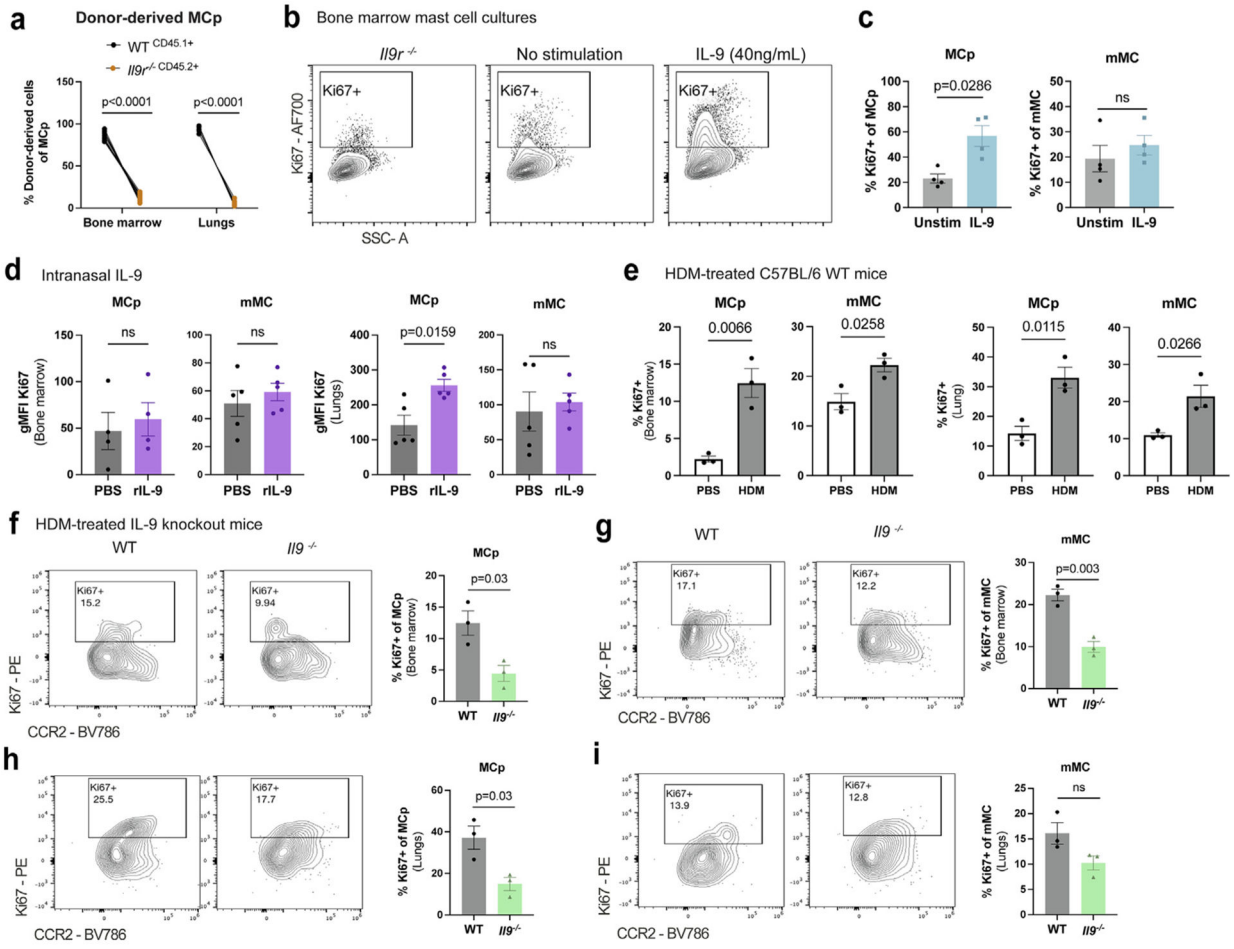

Fig. 3.

