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Nat Immunol. Author manuscript; available in PMC 2011 July 14.

Published in final edited form as:

Nat Immunol. 2010 June ; 11(6): 527–534. doi:10.1038/ni.1867.

The transcription factor PU.1 is required for the development of interleukin 9-producing T cells and allergic inflammation

Hua-Chen Chang[1,*], **Sarita Sehra**[1,*], **Ritobrata Goswami**[1,2], **Weiguo Yao**[1], **Qing Yu**[1], **Gretta L. Stritesky**[1,2], **Rukhsana Jabeen**[1], **Carl McKinley**[1], **Ayele-Nati Ahyi**[1,2], **Ling Han**[1], **Evelyn T. Nguyen**[1], **Michael J. Robertson**[3], **Narayanan B. Perumal**[4], **Robert S. Tepper**[1], **Stephen L. Nutt**[5], and **Mark H. Kaplan**[1,2]

[1] Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202

[2] Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202

[3] Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202

[4] School of Informatics, Indiana University-Purdue University, Indianapolis, Indianapolis, IN 46202

[5] The Walter and Eliza Hall Institute of Medical Research, Victoria 3050, Australia.

Abstract

$CD4^+$ T helper cells acquire effector phenotypes that promote specialized inflammatory responses. We show that the ETS family transcription factor, PU.1 was required for the development of an interleukin 9 (IL-9)-secreting subset of T_H cells. Decreasing PU.1 expression either by conditional deletion in murine T cells or siRNA in human T cells impaired IL-9 production, while ectopic PU.1 expression promoted IL-9 production. Mice with PU.1-deficient T cells developed normal T_H2 responses *in vivo*, but exhibited attenuated allergic pulmonary inflammation corresponding to decreased *Il9* and chemokine expression in peripheral T cells and in lungs as compared to wild-type mice. Together, these data suggest a critical role for PU.1 in generating the T_H9 phenotype and in the development of allergic inflammation.

Introduction

T helper (T_H) cells promote the development of specific types of inflammation following differentiation into various effector subsets. The cytokine microenvironment present when a naive T_H cell is activated determines the developmental pathway. Interleukin 4 (IL-4 [http://

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Correspondence should be addressed to MHK (mkaplan2@iupui.edu). .

*These authors contributed equally.

AUTHOR CONTRIBUTIONS MHK designed and supervised the study and wrote the manuscript. HCC, RG, RJ and LH did experiments in Figures 1-3 and Supplementary Fig. 1. QY, RG and GLS did experiments in Figure 4 and Supplementary Figure 2. WY, MJR and RST obtained human samples and WY performed all experiments in Figure 5. SS, ETN, CM and ANA performed experiments in Figure 6, 7 and Supplementary Fig. 3. NBP provided bioinformatics analysis. SLN provided mice.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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www.signaling-gateway.org/molecule/query?afcsid=A001262]) promotes the differentiation of T_H2 cells and allergic inflammation. IL-12 promotes T_H1 differentiation, while inflammatory cytokines, including transforming growth factor-β (TGF-β1), IL-6, IL-1 and IL-23, stimulate the development of T_H17 cells, both cell types contribute to autoimmune inflammation[1–3]. T_H differentiation and the establishment of effector phenotypes are governed by a network of transcription factors that regulate the expression of cytokines and other effector genes. For example, T_H2 development requires the IL-4–induced activation of STAT6, followed by the subsequent induction of GATA-3, a T_H2 lineage-promoting factor. The ability of T_H2 cells to respond to subsequent T cell receptor (TCR) stimulation with a rapid induction of T_H2-specific cytokines further requires NFAT family members, Jun family members, c-maf, and IRF4, likely among other factors, to transactivate cytokine loci[4–6].

