HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2010 September 01.

Published in final edited form as:

Nat Immunol. 2010 March ; 11(3): 250–256. doi:10.1038/ni.1846.

Regulation of IL-9 expression by IL-25 signaling

Pornpimon Angkasekwinai[1,2], **Seon Hee Chang**[1], **Manoj Thapa**[1], **Hiroshi Watarai**[3], and **Chen Dong**[1]

[1]Department of Immunology, University of Texas and MD Anderson Cancer Center, Houston, Texas 77030, USA. [2]Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathum-thani 12121,Thailand [3]Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, 1-7-22, Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045, Japan

Abstract

The physiological regulation of interleukin 9 (IL-9) expression, a cytokine traditionally regarded as being T_H2 associated, remains unclear. Here, we show that IL-9-expressing T cells generated *in vitro* in the presence of TGF-β and interleukin 4 (IL-4) express high levels of interleukin 17 receptor (IL-17R) B mRNA, the receptor for interleukin 25 (IL-25). Treatment of these cells with IL-25 enhances IL-9 expression *in vitro*. Moreover, transgenic and retroviral over-expression of IL-17RB in T cells results in IL-25-induced IL-9 production that is IL-4 independent. *In vivo*, the IL-25-IL-17RB pathway regulates IL-9 expression in allergic airway inflammation. Thus, IL-25 is a newly identified regulator of IL-9 expression.

Introduction

Interleukin-9 (IL-9) is regarded as a cytokine produced by activated T_H2 lymphocytes and is involved in T_H2-associated diseases such as asthma and parasite infection 1–5. However, IL-9 seems to be regulated differently from other classical T_H2-derived cytokines. For instance, TGF-β strongly induces IL-9 production 6. Although interleukin 4 (IL-4) is a critical T_H2 inducer, IL-4 alone has a minimal effect on IL-9 expression during naïve T cell differentiation 6. However, adding IL-4 together with TGF-β greatly enhances IL-9 production 6–8 and inhibits the production of classical T_H2 cytokines, IL-4, interleukin 5 (IL-5), and interleukin 13 (IL-13) 7,8. These cells were therefore called T_H9 cells to distinguish them from classical T_H2 cells. Interleukin 10 (IL-10) is produced by both T_H9 and classical T_H2 cells. Because IL-9 expression was only partially reduced in IL-4-

Correspondence: Chen Dong, Ph.D, cdong@mdanderson.org.

Author Contributions

P.A and C.D designed the research, analyzed and interpreted the results; P.A, S.H.C., M.T did the experiments; and P.A. and C.D prepared the manuscript.

The authors have no conflicting financial interests.

deficient mice 9, IL-4-independent IL-9 regulation was proposed. Moreover, the regulation of IL-9 expression *in vivo* remains unclear.

The interleukin 17 (IL-17) family consists of six cytokines and the IL-17 receptor family is comprised of IL-17 receptor A, B, C, D, and E. IL-17 receptor B (IL-17RB) binds interleukin 25 (IL-25) and this interaction was initially reported to regulate type-2 immune responses *in vivo* 10,11. Recently it was reported that mouse and human T_H2 cells, but not T_H1 or T_H17 cells express IL-17RB 12,13. IL-25 functions to promote T_H2 polarization in an IL-4-dependent manner.

In this study, we have further explored the regulation of IL-9 expression by IL-25 and IL-17RB. We detected IL-17RB expression not only in T_H2 but also in T_H9 cells and noticed that IL-25 treatment of differentiated T_H9 cells enhanced IL-9 production. Over-expression of IL-17RB in T cells strongly induced IL-9 production as well as T_H2 cytokine expression in response to IL-25. Whereas the induction of T_H2 cytokines was IL-4-dependent, that of IL-9 was not. In addition, IL-9 production *in vivo* was found to be greatly enhanced in IL-17RB transgenic mice challenged with an allergen while IL-25 deficiency resulted in remarkable reduction of IL-9 associated with the attenuation of allergic responses. These results thus establish IL-25 as a regulator of IL-9 expression.

Results

IL-17 receptor B (IL-17RB) expression in T_H9 cells

IL-17RB mRNA is expressed by naïve T and T_H2 cells but not by T_H1 or T_H17 cells 12. To further understand the regulation of IL-17RB during T cell differentiation, we analyzed its expression on activated T cells treated with T_H1- or T_H2-inducing cytokines with or without neutralizing antibodies by real-time RT-PCR analysis. Although IL-12 and interferon γ (IFN-γ) induced the expression of interleukin 12 receptor (IL-12R)β2, IL-17RB mRNA expression was inhibited (Fig. 1a). In contrast, IL-4, in the presence or absence of anti-IFN-γ, greatly enhanced IL-17RB mRNA expression and suppressed mRNA expression of IL-12Rβ2 (Fig. 1a). Since IL-4 and TGF-β drive T_H9 cell differentiation, we analyzed the expression of IL-17RB upon treatment of IL-4 and/or TGF-β. IL-4 or TGF-β enhanced IL-17RB mRNA expression, and treatment with both cytokines induced the highest amounts of IL-17RB mRNA (Fig. 1b). Because IL-4 and TGF-β both regulate IL-17RB expression, we further assessed the IL-17RB mRNA expression in differentiating T_H9, T_H1, T_H2 and T_H17 cells by real-time RT-PCR on day 1 and day 2 following activation. While we detected no change in the expression of IL-17RA and IL-17RC in different polarizing conditions, IL-17RB mRNA was greatly up-regulated in T_H2 and T_H9 polarized cells (Fig. 1c). In addition, fully differentiated T_H9 cells, which expressed IL-9 but not IL-4, and T_H2 cells that expressed IL-4 but not IL-9, showed similarly enhanced IL-17RB mRNA expression compared with naïve T cells (Fig. 1d). To confirm these results, we analyzed IL-17RB cell-surface expression on T_H1, T_H2 and T_H9 cells by using an anti-IL-17RB antibody 14. Consistent with the abovementioned data, IL-17RB expression was not detected on T_H1 cells but was observed on T_H2 and T_H9 cells (Supplementary Fig.1). Thus, IL-17RB is expressed in both T_H2 and T_H9 cells.

IL-25 enhances IL-9 expression in T_H9 cells

Expression of IL-17RB in T_H9 cells prompted us to investigate whether IL-25 might regulate T_H9 differentiation or function. Naïve T cells were isolated and activated with anti-CD3 and anti-CD28 with or without IL-25 in the presence or absence of TGF-β and analyzed for IL-9 production. While treatment with IL-4 and TGF-β drove T_H9 differentiation, IL-9 production was not detected after IL-25 stimulation with or without TGF-β, suggesting that IL-25 does not initiatiate T_H9 polarization in this system (Fig. 1e). Because TGF-β together with IL-4 induced the highest expression of IL-17RB, we tested whether IL-25 might enhance the IL-9 inducing effect of TGF-β and IL-4. In naïve T cells differentiated under T_H9 conditions IL-25 treatment led to significantly (P=0.047) enhanced IL-9 and IL-10 secretion (Fig. 1f). Consistent with a previous report 8, we found that, upon IL-25 treatment, IL-5-producing T_H2 cells appear distinct from IL-9-secreting T cells as assessed by intracellular cytokine staining (Fig. 1g) or IL-9 mRNA expression (Fig. 1h). Because GATA3 expression was not affected by IL-25 treatment (Fig. 1h), IL-25 appears to regulate IL-9 expression independent of this T_H2-regulating factor. Altogether, our data suggest that IL-17RB is expressed in T_H9 cells and IL-25 enhances the production of IL-9 in the presence of TGF-β and IL-4.

IL-25-mediated *in vivo* pathology is abrogated in mice lacking IL-17RA 15. To test whether IL-17RA expression in T cells is essential for IL-25 function, we isolated naïve T cells from wild-type and IL-17RA-deficient mice, activated them as above in the presence of anti-IFN-γ with or without IL-25, and assessed their cytokine profile. We found that IL-17RA deficiency resulted in impaired IL-25-mediated T_H2 differentiation (Fig. 2a). Addition of IL-25 to T cells cultured in T_H9 conditions significantly (P = 0.004) enhanced IL-9 secretion in wild-type cells but had no effect on IL-17RA-deficient cells (Fig. 2b), indicating that IL-17RA is required for the IL-25 effect on IL-9 production. To determine whether IL-17RB is required as well for the IL-25-mediated T_H9 differentiation, we added soluble IL-17RB protein during T_H9 differentiation. The soluble IL-17RB profoundly inhibited IL-25-enhanced IL-9 production as well as IL-5 and IL-13 production (Fig. 2c). Thus, our data suggest that IL-17 receptor A and B are required for IL-25-mediated regulation of T_H2 and T_H9 cytokine expression.

Enhanced IL-17RB signaling induces IL-9 expression

Although naïve T cells express IL-17RB 12,T_H9 cells express higher amounts. Because IL-25 regulates IL-9 expression in the presence of TGF-β and IL-4 but not on its own or together with TGF-β, we next tested whether increasing IL-17RB expression in naïve T cells could induce IL-9 expression. We thus generated transgenic IL-17RB mice using the CD4 mini-gene 16 (Fig. 3a). Five founder lines were obtained, and four of them were transmitted to progeny. The expression of IL-17RB mRNA in naïve $CD4^+$ T cells from four transgenic lines was compared to their control littermates (Fig. 3a). Lines 1, 3 and 5, expressing higher levels of IL-17RB mRNA were selected for further characterization. Surface expression of IL-17RB protein on $CD4^+$ and $CD8^+$ T cells from CD4-IL-17RB transgenic mice was confirmed by staining with an anti-IL-17RB antibody (Supplementary Fig.2). The CD4-IL-17RB transgenic mice exhibited normal immune cell populations in lymphoid organs (data not shown).