IL-9 enhances MC progenitor proliferative capacity. (A) WT (CD45.1+) and *Il9r*$^{-/-}$ (CD45.2+) bone marrow cells were transferred to lethally irradiated Boy/J x C57BL/6J F1 mice and after 3 months to allow repopulation of the immune system, mice were treated with HDM for 6 weeks. Flow cytometry analysis of CD45.1+ and CD45.2+ of lung MCp. (n = 10). (B–C) BMMC from WT mice were cultured for 2 weeks in IL-3 and SCF in RPMI. BMMC were harvested and stimulated with IL-9 (40 ng/ml) for 2 hours to assess intracellular Ki67 using flow cytometry. (B) flow cytometry plots of Ki67 staining in BMMC with WT and *Il9r*$^{-/-}$ BMMC. (C) Flow cytometry analysis of Ki67 frequencies in MCp and mMC (n = 4). (D) WT mice were intranasally treated with rIL-9 for 3 days. Flow cytometry of Ki67 gMFI was measured from bone marrow and lung MC (n = 5); E, flow cytometry analysis of Ki67 was assessed in lung MC from 6-week HDM-treated WT mice or PBS controls (n = 3). (F–I) *Il9*$^{-/-}$ and WT mice were treated intranasally with HDM 3x/week for 6 weeks. Ki67 expression was assessed via flow cytometry in (F–G) bone marrow and (H–I) lung MC (n = 3). Each data point represents an individual mouse. Data are representative of two independent experiments with similar results. Error bars indicate ± standard error of mean. Statistical significance was determined by analysis of variance, followed by Sidak's multiple comparison test (A), Mann-Whitney U test (C–D), and Student's t test (E–I). CD = clusters of differentiation; gMFI = geometric mean

fluorescence intensity; HDM = house dust mite; IL = interleukin; MC = mast cell; MCp = MC progenitors; mMC = mature MC; ns = not significant; PBS = phosphate buffered saline; PE = R-phycoerythrin; r = recombinant; SSC = side scatter; WT, wild type.

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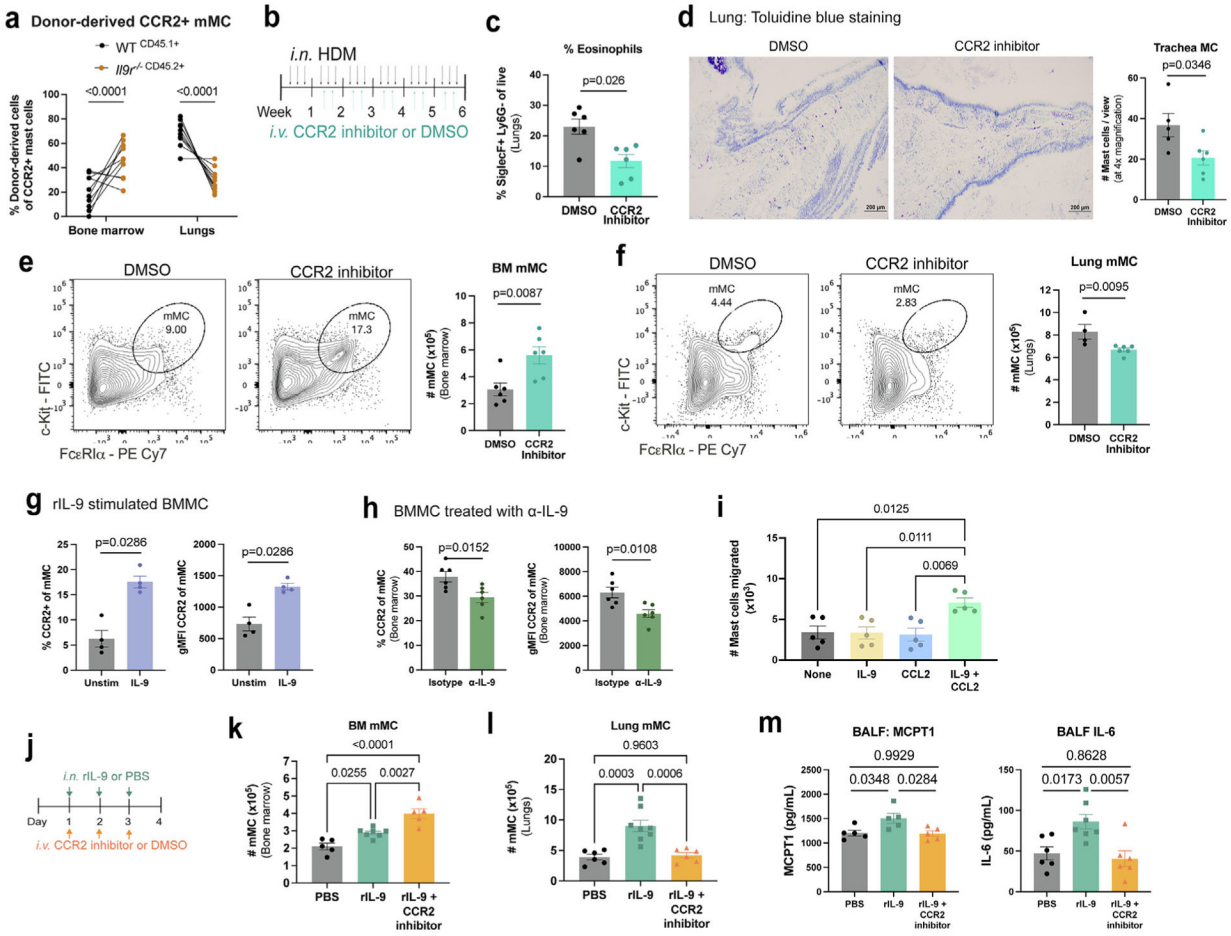