We have described that the transcription factor PU.1 [http://www.signaling-gateway.org/molecule/query?afcsid=A001950] also contributes to the T_H2 phenotype by promoting heterogeneity of T_H2 cytokine secretion[7,8]. PU.1 was specifically expressed in subpopulations of T_H2 cells that had low IL-4 expression. Ectopic expression of PU.1 reduced production of most T_H2 cytokines, while shRNA that decreased PU.1 expression, or PU.1-deficiency, increased overall cytokine production specifically in populations of cells that secreted two or more cytokines[7,8]. PU.1 functions, at least in part, by limiting the ability of GATA-3 and IRF4 to interact with cytokine loci targets[7–9].

IL-9 [http://www.signaling-gateway.org/molecule/query?afcsid=A001559] has long been thought to be a T_H2 cytokine as it promotes allergic inflammation and is associated with various T_H2 responses[10]. Transgenic expression of *Il9* results in allergic inflammation[11–13]. IL-9 promotes mucus production from lung epithelial cells and pulmonary mastocytosis[14–17]. While allergic inflammation can develop in *Il9*$^{-/-}$ mice using a standard sensitization-challenge model, blockade of IL-9 in a similar model results in decreased allergic inflammation[18,19]. IL-9 expression is increased in lungs of asthmatic patients[20,21], and blocking antibodies are being developed as a therapy for atopic disease[22]. IL-9 may also contribute to extracellular parasite immunity[23,24]. Most recently, IL-9 has been shown to promote the development of T_H17 cells, while increasing Treg activity[25,26], suggesting that IL-9 biology may be more complex.

In addition to established T_H subsets, reports have described an IL-9-secreting population of T cells[27]. T_H2 cells and Treg cells can produce IL-9 (ref. [28]), and culture with TGF-β1 may increase IL-9 production from multiple cell types[26]. A T_H subset putatively called T_H9 cells are derived in culture with a combination of TGF-β1 and IL-4 (refs. [24,29]). These cells are related to T_H2 cells in that they require STAT6 and GATA-3 for development, but have reduced expression of T_H2 cytokines[24,29]. Transcription factors that specifically control the IL-9–secreting T cell phenotype have not been identified.

In this report, we identified PU.1 as a regulator of IL-9 production in T cells. PU.1-deficient T cells had diminished IL-9 production and ectopic expression of PU.1 increased IL-9 production from T_H2 or T_H9 cultures. Diminishing PU.1 expression in human IL-9–secreting T cell cultures also decreased IL-9 production. In mice that lack PU.1 only in T

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cells, allergic airway inflammation was attenuated and this observation correlated with decreased IL-9 and PU.1-induced chemokine production in the lung. These results suggest that PU.1 is a critical regulator of the IL-9–secreting T cell phenotype and is important for the development of allergic inflammation.

RESULTS

PU.1 promotes the development of IL-9–secreting T cells

Initial descriptions of the IL-9–secreting T_H cells (hereafter called T_H9 for simplicity) did not identify a transcription factor that distinguished these cells as a distinct lineage[24,29]. We have shown that PU.1 limits T_H2 cytokine production in T cells[7,8], suggesting that it could be involved in the T_H9 phenotype. To test this hypothesis, we differentiated naive T cells from wild-type and PU.1-deficient (*Sfpi1*$^{lck-/-}$) mice under T_H9 conditions (TGF-β1 plus IL-4). We observed that IL-9 secretion, mRNA expression and IL-9 intracellular staining were decreased in T_H9 cultures that lacked PU.1 expression (Fig. 1a–c).