Cytokine expression was analyzed in naive T cells isolated from CD4-IL-17RB transgenic mice or littermate controls activated with anti-CD3 and anti-CD28, with or without IL-25. Consistent with our previous report on the role of IL-25 in promoting T_H2 differentiation12, IL-25 treatment induced an increase in the frequency of IL-5-positive cells while the frequency of IFN-γ expressing cells was reduced in CD4-IL-17RB transgenic T cells (Fig. 3b). Using ELISA, we found enhanced production of T_H2 cytokines, including IL-5 and IL-13, and decreased IFN-γ production in two independent transgenic lines compared to control cells (Fig. 3c). IL-4 production was decreased, possibly due to the rapid consumption of IL-4 in the culture of IL-17RB transgenic T cells. Indeed, anti-IL-4 receptor (IL-4R) α treatment resulted in enhanced IL-4 protein expression in the culture supernatants (Supplementary Fig. 3). Consistently with that, we found up-regulation of Th2 cytokine gene expression, as assessed by quantitative RT-PCR (Fig. 3d). We could not detect IL-9 expression in IL-25-treated wild-type T cells; however we found a strong induction of IL-9 expression in IL-17RB transgenic T cells treated with IL-25. Likewise, when wild-type T cells were infected with a retrovirus expressing IL-17RB or an empty vector containing IRES-GFP (Supplementary Fig. 4) IL-25 treatment induced IL-9 secretion only in GFP^+ IL-17RB-expressing T cells (Fig. 3e). In addition, T cells expressing IL-9 were distinct from IL-4-producing T cells. Our results using a transgenic mouse models and retroviral transduction suggest that over-expression of IL-17RB on naïve T cells allows IL-25 to induce IL-9-producing T cells.

Because IL-17RB and IL-17RA both are critical for IL-25 function, we further examined the signaling mechanisms by which IL-25 regulates IL-9 expression. Isolated naïve T cells from wild-type or IL-17RA-deficient mice were retrovirally transduced with IL-17RB or control IRES-GFP and were cultured with IL-25. Cytokine gene expression was examined by real-time RT-PCR in purified GFP^+ cells restimulated with anti-CD3. While IL-17RB overexpressing T cells up-regulated IL-13 and IL-9 expression, this effect was completely absent in IL-17RA-deficient T cells (Fig. 4a).

Because IL-25 regulation of IL-9 expression seems to require both IL-17RA and IL-17RB, we next tested the physical interaction between IL-17RA and IL-17RB. When IL-17RA and HA-tagged or wild-type IL-17RB were expressed simultaneously in 293T cells, IL-17RA co-immunoprecipitated with IL-17RB (Fig. 4b **and** Supplementary Fig. 5), suggesting the association of IL-17RA and IL-17RB as a signaling complex for IL-25. As IL-25 was initially found to bind to IL-17RB but not IL-17RA 10, it is not clear whether IL-17RB only mediates ligand recognition or additionally transduces signals in T cells. We showed that IL-17RA binds to Act1 adaptor protein through the SEFIR (similar expression to fibroblast growth factor genes and IL-17Rs) domain 17. Although the SEFIR domain in IL-17RB is critical for IL-25-mediated induction of G-CSF in embryonic fibroblast cells 18,19, the importance of this domain in T cells is unclear. Over-expression of IL-17RB containing a truncated cytoplasmic domain in activated T cells did not regulate IL-9 or other classical T_H2 cytokine expression following IL-25 treatment (Fig. 4c). Altogether, our data suggest that IL-25 signaling via an IL-17RA and IL-17RB complex in T cells regulates T_H2 and T_H9 differentiation.

IL-25–mediated IL-9 production is IL-4-independent

Because over-expression of IL-17RB induced IL-9 expression in the absence of TGF-β and IL-4, we next analyzed whether endogenous expression of TGF-β or IL-4 in CD4-IL-17RB transgenic T cells contributes to IL-9 production. We isolated naïve T cells from CD4-IL-17RB transgenic mice or littermate controls, activated them with or without IL-25 in the presence of neutralizing antibodies against TGF-β, IL-4, both TGF-β and IL-4, IL-13, or IL-4Rα. IL-25 treatment induced substantial amounts of IL-9 in CD4-IL-17RB transgenic T cells; TGF-β blockade resulted in a reduction in IL-9 production but no change in IL-5 expression (Fig. 5a). There was no further reduction of IL-9 production with the inclusion of other antibodies. In contrast, while treatment with antibodies to IL-4 and/or IL-13 or IL-4Rα affected the IL-5 expression, these agents did not significantly reduce IL-9 cytokine expression (Fig. 5a).

To confirm the above results, we assessed IL-9 expression in purified naïve T cells from IL-4-deficient mice retrovirally transduced with IL-17RB. Upon IL-25 stimulation of these cells, we found a strong reduction of conventional T_H2 cytokines expression in IL-4-deficient versus wild-type T cells, but no change in the mRNA expression level of IL-9 (Fig. 5b). Thus, our data suggest that IL-25 signaling promotes IL-9 expression through TGF-β-dependent but IL-4-independent mechanisms. Thus, TGF-β not only regulates IL-17RB expression but may also act together with IL-25 signaling to induce IL-9 expression.

IL-9 production *in vivo*

We next analyzed the *in vivo* IL-9 production in CD4-IL-17RB transgenic mice by employing an allergen-induced allergic lung disease model. CD4-IL-17RB transgenic or control littermate mice were repeatedly challenged (4 doses every other day) with the *Aspergillus* protease and chicken ovalbumin (OVA) protein allergens intranasally. 24 h after the last challenge, mice were sacrificed and BAL fluid cells were collected for analysis of inflammatory cell infiltration. CD4-IL-17RB transgenic mice showed increased total cell numbers in the BAL fluid when compared with wild-type control mice (Fig. 6a). Lung-infiltrating cells consisted predominantly of eosinophils and $CD4^+$ T cells. Analysis of OVA-specific cytokine production in wild-type and transgenic splenocytes revealed no change in OVA-specific IL-4 production, possibly due to consumption by T cells in the culture, but showed increased IL-5 and IL-13 production in cells from CD4-IL-17RB transgenic mice (Fig. 6b). Analysis of lung cells and draining lymph node cells from allergen challenged CD4-IL-17RB transgenic or wild-type mice stimulated with PMA and ionomycin showed similar results (Supplementary Fig. 6). In addition, although we could not detect OVA-specific IL-9 expression in allergen-challenged wild-type mice, we consistently observed significant ($P = 0.03$) levels of OVA-specific IL-9 cytokine production in CD4-IL-17RB transgenic mice (Fig. 6b).

Although IL-9 is known to be important in allergic inflammation 4,5,20, its role in IL-25-mediated pathology has not been directly analyzed. To further address the role of IL-9 in allergic inflammation in CD4-IL-17RB transgenic mice, we assessed lung inflammation and T_H2 cytokine production in transgenic mice administered an anti-IL-9 blocking antibody during the induction of allergic lung disease. IL-9 blockade resulted in a significant

reduction of total cells and eosinophils in the airway (Fig. 6c). Moreover, we found a strong reduction of OVA-specific IL-5 and IL-13 production in splenocytes from anti-IL-9 treated CD4-IL-17RB transgenic mice (Fig. 6d). These data indicate that IL-17RB over-expression up-regulates IL-9 and conventional T_H2 cytokines *in vivo* and that IL-25-mediated IL-9 functions to regulate allergic lung inflammation.

IL-25 deficiency attenuates allergic lung inflammation

We next examined whether IL-25 is required for IL-9 expression in an allergen-induced allergic mouse model. We generated IL-25 mutant mice through mouse ES cells gene-targeting (Supplementary Fig. 7). Two loxP sites were introduced into the IL-25 gene locus, flanking exons 2 to 3 and germline IL-25-deficient mice were produced by breeding floxed and CMV-Cre mice, IL-25 mRNA could not be detected by real-time PCR in the lung tissue of IL-25-deficient mice (Supplementary Fig. 8c).

We found a strong reduction of infiltrating inflammatory cells and classical T_H2 cytokines in the BAL fluid of IL-25-deficient mice after repeated allergen challenge (Supplementary Fig. 8a and 8b). Although we could not detect IL-9 protein expression in the BAL fluids or splenocytes from either wild-type or IL-25-deficient mice, mRNA expression analysis from individual lungs revealed a significant ($P = 0.02$) reduction in IL-9 mRNA expression in IL-25-deficient mice (Supplementary Fig. 8c).