Fig. 4.

IL-9 promotes CCR2-dependent MC migration from the bone marrow to the allergic lung. (A) WT (CD45.1+) and *Il9r*$^{-/-}$ (CD45.2+) bone marrow cells were transferred to lethally irradiated BoyJ x C57BL/6J F1 mice, allowed to reconstitute immune cells, and treated with HDM for 6 weeks. Flow cytometry analysis of CCR2+ lung MC. (n = 10) Data are representative of two independent experiments with similar results. (B–F) HDM-treated WT mice were treated with CCR2 inhibitor or DMSO control as indicated in (B); C, eosinophil (CD11b^{+} SiglecF^{+} Ly6G^{-}) frequencies were determined by flow cytometry. (D) MC in the trachea were assessed using toluidine blue staining. MC numbers were assessed in the bone marrow (E) and lungs (F) by flow cytometry (n = 6). Data are representative of two independent experiments with similar results. (G) Flow cytometry analyses of CCR2 expression in BMMC treated with IL-9 (40 ng/ml) for 2 hours (n = 4). Data are representative of two independent experiments. (H) Flow cytometry analyses of CCR2 expression in bone marrow MC from HDM-treated WT mice treated with anti-IL-9 or an isotype control (n = 6). Data are representative of three independent experiments with similar results. (I) BMMC migration assay toward cytokines and/or chemokines (n = 5). Data are representative of three independent experiments with similar results. (J–M) WT mice, treated intranasally with IL-9 for 3 days, were also intravenously treated with CCR2 inhibitor for 3 days indicated in (J). Flow cytometry analysis of bone marrow (K) and

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lung (L) MC. (M) MCPT1 and IL-6 protein expression was determined via enzyme-linked immunosorbent assay (n = 5–8). Data are representative of two independent experiments with similar results. Error bars indicate ± standard error of mean. Statistical significance was determined by analysis of variance, followed by Tukey's multiple comparison test (A, I, K, and L) or Mann-Whitney U test (C, E–H, and M). CD = clusters of differentiation; DMSO = dimethyl sulfoxide; gMFI = geometric mean fluorescence intensity; HDM = house dust mite; IL = interleukin; MC = mast cell; MCp = MC progenitors; MCPT 1 = MC protease 1; mMC = mature MC; ns = not significant; PBS = Phosphate buffered saline; PE = R-phycoerythrin; r = recombinant; SSC = side scatter; WT, wild type.