PU.1 expression was low in T_H1 cells, and in T_H2 populations was expressed in IL-4^{lo} but not IL-4^{hi} cells[8]. To compare the expression of PU.1 in additional T_H subsets, we generated T_H1, T_H2, T_H17 and T_H9 cells and analyzed RNA for expression of PU.1. We observed that T_H1 and T_H17 had the lowest expression of *Sfpi1* mRNA, T_H2 cells had 3–5-fold more abundant expression, and T_H9 cells expressed approximately 3-fold more *Sfpi1* than T_H2 cells (Fig. 1d). Inducible Treg cultures expressed even more *Sfpi1* mRNA than in T_H9 cultures (Supplementary Fig. 1a), suggesting that *Sfpi1* might be a TGF-β-induced gene. The expression of *Sfpi1* was not STAT6-dependent as determined by comparing wild-type and *Stat6*$^{-/-}$ T_H9 cultures, (data not shown). As both iTreg and T_H17 cultures have been suggested to produce IL-9 (refs. [25,26,28]), we also directly compared cells cultured under these conditions to T_H9 cultures. By intracellular staining and ELISA we observed low or undetectable amounts of IL-9 produced by iTreg cultures (Fig. 1e and data not shown). In comparing T_H9 and T_H17 cultures, we observed little IL-9 production in T_H17 cultures assays by intracellular staining or ELISA, though there was a low amount of IL-17 produced from T_H9 cultures (Fig. 1f,g). As IL-9 was observed in T_H2 cultures, we separated T_H2 populations into IL-4^{hi} and IL-4^{lo} populations[8], and observed that *Il9* expression segregated with *Sfpi1* expression in the IL-4^{lo} cells and segregated away from *Il4* expression (Fig. 1h).

To define if PU.1 was a determining factor in promoting IL-9 secretion, we ectopically expressed PU.1 using a bicistronic retrovirus in T_H2, T_H9 and iTreg cultures and examined cytokine secretion and gene expression in sorted EGFP$^+$ cells. Increased PU.1 expression in T_H2 or T_H9 cultures further induced IL-9 production, assessed by both ELISA and intracellular cytokine staining, and measurement of *Il9* mRNA in these cultures (Fig. 2a-c). Ectopic PU.1 expression in T_H2 cells, as previously described, reduced the expression of T_H2 cytokines (Fig. 2c). While ectopic PU.1 expression could decrease *Foxp3* expression in iTreg cultures (Supplementary Fig. 1b), it did not induce *Il9* expression in these cells (data not shown). Inducible Treg generation was normal in PU.1-deficient cells, and the ability of IL-4 to repress Foxp3 was not significantly impaired in the absence of PU.1 expression (Supplementary Fig. 1c,d). Moreover, there were no significant increases in CD4$^+$Foxp3$^+$ cells in a variety of *Sfpi1*$^{lck-/-}$ lymphoid organs, compared to wild-type mice

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(Supplementary Fig. 1e,f), suggesting that PU.1-deficiency does not affect Foxp3 expression. These results suggest that PU.1 promotes the IL-9–secreting T cell phenotype, but that additional IL-4-induced signals are required to generate T_H9 cells.

Distinct populations secrete IL-10 and IL-9

Previous reports on IL-9–secreting T cells suggested that they also express IL-10 (refs. [24,29]). In contrast, our previous report demonstrated that IL-9 and IL-10 expression in T_H2 cells were reciprocally regulated[9]. To further examine this issue, we differentiated T_H2 and T_H9 cells from cultures of naïve $CD4^+$ T cells using IL-4 or a combination of IL-4 and TGF-β1, respectively. While IL-10 was detected in T_H9 cells, production was much lower than from T_H2 cultures (Fig. 3a,b). Moreover, additional treatments of T_H9 cultures had reciprocal effects on IL-9 and IL-10 production. We observed an increase of IL-9 production from T_H9 cultures when they were differentiated in the presence of anti-IL-10 concomitant with a decrease in IL-10 production (Fig. 3c). Addition of IL-9 to T_H2 cultures did not affect IL-10 production (data not shown). Addition of TGF-β1 to differentiated T_H9 cultures, during anti-CD3 restimulation, increased IL-9 production, but decreased IL-10 production (Fig. 3d). While PU.1-deficiency resulted in less IL-9 production (Fig. 1), it did not significantly affect IL-10 production from T_H9 cultures (Fig. 3e). Thus, while T_H9 cultures may produce IL-10, IL-9 and IL-10 are not coordinately regulated.