IL-9 production was previously reported in the BAL fluid of a different allergic lung disease model 21. According to this alternate senzitisation protocol, IL-25-deficient or wild-type mice were sensitized with OVA in alum at day 0 and 14, followed by intranasal challenge with OVA at day 14, 25, 26, and 27 and inflammatory cell infiltration in the BAL fluid was analysed twenty-four hours following the last challenge. We found a remarkable decrease in total cell infiltrates predominantly composed of eosinophils in the BAL fluid from IL-25-deficient mice (Fig. 7a). As previously described using this model, we could detect OVA-specific IL-9 besides conventional T_H2 cytokine in splenocytes from wild-type mice. However, IL-9 and conventional T_H2 cytokines production in IL-25-deficient mice was strongly decreased (Fig. 7b). Analysis of OVA-specific cytokine response in draining lymph node cells (Fig. 7c) and BAL fluid (Fig. 7d) from IL-25-deficient mice showed no detectable IL-9 and marked reduction of IL-4, IL-5, and IL-13. Consistent with protein expression, we found a significant reduction of IL-9 and T_H2 cytokine mRNA expression in the lungs of IL-25-deficient mice (Fig. 7e). Thus, our data suggest that IL-25 expression is required for IL-9 expression in allergic lung diseases *in vivo*.

Discussion

IL-9 was originally described as a T_H2-derived cytokine. Unlike classical T_H2 cytokines, IL-4 alone cannot induce IL-9 expression 6. Indeed, TGF-β was found to be a crucial factor for inducing IL-9 and maximal production could be achieved by adding IL-4. Moreover, recent data have suggested that IL-9-producing T cells are distinct from a conventional T_H2 lineage 7,8. However, the regulation of these cells remains unclear. Here, we found that IL-17RB is expressed by IL-9-producing T cells and regulates IL-9 production *in vitro* and *in vivo*.

IL-17RB is a single transmembrane protein that binds to IL-25 with high affinity. We previously showed that IL-17RB is expressed by T_H2 but not by T_H1 or T_H17 cells and IL-25 functions to promote the differentiation of T_H2 cells 12. In this study, we describe that T_H9 cells also expressed IL-17RB and IL-25 plays additional role in promoting T_H9 differentiation. . Because IL-25 could not replace IL-4 in inducing T_H9 differentiation and IL-4 induced IL-17RB expression, it is probable that optimal IL-4 induced IL-17RB expression on T cells is necessary to promote IL-9 production. Indeed, naïve T cells from CD4-IL-17RB transgenic mice secreted substantial amounts of IL-9 in response to IL-25 in the absence of IL-4. In contrast, TGF-β appears not only to regulate IL-17RB expression but also acts with IL-25 signaling to induce IL-9 expression. Future studies will be needed to elucidate how downstream signals of the receptors for TGF-β, IL-4 and IL-25 act in inducing and/or enhancing IL-9 expression.

In addition to IL-17RB, IL-17RA is essential for IL-25 function. IL-25-mediated type-2 immune responses requires IL-17RA and IL-17RB 15, however direct function of both receptors for IL-25 activities on T cells is unclear. Our data analysis of T_H cell differentiation *in vitro* indicate that IL-17RA and IL-17RB is essential for IL-25-enhanced T_H2 and T_H9 differentiation in vitro. Thus, endogenous expression of IL-17RA in T cells and inducible expression of IL-17RB may contribute to the determination of T_H lineages. The physical interaction of IL-17RA and IL-17RB indicate that IL-25 functions need association of both receptors to form receptor complex. In addition, the cytoplasmic domain of IL-17RB was required for IL-25 function in enhancing the production of Th2 cytokines and IL-9, suggesting that not only IL-17RB mediates ligand recognition, it contributes to signal transduction in T cells. Recent studies indicate the essential of adaptor protein Act1, also known as CIKS (originally derived from connection to IκB kinase and stress-activated protein kinases) in IL-25-mediated allergic inflammation 18,19. Requirement of Act1 in IL-25-enhanced Th2 and Th9 differentiation remains elusive. Dissecting the signal transduction pathways involved in IL-25 activities on T cells requires further investigation. Previous work has shown IL-17R-deficient mice show reduced airway inflammation in association with attenuated T_H2 responses 22; these defects may therefore not be due solely to a defect in IL-17 signaling but also to a defect in IL-25-signaling.

TGF-β, IL-2, and IL-4 can regulate IL-9 expression6,23. In CD4-IL-17RB transgenic T cells that secrete large amounts of IL-9 in response to IL-25, inhibition of IL-4 and IL-2 had little effect on IL-9 expression. However, neutralization of TGF-β abolished the production of IL-9. Furthermore, over-expression of IL-17RB in IL-4-deficient T cells resulted in normal induction of IL-9 expression, although it did not affect IL-5 and IL-13 expression. These data suggest that IL-25 uses different mechanisms to mediate T_H2 differentiation and IL-9 expression. IL-25 may promote T_H2 polarization through an IL-4-dependent pathway, whereas the regulation of IL-9 expression by IL-25 is independent of IL-4 but dependent of TGF-β signaling. Our *in vitro* data not only indicate IL-25 as a novel IL-9 regulator, but also support the notion that IL-9 and T_H2 cytokines expression is regulated by common but also by distinct regulatory mechanisms.

Our *in vivo* experiments confirm the importance of IL-25 in regulating IL-9 expression. Because IL-25 is an important regulator in allergic asthma 12,13,24,25 and IL-9 is a key

player in airways inflammation and asthma 4,5,26, IL-25 may contribute to IL-9-mediated lung inflammation. In mouse model of allergic lung diseases, the enhanced IL-9 expression in IL-17RB transgenic mice and reduced IL-9 expression in IL-25-deficient mice indicate the importance of IL-25 in the regulation of IL-9. Inhibition of IL-9 production in IL-17RB transgenic mice that led to reduced allergic inflammation in these mice clearly suggest that IL-25 is one regulator of IL-9 in vivo. Because of IL-25 can regulate the production of Th2 cytokines from non-T-non-B cells and the differentiation of Th2 and Th9 in allergic diseases, it may serve as an important therapeutic target for treatment of allergy and asthma.

In conclusion, we have provided *in vitro* and *in vivo* evidence that IL-25 acts as a regulator of IL-9-producing T cells through the expression of IL-17RB on these cells. Our results indicate an important function of IL-25 in coordinating both conventional T_H2 cytokine and IL-9 expression. Further research on the regulation of T_H9/T_H2 subsets and their function in immune-mediated diseases are required to understand the complex effector mechanisms involved in immune responses.

Methods

Generation of CD4-IL-17RB transgenic mice

A full-length cDNA encoding mouse IL-17RB was cloned into SalI site of the CD4 mini-gene plasmid, which contains CD4 promoter/enhancer and the first intron without the silencer element (a gift from D. Littman, New York University School of Medicine, New York, NY) 16. The IL-17RB transgene construct was digested with NotI and microinjected into C57Bl/6 mice at the Transgenic Mouse Facility at MD Anderson Cancer Center (Houston, Texas). Transgene integration was analyzed by genomic PCR by using the following primer: forward: 5’-GCTCAGATTCCCAACCAACAAGAG-3’, reverse: 5’-CAGGGCCATCTCCAGAGT-3’. Five transgenic founders were obtained and bred with C57Bl/6 mice. Subsequent screening of CD4-IL-17RB transgenic mice was carried out by genomic PCR using the following primers: forward: 5’-ATGGTCCAACACACACTCACTCCA-3’, reverse: 5’-ACAGACAAAGAAGGGCTGTCCTCA-3’. Three lines expressing highest IL-17RB mRNA levels were further characterized.

Generation of IL-25 knockout mice

IL-25-deficient mice were generated by introducing a pair of loxP sites into the IL-25 locus in 129/TC1 embryonic stem cell line. The targeting vector contained NeoR as a positive selection marker and Diphtheria toxin A as a negative selection marker. Targeted ES clones were selected and injected into C57BL/6 blastocyst to generate chimeras. High percentage chimeras were bred with female CMV-Cre transgenic mice to obtain IL-$25^{+/-}$ mice. Heterozygous mice were then bred to obtain homozygous knockout mice and wild-type littermates for experiments. The genotyping primers for IL-$25^{-/-}$ mice were, Forward: 5'-CTGCTCCAGTCAGCCTCTCT-3’, Reverse 1: 5'-AGCAGCTGGGCAAGTGAC-3’ and Reverse 2: 5'-AGGTGGAGAAAGTGCCTGT-3’. The primers Forward and Reverse1 surrounding the first loxP site amplify a 368-bp wild-type band, while the primers Forward and Reverse2 flanking the deleted exons giving a 500-bp knock out band. Mice 6–10 weeks

of age were used in experiments using protocols approved by Institutional Animal Care and Use Committee, MD Anderson Cancer Center.

Induction and analysis of asthma models

For allergen-induced asthma model, mice (5 mice per group) were subjected for intranasal antigen challenge. Mice were anesthetized with isofluorene and allowed to inhale a mixture containing *A.oryzae* and OVA every other day for four total challenges. An anti-IL-9 blocking antibody or control antibody was given intraperitoneally at the time of challenge in some experiments. 24h following the final challenge, mice were sacrificed for analysis of BAL fluid and antigen-specific T cell responses as previously described 12. For OVA-induced asthma model, mice were immunized with 100 μg OVA in alum at day 0 and 14, followed by intranasal challenge with 50 μg OVA at day 14, 25, 26, and 27. Twenty-four hours after last challenge, mice were sacrificed and analyzed for inflammatory cell infiltration in BAL fluid and antigen-specific T cell response as above.