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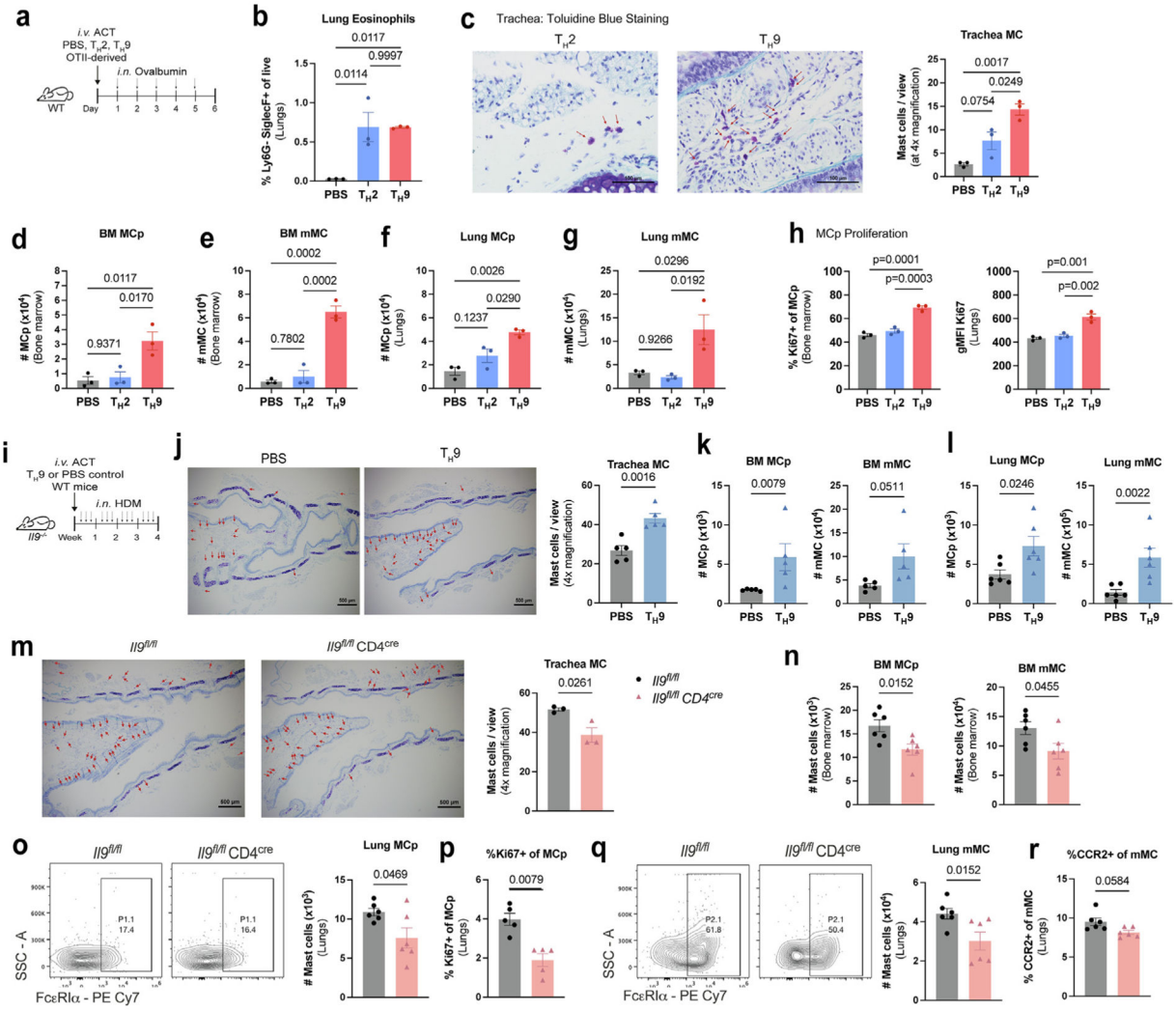

Fig. 5.