PU.1 is required for chromatin modifications of *Il9*

As T helper specification is associated with specific chromatin structure, we examined chromatin modifications at the *Il9* locus in naive T cells, and naive T cells cultured for five days under T_H1, T_H2, T_H9 or T_H17 conditions using chromatin immunoprecipitation (ChIP) assays. Total acetylation of both histone H3 and H4, and acetylation of H3K9 and H3K18 at two *Il9* CNS (Fig. 4a) were highest in T_H9 cultures compared to all other subsets (Fig. 4b), though all subsets demonstrated an increase in acetylation compared to naïve cells. T_H9 cells also had the lowest amount of trimethylated H3K27 (H3K27me3), a modification associated with repressed genes (Fig. 4b). Cultures of iTreg cells displayed histone acetylation comparable to T_H9 cells (Fig. 4c), in agreement with data on the *Il9* locus derived from genome-wide studies[30]. However, the *Il9* locus had increased amounts of H3K27me3 in iTreg cultures compared to T_H9 cultures (Fig. 4c). Thus, active *Il9* expression was associated with a combination of high H3 acetylation and low H3K27 methylation. To test if PU.1 was required for the establishment of these chromatin modifications, we differentiated wild-type and PU.1-deficient naive T cells under T_H9 conditions and analyzed chromatin modifications. We observed H3 acetylation, the modification that showed the greatest difference between T_H9 and other T_H cultures, was decreased in the absence of PU.1 at both CNS sites (Fig. 4d) and that this effect was specific for T_H9 cultures since PU.1-deficiency did not affect H3 acetylation of *Il9* in T_H2 or T_H17 cells (Supplementary Fig. 2). Moreover, the presence of the Cre transgene did not decrease *Il9* H3 acetylation (Supplementary Fig. 2). In contrast, H4 acetylation or H3K27 methylation were not affected by the absence of PU.1 (Fig. 4e). These results suggest that PU.1 is required for programming the *Il9* locus but plays a restricted role in the histone modifications that are affected.

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To determine if PU.1 was acting directly on the *Il9* gene we examined the CNS1 sequence for consensus PU.1 binding sites (5′-GGAA-3′ core sequence). We found two potential sites within sequences that were conserved among human, mouse and dog *Il9* promoters (Fig. 4f). Using DNA affinity precipitation assay (DAPA) with cell extracts from T_H2 or T_H9 cells, followed by immunoblot, we observed PU.1 binding to both sites, with stronger binding to site 2 and binding was decreased by incubation with a non-biotinylated competitor oligonucleotide (Fig. 4f and data not shown). More PU.1 binding was observed using T_H9 extracts, parallel to increased PU.1 protein expression in T_H9 extracts (Fig. 4f). We then tested if PU.1 bound to the *Il9* promoter using ChIP assays. Following differentiation of T_H1, T_H2 and T_H9 cells, PU.1 ChIP assays detected PU.1 binding to the *Il9* promoter in T_H9 cells, with no detectable binding in T_H1 or T_H2 cells (Fig. 4g). Thus, PU.1 binds to sites in the *Il9* promoter specifically in T_H9 cells.

PU.1 is required for IL-9 production in human T cells

The ability of conditions that promote the development of murine T_H9 cells to promote a human T_H9 phenotype has not been tested. To assess the derivation of human T_H9 cells, we cultured naive $CD4^+$ T cells purified from peripheral blood under T_H2 or T_H9 conditions. As with murine cells, the addition of TGF-β1 to T_H2 cultures decreased T_H2 cytokine production and increased production of IL-9 (Fig. 5a–c). Human T_H9 cultures also expressed more *SPI1* than T_H2 cultures (Fig. 5a). We then determined if PU.1 was required for IL-9 expression in human T_H9 cells by transfecting differentiated human T_H9 cultures with control or *SPI1*-specific siRNA before restimulation. Inhibiting *SPI1* expression resulted in a decrease in IL-9 production (Fig. 5d,e). These results suggest that the ability of PU.1 to promote the development of IL-9–secreting T cells is conserved in human and mouse cells.