T_H cell differentiation

Lymph nodes and spleens of mice were FACS-sorted as described 12 and stimulated with plate-bound anti-CD3 (1 μg/ml) plus soluble anti-CD28 (2 μg/ml) in the presence of indicated stimuli or neutralizing antibodies (20 μg/ml anti-TGF-β, 10 μg/ml anti-IL-4, 10 μg/ml anti-IL-13, 10 μg/ml anti-IL-2, 10 μg/ml anti-IL-4Rα, 10 μg/ml anti-IFN-γ, 10 μg/ml sIL-17RB) or with polarizing cytokines (10 μg/ml anti-IL-4, 10 ng/ml IL-12, and 50 U/ml hIL-2 for Th1, 10 μg/ml anti-IFN-γ, 10 ng/ml IL-4, and 50 U/ml hIL-2 for Th2, 20 ng/ml IL-6, 5 ng/ml TGF-β, anti-IFNγ and anti-IL-4 for Th17, 5 ng/ml IL-4 and 1 ng/ml TGF-β for Th9) with or without IL-25Ig (2 μg/ml). After 4 days of restimulation, cells were washed and restimulated with plate-bound anti-CD3 (1 μg/ml) for 4h, and cells were then collected for RNA extraction. For cytokine measurement by ELISA, culture supernatants were collected at 24h. For intracellular cytokine analysis, cells were restimulated with 500 ng/ml of ionomycin and 50 ng/ml of PMA in the presence of Golgi Stop (BD Pharmingen) for 5h. Cells were then permeabilized with Cytofix/Cytoperm Kit (BD Pharmingen) and analyzed for the expression of IL-4, IL-5 or IFN-γ (BD Pharmingen) or IL-9 (Biolegend). For detection of of IL-17RB surface expression, T cells after activation were stained with biotinylated anti-IL-17RB antibody 14, followed by streptavidin-APC.

Retroviral transduction

pGFP-RV-full-length-IL-17RB and pGFP-RV-mutated IL-17RB were generated. Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells from C57Bl/6 mice, IL-17R-deficient mice or IL-4-deficient mice were FACS-sorted and activated with plate-bound anti-CD3 and anti-CD28 in the presence of polarizing cytokines. Thirty-six hours after activation, cells were spin-infected with retrovirus expressing IL-17RB, or IL-17RB with deleted cytoplasmic domain, and control empty vector, and medium was replaced with IL-25 added after 4h of infection. Four days after activation, GFP^+ cells were FACS-sorted and gene expression was assessed by quantitative real-time PCR.

Immunoprecipitation

293T cells expressing HA-IL-17RB or wild-type IL-17RB and/or IL-17RA were washed once with ice cold phosphate-buffered saline and lysed in lysis buffer. Lysates were used for western blot or immunoprecipitation as previously described 17. For immunoprecipitation, cleared cell lysate was incubated with anti-HA antibody or anti-IL-17RB antibody (Santa Cruz Biotechnology Inc.) for 90 min followed by 4 washes in lysis buffer. Immunoprecipitates were denatured with 2x SDS sample buffer before SDS-polyacrylamide gel electrophoresis. Protein transfer was followed by overnight incubation with anti-HA, anti-IL-17RB (R&D Systems) or anti-IL-17RA (R&D) antibodies . After incubation with HRP-conjugated secondary antibody, the signal was detected with ECL reagent (Promega).

Real-Time RT-PCR analysis

Total RNA extracted using TRIzol reagent (Invitrogen) was used to generate cDNA using oligo-dT, random hexamers, and MMLV reverse transcriptase (Invitrogen). For quantitation of cytokine, cDNA samples were amplified in IQ™ SYRB® Green Supermix (Biorad Laboratories). The data were normalized to *Actb* reference. The primer pairs for analysis of *IL-4*, *IL-5*, *IL-13*, and *IFN*-γ as previously described were used 12. The primer pair for *IL-9* is Forward: 5'-CATCAGTGTCTCTCCGTCCCAACTGATG-3', Reverse: 5'-GATTTCTGTGTGG CATTGGTCAG-3'. The primer pair for *IL-17RB*: Forward: 5'-CCATCCCTCCAGAT GACAAC-3', Reverse: 5'-TGCTCCTTCCTTGCCTCCAAGTTA -3', IL-17RA: Forward: 5'-AGTGTTTCCTCTACCCAGCAC-3', Reverse: 5'-GAAAACCGCCACCG CTTAC-3', IL-17RC: Forward: 5'-GCTGCCTGATGGTGACAATGT-3', Reverse: 5'-TGGACGCAGGTACAGTAAGAAG-3'.

Statistical Analysis

Data are presented as mean value+SD. Data were analyzed using Student's t test (n=2 groups). A value of $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank S. Rivera, S. J. Kuschert and J. Parker-Thornburg for their help in generation of knockout and transgenic animals, D. Littman for the CD4 mini-gene plasmid, K. Murphy for GFP-RV vector, Amgen for IL-17-receptor deficient mice, J. van Snick for an anti-IL-9 staining antibody, B.Stockinger and M. Veldhoen for suggestions and the Dong lab members for their help and discussion. The work is supported by research grants from NIH (to CD). PA receives a fellowship from Royal Thai government and CD is a Trust Fellow of the MD Anderson Cancer Center and a Leukemia and Lymphoma Society Scholar.

References

1. Van Snick J, et al. Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40). J Exp Med. 1989; 169:363–368. [PubMed: 2521242]

2. Gessner A, Blum H, Rollinghoff M. Differential regulation of IL-9-expression after infection with Leishmania major in susceptible and resistant mice. Immunobiology. 1993; 189:419–435. [PubMed: 8125519]

3. Else KJ, Hultner L, Grencis RK. Cellular immune responses to the murine nematode parasite Trichuris muris. II. Differential induction of TH-cell subsets in resistant versus susceptible mice. Immunology. 1992; 75:232–237. [PubMed: 1532377]

4. Temann UA, Geba GP, Rankin JA, Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. J Exp Med. 1998; 188:1307–1320. [PubMed: 9763610]

5. McLane MP, et al. Interleukin-9 promotes allergen-induced eosinophilic inflammation and airway hyperresponsiveness in transgenic mice. Am J Respir Cell Mol Biol. 1998; 19:713–720. [PubMed: 9806735]

6. Schmitt E, et al. IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. J Immunol. 1994; 153:3989–3996. [PubMed: 7930607]

7. Dardalhon V, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(−) effector T cells. Nat Immunol. 2008; 9:1347–1355. [PubMed: 18997793]

8. Veldhoen M, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. Nat Immunol. 2008; 9:1341–1346. [PubMed: 18931678]

9. Monteyne P, et al. IL-4-independent regulation of in vivo IL-9 expression. J Immunol. 1997; 159:2616–2623. [PubMed: 9300680]

10. Lee J, et al. IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. J Biol Chem. 2001; 276:1660–1664. [PubMed: 11058597]

11. Fort MM, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001; 15:985–995. [PubMed: 11754819]

12. Angkasekwinai P, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. J Exp Med. 2007; 204:1509–1517. [PubMed: 17562814]

13. Wang YH, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC activated Th2 memory cells. J Exp Med. 2007; 204:1837–1847. [PubMed: 17635955]

14. Terashima A, et al. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. J Exp Med. 2008; 205:2727–2733. [PubMed: 19015310]

15. Rickel EA, et al. Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. J Immunol. 2008; 181:4299–4310. [PubMed: 18768888]

16. Sawada S, Scarborough JD, Killeen N, Littman DR. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell. 1994; 77:917–929. [PubMed: 8004678]

17. Chang SH, Park H, Dong C. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. J Biol Chem. 2006; 281:35603–35607. [PubMed: 17035243]

18. Swaidani S, et al. The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation. J Immunol. 2009; 182:1631–1640. [PubMed: 19155512]

19. Claudio E, et al. The adaptor protein CIKS/Act1 is essential for IL-25-mediated allergic airway inflammation. J Immunol. 2009; 182:1617–1630. [PubMed: 19155511]

20. Kung TT, et al. Effect of anti-mIL-9 antibody on the development of pulmonary inflammation and airway hyperresponsiveness in allergic mice. Am J Respir Cell Mol Biol. 2001; 25:600–605. [PubMed: 11713102]

21. Salek-Ardakani S, et al. OX40 (CD134) controls memory T helper 2 cells that drive lung inflammation. J Exp Med. 2003; 198:315–324. [PubMed: 12860930]

22. Schnyder-Candrian S, et al. Interleukin-17 is a negative regulator of established allergic asthma. J Exp Med. 2006; 203:2715–2725. [PubMed: 17101734]

23. Houssiau FA, Renauld JC, Fibbe WE, Van Snick J. IL-2 dependence of IL-9 expression in human T lymphocytes. J Immunol. 1992; 148:3147–3151. [PubMed: 1533652]
24. Ballantyne SJ, et al. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. J Allergy Clin Immunol. 2007; 120:1324–1331. [PubMed: 17889290]
25. Tamachi T, et al. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. J Allergy Clin Immunol. 2006; 118:606–614. [PubMed: 16950278]
26. Ying S, Meng Q, Kay AB, Robinson DS. Elevated expression of interleukin-9 mRNA in the bronchial mucosa of atopic asthmatics and allergen-induced cutaneous late-phase reaction: relationships to eosinophils, mast cells and T lymphocytes. Clin Exp Allergy. 2002; 32:866–871. [PubMed: 12047433]