T cell IL-9 is a central source of IL-9 mediating MC expansion in the allergic lung. (A–H): A, WT mice transferred with OTII-derived T_H2 or T_H9 cells were treated with OVA for 5 days; B, lung eosinophil frequencies were determined by flow cytometry. MC numbers were assessed in the trachea (C) using toluidine blue staining and in the bone marrow (D–E) and lungs (F–G) using flow cytometry. (H) Flow cytometry analysis of Ki67 expression in MCp. ($n = 3$). Data are representative of three independent experiments with similar results. (I–L) WT-derived T_H9 cells or PBS were transferred to *Il9*$^{-/-}$ recipient mice, and subsequently treated with HDM as shown in (I); (J) MC numbers were assessed in the trachea using toluidine blue staining. 4x magnification with Scale = 500 μm. (K–L) Flow cytometry analysis of bone marrow (K) and lung (L) MC numbers ($n = 5$–6). (M–Q) *Il9*$^{fl/fl}$ *CD4*^{cre} mice and littermate controls (*Il9*$^{fl/fl}$) were treated with HDM for 6 weeks M, toluidine blue staining of trachea. 4x magnification with Scale = 500 μm; N, flow cytometry analysis of MCs in the bone marrow; O, representative flow cytometry contour plots of lung MCp; P, Ki67 expression in lung MCp; Q, representative flow cytometry contour plots of lung mMC; R, CCR2 expression in lung mMC ($n = 6$). Data are representative of

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two independent experiments with similar results. Error bars indicate ± standard error of mean. Statistical significance was determined by analysis of variance, followed by Tukey's multiple comparison test (B–H) or Mann-Whitney U test (J–R). ACT = adoptive cell transfer; CD = clusters of differentiation; HDM = house dust mite; IL = interleukin; MCp = mast cell progenitors; MCPT 1 = mast cell protease 1; mMC = mature mast cells; ns = not significant; OVA = ovalbumin; PBS = phosphate buffered saline; PE = R-phycoerythrin; SSC = side scatter; T_H = T helper; WT, wild type.