T_H9 cells and allergic inflammation

The role of IL-9 in allergic inflammation is still unclear. To begin to explore this area further we examined IL-9 production from anti-CD3–stimulated peripheral blood mononuclear cells (PBMCs) from atopic (positive for at least one allergen-specific IgE of ten examined) and non-atopic (no allergen-specific IgE) children with ages ranging from 18-30 months. This cohort has been described in more detail elsewhere[31]. Importantly, while these children show signs of developing atopy, they have not had episodes of wheezing and are not considered to be asthmatic. IL-9 production was significantly higher from atopic patients than from non-atopic patients (Fig. 5f).

To further examine a role for PU.1-dependent T cell function in allergic inflammation, wild-type and *Sfpi1*$^{lck-/-}$ mice were subjected to a standard protocol for the development of ovalbumin (Ova)-induced allergic airway inflammation. Mice were sensitized with Ova in alum and challenged intranasally with Ova in PBS. We first tested the generation of T_H responses in the periphery by stimulating splenic cells with Ova or anti-CD3. Consistent with our previous *in vitro* analyses[7,8] there was no impairment of T_H2 cytokine responses in *Sfpi1*$^{lck-/-}$ mice, compared to wild-type mice (Fig. 6a). Production of T_H17 cytokines was also unimpaired by the absence of PU.1 (Supplementary Fig. 3). The generation of antigen-

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specific IgE and IgG1 was not significantly different between wild-type and *Sfpi1*$^{lck-/-}$ mice (data not shown).

In contrast to the normal development of peripheral responses, inflammation in the lung was decreased in mice with PU.1-deficient T cells, compared to wild-type mice (Fig. 6b). *Sfpi1*$^{lck-/-}$ mice also demonstrated significantly decreased airway hyperresponsiveness in response to methacholine challenge than wild-type mice (Fig. 6c). Analysis of bronchoalveolar lavage cellular composition demonstrated increased infiltration in wild-type and *Sfpi1*$^{lck-/-}$ mice compared to non-challenged mice, but total cell numbers, and the cell numbers of specific cell populations, including eosinophils, were decreased in *Sfpi1*$^{lck-/-}$ mice compared to wild-type mice (Fig. 6d). To confirm that Lck-Cre-mediated deletion of the *Sfpi1* allele was not affecting PU.1-dependent development of dendritic cells important for the development of inflammation, we examined CD11b^{+} and CD103^{+} subpopulations of CD11c^{+} MHC class II^{+} cells in bronchoalveolar lavage (BAL) and lung cell suspensions of non-sensitized and non-challenged mice and observed no significant differences between wild-type and *Sfpi1*$^{lck-/-}$ mice (data not shown). There were decreased amounts of IL-9 and T_H2 cytokines present in BAL fluid (Fig. 6e). In contrast, amounts of interferon-γ and IL-17 present in the BAL were not significantly affected (data not shown).

To demonstrate that decreases in IL-9 could result in a similar phenotype, we used the Ova-induced allergic airway inflammation model for wild-type mice treated with control or anti-IL-9 administered intravenously at the first, third and fifth challenges. We observed decreased inflammation and cell numbers of allergic inflammatory cells in mice treated with anti-IL-9, compared to control Ab-treated mice (Fig. 6f,g). This result confirms a role for IL-9 in this model established using a similar protocol in BALB/c mice[18].