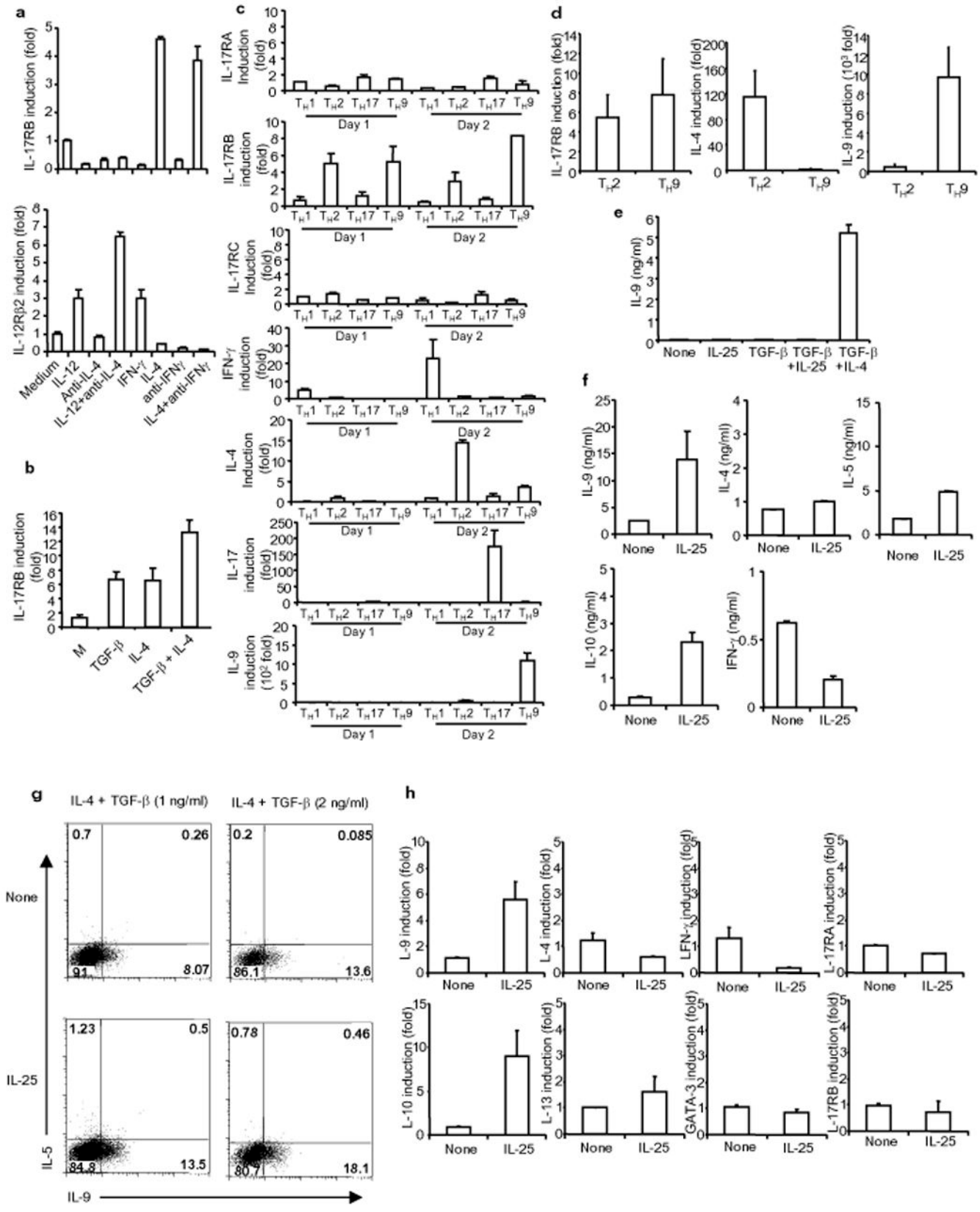


Figure 1. IL-17RB expressed by T_H9 cells enhances IL-9 production

(a) Real-time RT-PCR of IL-17RB mRNA expression in T cells activated with the indicated cytokines or antibodies **(b)** Real-time RT-PCR of IL-17RB mRNA expression in T cells activated in the presence or absence of IL-4 and/or TGF-β for 2 days. **(c)** Real-time RT-PCR of IL-17RB mRNA expression at the indicated time points in naive T cells differentiated under T_H1, T_H2, T_H17, and T_H9 conditions. Fold induction was calculated using Th1 cells as control and normalizing with actin expression. **(d)** Real-time PCR of IL-17RB mRNA expression in naive T cells differentiated under T_H2 and T_H9 condition for 5 days and restimulated with anti-CD3 for 4 hours. Naïve T cells were used as control. **(e)** Enzyme-linked immunosorbent assay (ELISA) of IL-9 production in activated naïve T cells cultured with or without IL-25 in the presence or absence of TGF-β or in the presence of IL-4 and TGF-β for 4 days, followed by restimulation with anti-CD3. ELISA **(f)** intracellular cytokine staining **(g)** or real-time PCR by setting no treatment as control **(h)** of cytokine production in naïve T cells stimulated with anti-CD3 and anti-CD28 in the presence of IL-4 and TGF-β with or without IL-25 for 4 days. . Data shown are a representative of at least two independent experiments.

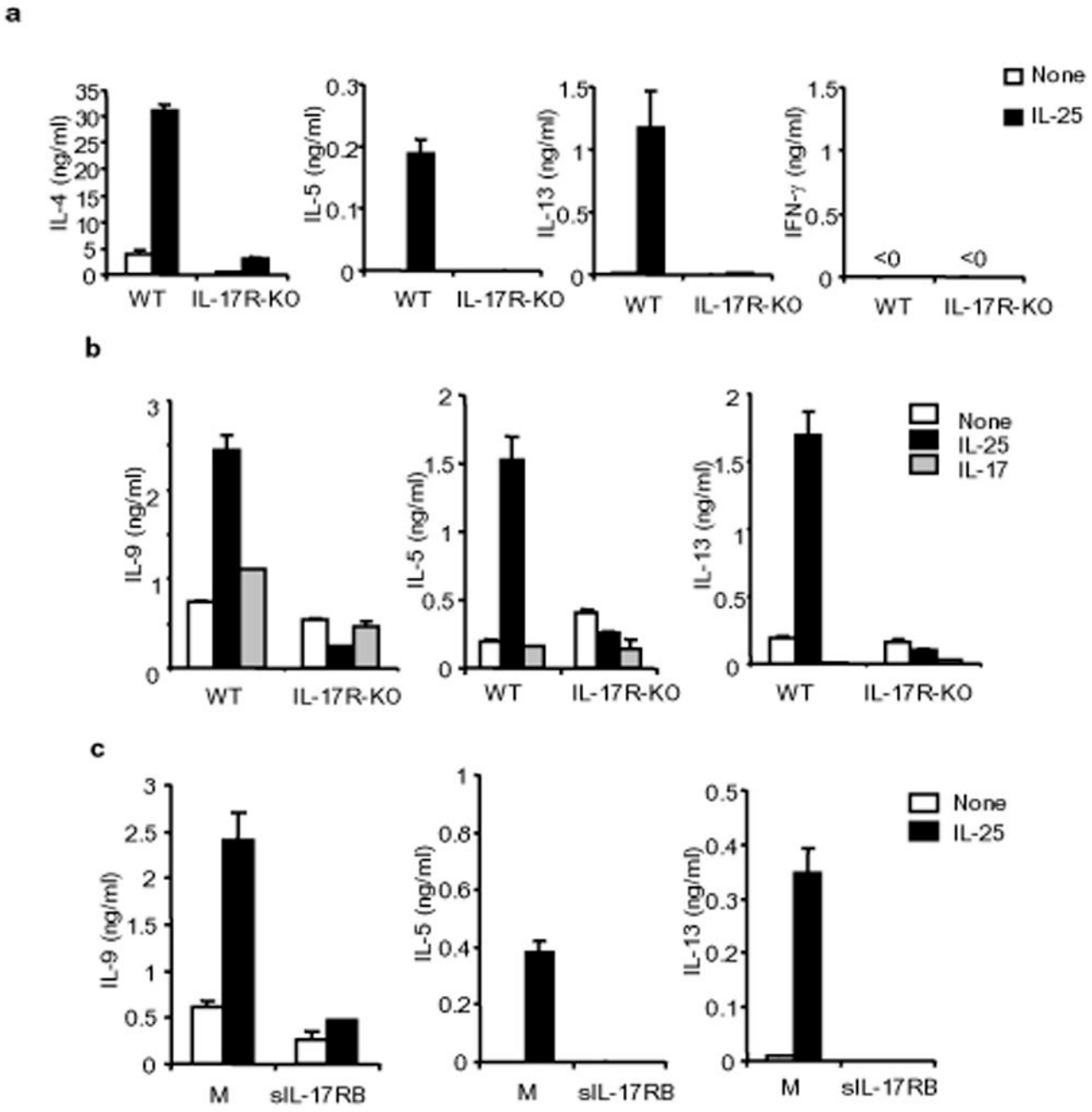
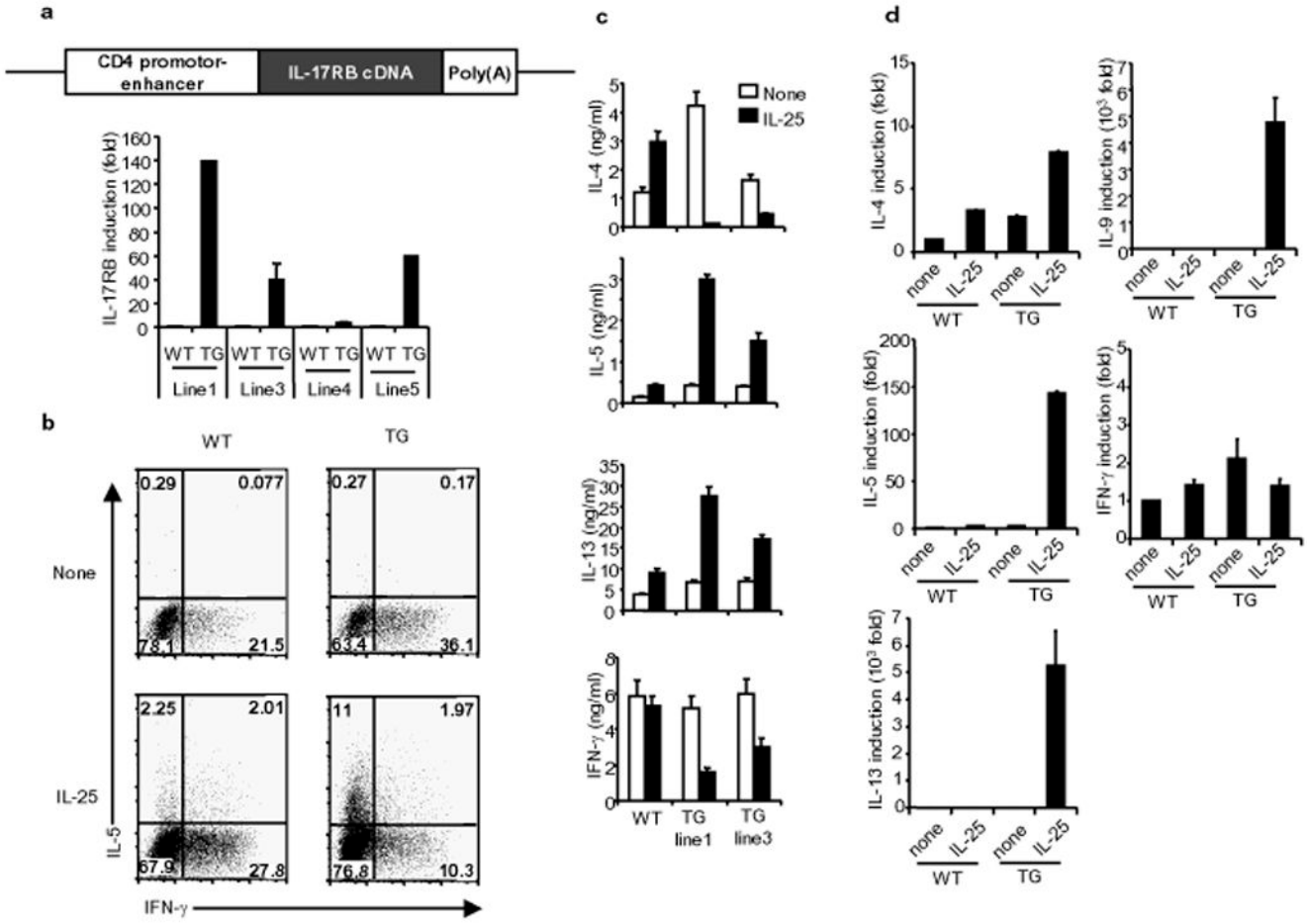