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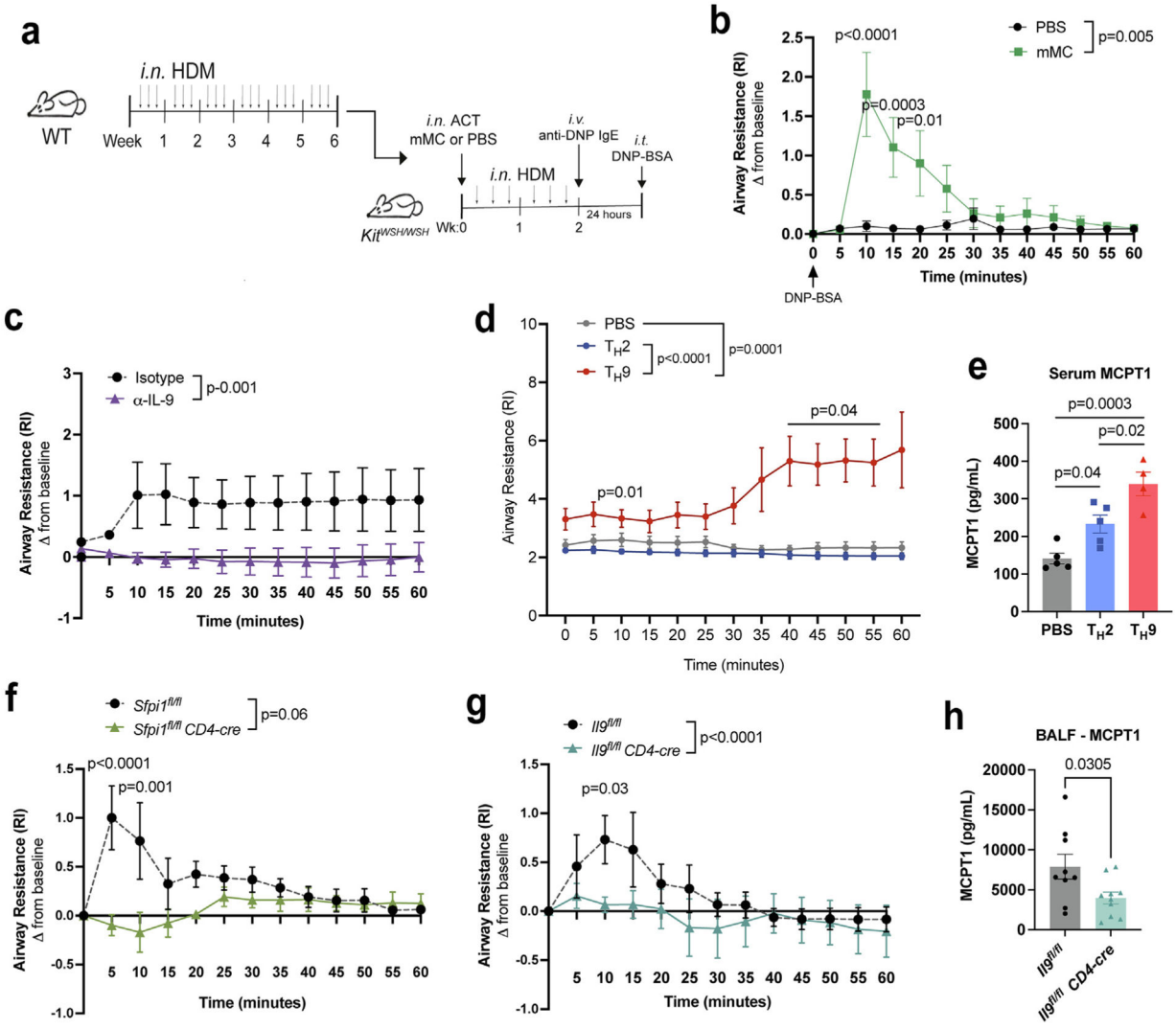

Fig. 6.

T cell IL-9 promotes antigen-induced mMC-mediated airway reactivity. (A–B) *Kit*$^{WSH/WSH}$ mice were adoptively transferred with HDM-sensitized mMC from WT mice and subsequently treated with HDM for 2 weeks. Following passive sensitization to 2,4-Dinitrophenyl (DNP) and challenge intratracheally with DNP-BSA, airway reactivity was measured for one hour. Experimental design indicated in (A) and results in (B) ($n = 5$). Data are representative of two independent experiments with similar results. (C) HDM-treated WT mice were intravenously treated with α-IL-9 or isotype control for the last 2 weeks of treatment. Airway resistance was monitored in (C) ($n = 4$–5). Data are representative of two independent experiments with similar results. (D–E) WT mice transferred with OTII-derived T_H2 or T_H9 cells were treated with OVA for 6 days. Airway resistance was monitored for 60 minutes in (D) (PBS: $n = 7$, T_H2: $n = 10$, and T_H9: $n = 10$). Data are pooled from three independent experiments; E, serum MCPT1 expression was assessed via enzyme-linked immunosorbent assay ($n = 5$); F, airway resistance for HDM-treated *Sfpi1*$^{fl/fl}$*CD4*^{cre} and *Sfpi1*$^{fl/fl}$ mice ($n = 5$). Data are representative of two independent experiments with similar results. (G–H): G, airway resistance for HDM-treated *Il9*$^{fl/fl}$*CD4*^{cre} and *Il9*$^{fl/fl}$ mice; H,

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MCPT1 protein expression was determined via enzyme-linked immunosorbent assay (n = 5). Data are representative of two independent experiments with similar results. Error bars indicate ± standard error of mean. Statistical significance was determined by analysis of variance, followed by Tukey's multiple comparison test (E), Sidak's multiple comparison test (B, D, F, and G), Student's t test in (H), and Mann-Whitney U test of area under the curve (B–D, F, and G). ACT = adoptive cell transfer; BALF = bronchoalveolar lavage fluid; CD = clusters of differentiation; HDM = house dust mite; Ig = immunoglobulin; IL = interleukin; MCp = mast cell progenitors; MCPT 1 = mast cell protease 1; mMC = mature mast cells; ns = not significant; OVA = ovalbumin; PBS = phosphate buffered saline; SSC = side scatter; T_H = T helper; WT, wild type.