Figure 2. IL-17RA expression in T cells is essential for IL-25 function

(a) ELISA of cytokines production in naïve T cells isolated from wild-type or IL-17R KO mice and activated with anti-CD3 and anti-CD28 in the presence of an anti-IFN-γ antibody with or without IL-25 for 4 days, followed by restimulation with anti-CD3 for 24 hrs. **(b)** ELISA of cytokines production in naïve T cells activated as above in the presence of IL-4 and TGF-β with or without IL-25 or with IL-17. **(c)** ELISA of cytokines production in naïve T cells activated as above with IL-4 and TGF-β with or without IL-25 in the presence or

absence of soluble IL-17RB. Data shown are a representative of at least two independent experiments.



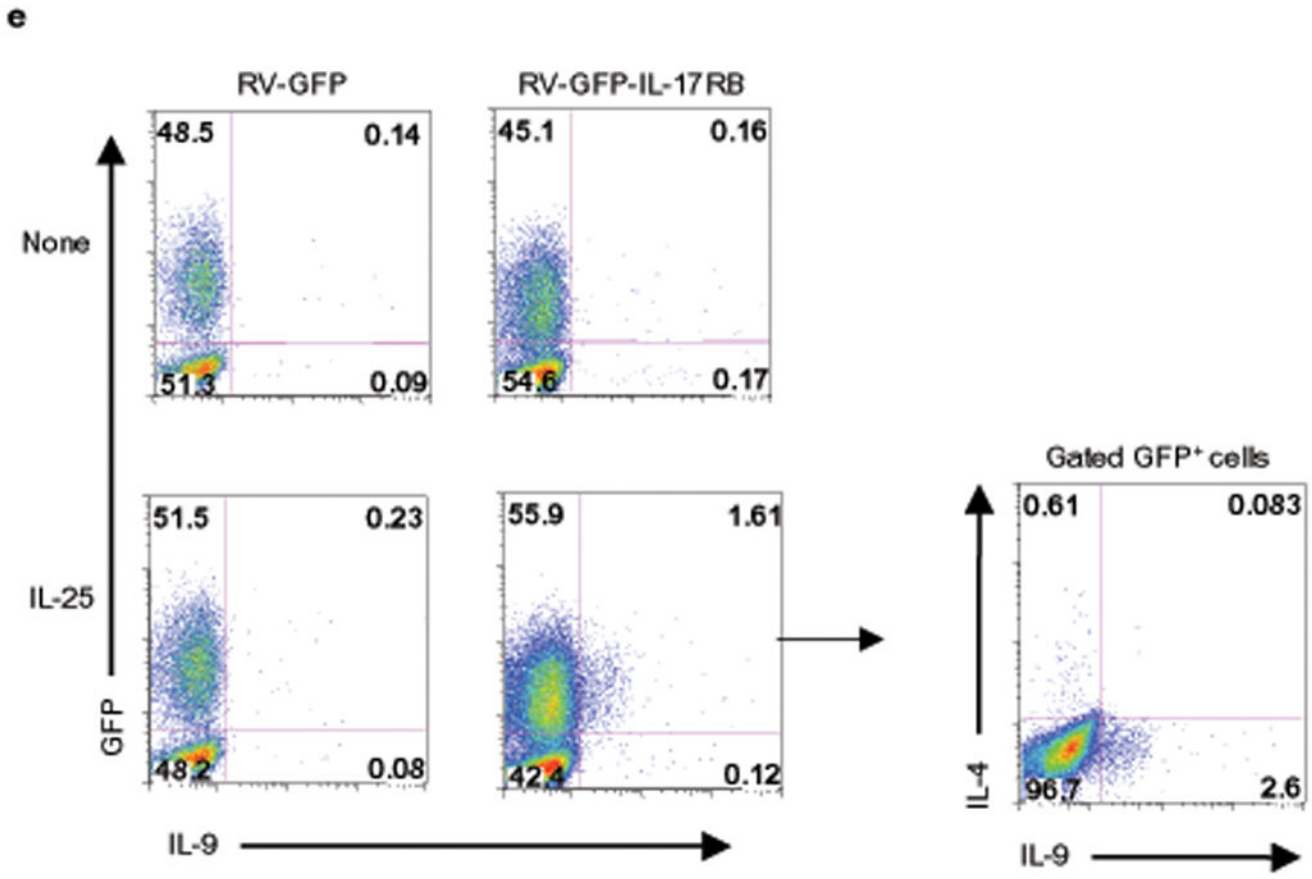


Figure 3. Enhanced IL-17RB signaling induces IL-9 expression

(a) Schematic diagram of CD4-IL-17RB transgenic construct and mRNA expression of IL-17RB in naïve T cells isolated from wild type or different lines of transgenic mice as assessed by real-time RT-PCR. **(b)** Frequency of IL-5 and IFN-γ-producing cells in naïve T cells isolated from wild-type or CD4-IL-17RB (TG) mice and activated with anti-CD3 and anti-CD28 in the presence or absence of IL-25 for 4 days, followed by 5 hrs of PMA and ionomycin stimulation in the presence of Golgi Stop. ELISA **(c)** or by real-time PCR **(d)** of cytokine production in naïve T cells from wild type or CD4-IL-17RB TG mice activated as above for 4 days, followed by restimulation with anti-CD3. **(e)** Intracellular IL-9 and IL-4 cytokine staining and GFP expression in wild-type naïve T cells retrovirally transduced with IL-17RB or control viral vector (RV-KM) followed by treatment with IL-25 for 4 days. Data shown are a representative of at least three independent experiments.

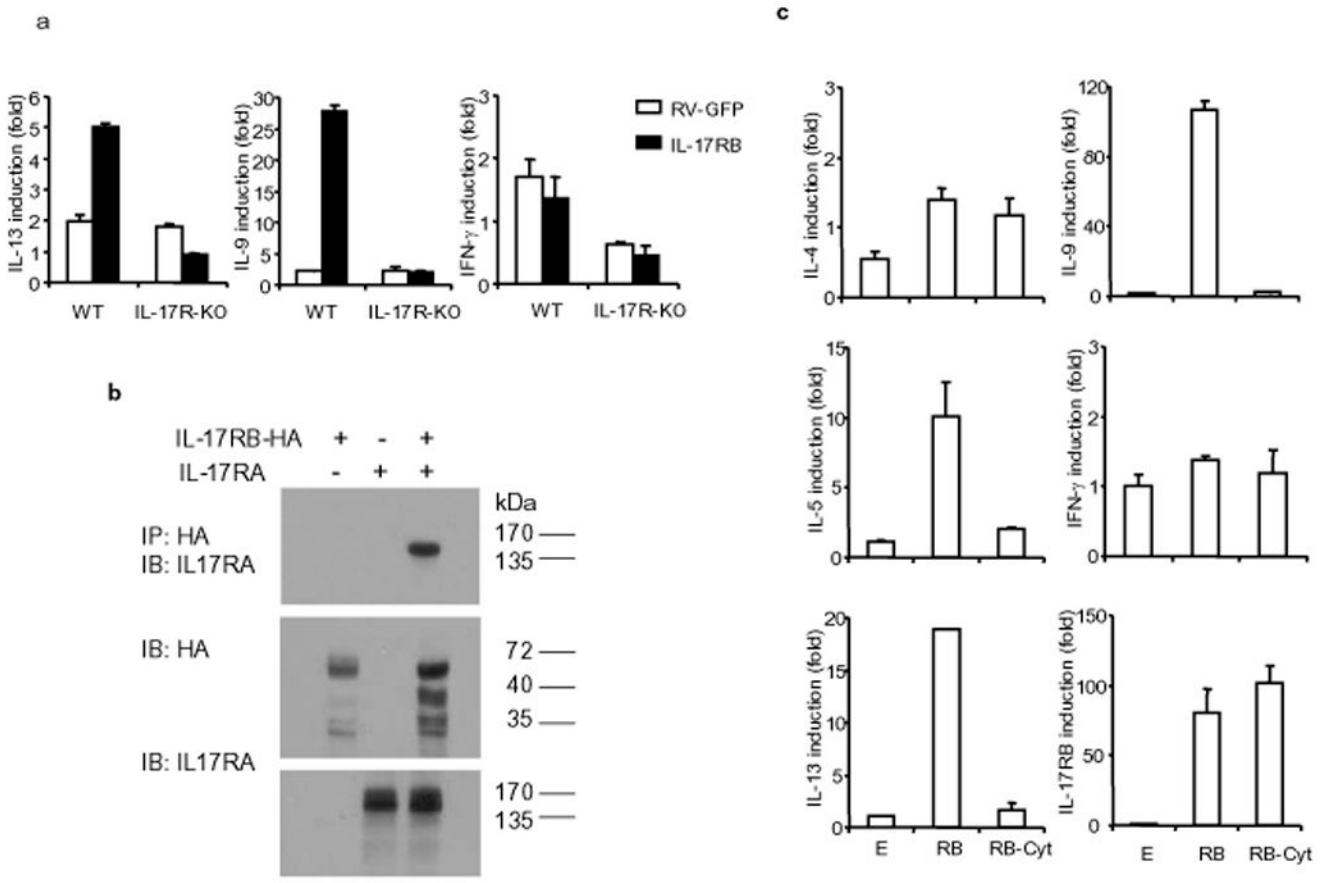


Figure 4. IL-17RA and IL-17RB are essential for IL-25-mediated IL-9 expression

(a) Real-time PCR of cytokines mRNA expression in wild-type and IL-17R KO T cells retrovirally transduced with IL-17RB or control viral vector (RV-GFP) and cultured with IL-25 for 4 days. Sorted GFP+ cells were activated with anti-CD3 for 4 hrs prior to mRNA isolation. **(b)** Immunoblot analysis of IL-17RA or HA-tagged IL-17RB of lysates from 293T cells transfected with IL-17RA or HA-tagged IL-17RB or both and immunoprecipitated with anti-HA antibody. **(c)** Real-time PCR of cytokine mRNA expression in wild-type T cells retrovirally transduced with full length IL-17RB (RB) or IL-17RB lacking the cytoplasmic domain (RB-cyt) or control viral vector (E) and activated for 4 days. Data shown are a representative of at least two independent experiments.

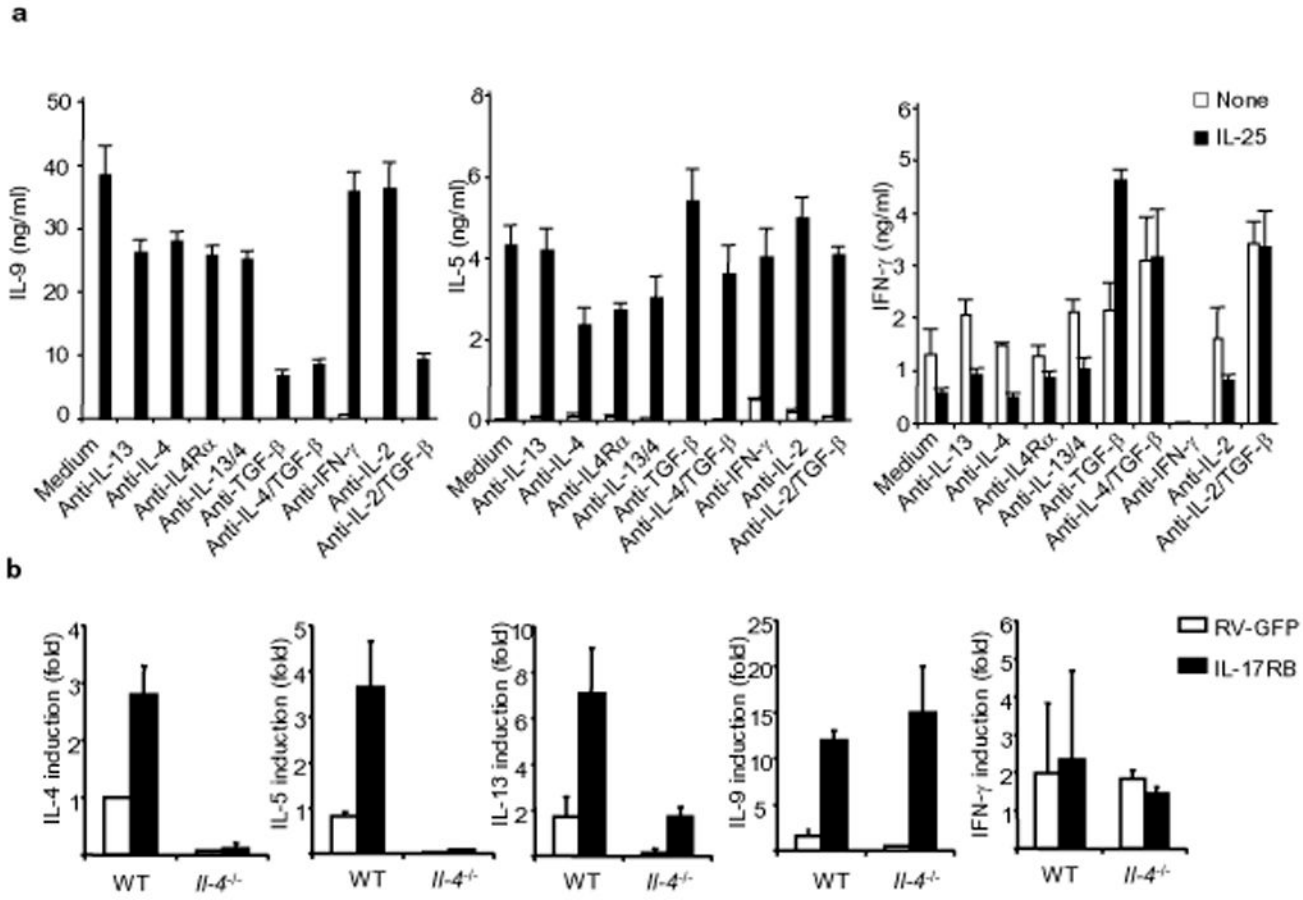


Figure 5. IL-9 production induced by enhanced IL-17RB signaling requires TGF-β but not IL-4

(a) ELISA of cytokines production in naïve T cells isolated from CD4-IL-17RB transgenic stimulated with anti-CD3 and anti-CD28 in the presence or absence of IL-25 with the indicated neutralizing antibodies for 4 days, followed by reactivation with anti-CD3 for 24 hrs. **(b)** Real-time RT-PCR analysis of cytokine expression in wild-type or IL-4-deficient T cells retrovirally transduced with IL-17RB or control vector and treated with IL-25 for 4 days. Sorted GFP+ cells were activated with anti-CD3 for 4 hrs prior to mRNA isolation. Data shown are a representative of at least two independent experiments.