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Table 1.

Reagents for flow cytometry, cell culture, and ELISA.

Flow cytometry antibodies					
Antigen/Name	**Clone**	**Fluorochrome**	**Company**	**Dilution in FACS buffer**	**Catalog number**
Fixable viability dye		eFluor 780	eBioscience	1:1500	65–0865-14
Mouse lineage antibody cocktail:		PerCP-Cy5.5	BD Biosciences	1:25	561317
CD3e	145–2C11				
CD11b	M1/70				
CD45R/B220	RA3–6B2				
Ly-76	TER-119				
Ly6G and Ly6C	RB6–8C5				
Mouse CD45	30-F11	PE/Dazzle 594	Biolegend	1:250	103145
Mouse CD45.1	A20	BV421, BV650	Biolegend	1:200	110735
Mouse CD45.2	104	BV605, APC	Biolegend	1:200	109841
Mouse CD49b	HMa2	PE	BioLegend	1:250	103506
Mouse CD49b	Dx5	BV421	BD Biosciences	1:200	563063
Mouse c-Kit	2B8	FITC, APC	BioLegend	1:100	105805, 105811
Mouse ST2/IL-33R	U29–93	BV510	BD Biosciences	1:100	745080
Mouse FCERI	MAR-1	PE-Cy7	BioLegend	1:200	134317
Mouse FcyRII/III	93	BV510	BioLegend	1:200	101333
Mouse Integrin β7	FIB504	APC, BV711	BioLegend	1:200	321207, 321239
Mouse CCR2	475301	BV786	BD Biosciences	1:200	747966
Mouse Ki67	SolA15	PE-eFluor610, AF700	Invitrogen	1:200	61–5698-82, 56–5698-82
Mouse CD3	145–2C11	PerCP-Cy5.5	BD Biosciences	1:200	551163
Mouse IL-4	11B11	AF647	BioLegend	1:200	504110
Mouse IL-9	RM9A4	PE	BioLegend	1:200	514104
Mouse IL-13	eBio13A	AF488	eBioscience	1:200	53–7133-82
Mouse Ly6G	RB6–8C5	FITC	BD Biosciences	1:200	553126
Mouse SiglecF	E50–2440	PE	BD Biosciences	1:200	552126
Mouse CD64	X54–5/7.1	FITC	BioLegend	1:200	139316
Mouse Mertk	2B10C42	PE	BioLegend	1:200	151506
Mouse CD11b	M1/70	PerCP-Cy5.5	eBioscience	1:200	45–0112-82
Mouse CD11c	N418	PE-Cy7	eBioscience	1:200	25–0114-82
Reagents for T helper cell differentiation					
Name (Clone)	**Company**	**Catalog Number**			
anti-mouse CD3 (145–2C11)	BioXCell	BP-0001–1			
anti-mouse CD28(37.51)	BioXCell	BE0015–1			
anti-mouse IFNy (XMG1.2)	BioXCell	BE0055			
mouse IL-4 (11B11)	BioXCell	BE0045			
mouse IL-2 (JES6–5H4)	BioXCell	BE0042			

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Human TGF-β1	Miltenyi Biotec	130–095-067
Reagents for mast cell cultures		
mouse IL-3	Biolegend	575504
mouse SCF	Biolegend	579706
ELISA kits		
Name	**Company**	**Catalog Number**
LEGEND MAX Mouse OVA-specific IgE ELISA	BioLegend	439807
Mouse MCPT1 ELISA	Invitrogen	88–7503-88
Mouse IL-9 ELISA MAX Deluxe	BioLegend	442704

CD = clusters of differentiation; IL = interleukin.