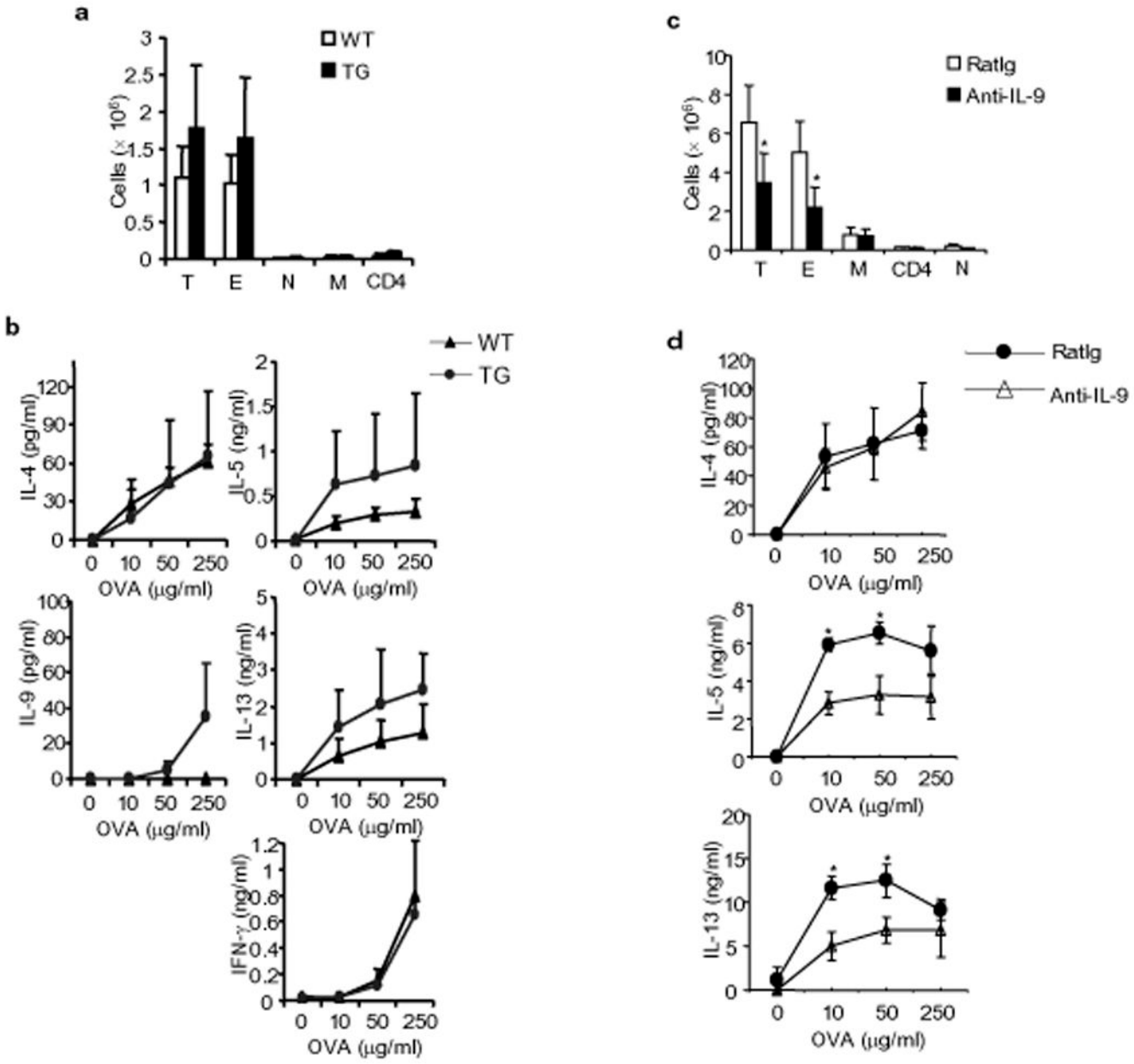


Figure 6. IL-25-regulated IL-9 expression in CD4-IL-17RB transgenic mice exacerbates allergic lung diseases

(**a**) Total cell counts in BAL from CD4-IL-17RB transgenic mice (TG) or wild-type littermate controls intranasally challenged with *A. oryzae* and OVA allergens every other day for 4 allergen challenges in total. Four to five mice were analyzed in each group. (**b**) ELISA of OVA-specific cytokine production in splenocytes from wild-type and CD4-IL-17RB transgenic mice allergen challenged as in **a**. Cells were re-stimulated for 72 hours with different concentrations of OVA *ex vivo*. (**c**) Total cell counts in BAL from CD4-IL-17RB transgenic mice intranasally challenged with *A. oryzae* and OVA allergens every other day for 4 allergen challenges in total, with anti-IL-9 blocking antibody or a Rat IgG control antibody administered at the time of challenge. Four to five mice were analyzed in

each group. (**d**) ELISA of OVA-specific cytokine production in splenocytes from CD4-IL-17RB transgenic mice allergen challenged as in **c**. Data are a representative of at least two independent experiments.

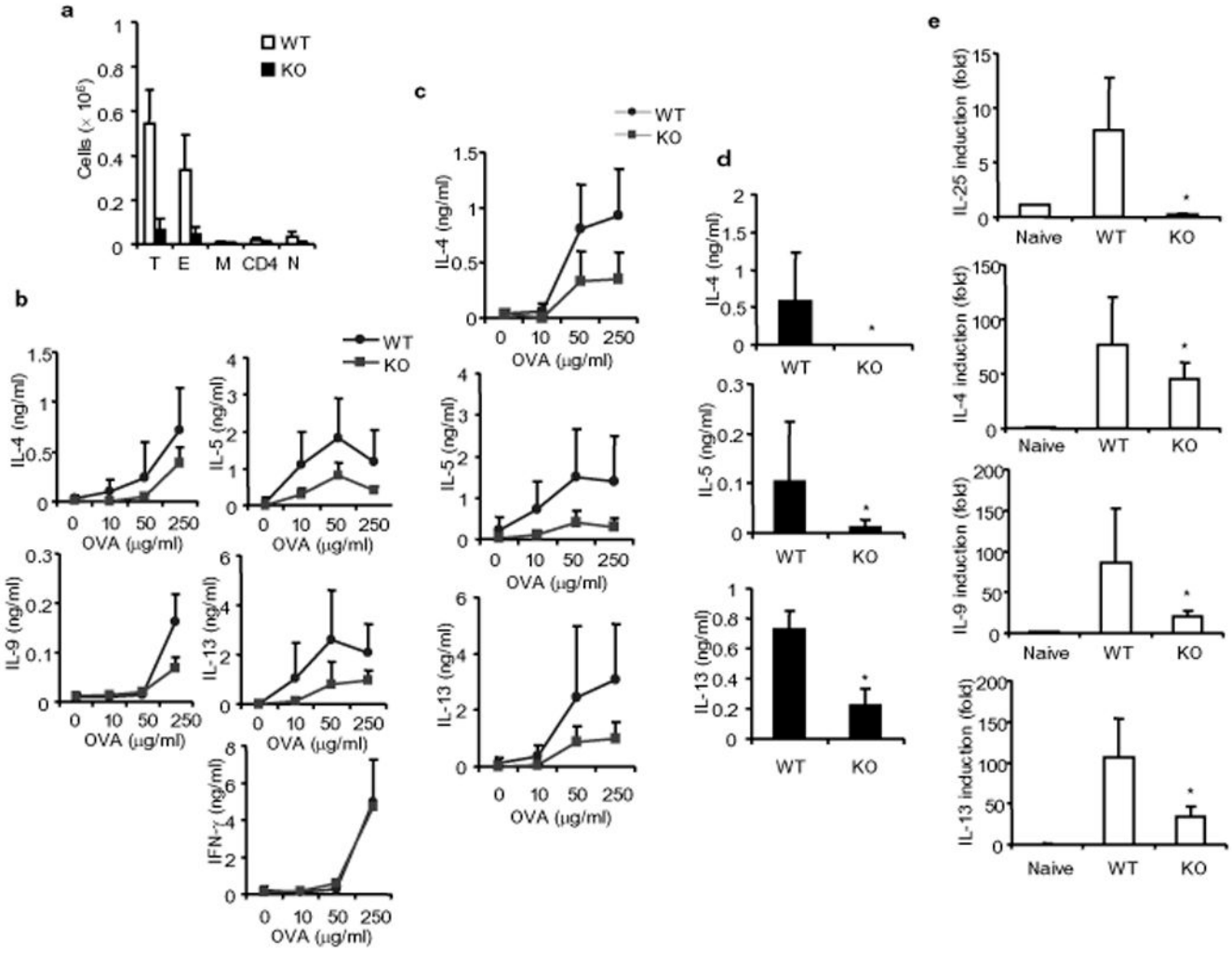


Figure 7. IL-25-deficiency leads to reduced IL-9 expression and airway inflammation in a mouse model of chronic allergic lung disease

(**a**). Total cell counts in BAL fluid of IL-25KO mice or wild-type littermate controls sensitized with OVA in alum at day 0 and 14, followed by intranasal challenge with OVA at day 14, 25, 26, and 27. ELISA of OVA-specific cytokine production in splenocytes (**b**) and draining lymph node cells (**c**) or BAL fluid (**d**) from wild-type or IL-25KO mice allergen challenged as in **a**. Cells were re-stimulated for 72 hours with different concentrations of OVA *ex vivo*,* $p < 0.05$. (**e**) Real-time PCR analysis of cytokine expression in lungs harvested from wil-type or IL-25 KO mice. * $p< 0.05$. Data are a representative of at least three independent experiments.