To determine if *Il9* expression was lower in T cells from sensitized *Sfpi1*$^{lck-/-}$ mice than in wild-type mice, we used qPCR to assess mRNA abundance in antigen-stimulated splenocyte cultures. We observed diminished *Il9* mRNA in *Sfpi1*$^{lck-/-}$ cultures (Fig. 7a). It was possible that PU.1 was regulating other genes associated with a T_H9 phenotype. We have previously shown that PU.1 expression segregated with expression of *Ccl22* in IL-4^{lo} cells, and that ectopic PU.1 expression increased CCL22 production[8]. Consistent with those observations, we observed that chemokines associated with allergic inflammation, *Ccl17* and *Ccl22*, were less abundantly produced from antigen-stimulated *Sfpi1*$^{lck-/-}$ cultures as compared to wild-type cultures (Fig. 7a). Conversely, ectopic expression of PU.1 induced the expression of both chemokines in T_H9 cultures (Fig. 7b). To determine if the deficiency of chemokine production from T cells was reflected in the allergic inflammation model, we analyzed whole lung homogenates for *Ccl17* and *Ccl22* mRNA. While mRNA for both chemokines was present in tissue from sensitized and challenged mice, both were decreased in *Sfpi1*$^{lck-/-}$ lung tissue as compared to wild-type (Fig. 7c). These results suggest that PU.1 in T cells regulates allergic inflammation by inducing the expression of IL-9 and pro-allergic chemokines.

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DISCUSSION

The defined phenotypes of effector T helper subsets, once classified simply as T_H1 and T_H2, have been expanded to include T_H17, T_{FH} and inducible Treg cells. Most recently, an IL-9–secreting T cell, putatively termed T_H9, was identified as arising from a cytokine combination of TGF-β1 and IL-4 (refs. [24,29]). Distinguishing these subsets has become more difficult as the plasticity between the phenotypes has become apparent[32]. However, each of the lineages express transcription factors that are lineage-determining factors or that promote particular aspects of the lineage genetic program. The T_H9 lineage, if it is indeed a separate lineage, is related to T_H2 by the developmental requirement for IL-4 and the downstream transcription factors STAT6 and GATA3 (refs. [24,29]). In this report, we have identified PU.1 as a transcription factor that induces the T_H9 phenotype by promoting the expression of IL-9 and pro-allergic chemokines.

In our previous analyses of PU.1 in T_H2 cells we noted that expression was segregated to a subpopulation of T_H2 cells that expressed low amounts of IL-4 and other T_H2 cytokines[8]. This observation corresponded with the ability of ectopically expressed PU.1 to inhibit the production of T_H2 cytokines, and of decreasing PU.1 expression, either by siRNA or gene deletion, to increase T_H2 cytokine production[7,8]. This function was at least partly by interfering with factors that promote the T_H2 phenotype including GATA3 and IRF4 (ref. [8,9]). The data presented in this report suggests that the IL-4^{lo} T_H2 subset that we previously characterized might contain some T_H9 cells. Given the apparent plasticity of T_H2 cells to acquire a T_H9 phenotype[24], these two cell types might exist in a continuum where increasing PU.1 expression would lead to decreasing T_H2 cytokines and increasing characteristics of the T_H9 phenotype, with intermediate phenotypes being possible.

Our data indicate that PU.1 can bind the *Il9* promoter directly in T_H9 cells, and that conserved PU.1 binding sites exist within CNS1. While ectopic expression of PU.1 increased characteristics of T_H9 cells and converted T_H2 cells to a T_H9 phenotype, it had more modest effects on the induction of *Il9* in iTreg, T_H1 or T_H17 cultures (data not shown). These results might suggest that while PU.1 promotes the T_H9 phenotype, it may be most active in cells where additional factors are present to mediate these effects. This scenario is not uncommon in the T_H differentiation paradigm. As described above, multiple factors contribute to the T_H2 phenotype, and multiple factors including T-bet, STAT4, Hlx and Runx3 contribute to the T_H1 genetic program[33–35]. One possible context requirement might be decreased expression of IRF4, which we have demonstrated represses *Il9* in T_H2 cultures and is further required for the development of T_H17 cells[9,36]. Preliminary observations suggest that *Irf4* expression is less in T_H9 cells than in T_H2 cells (data not shown). The chromatin modifications present at the *Il9* locus in each of these cell types may also reflect the ability of PU.1 to induce *Il9* mRNA. The activity of the *Il9* locus correlated with the combination of increased histone acetylation and reduced H3K27 methylation. However, in iTreg cultures that had a comparable extent of histone acetylation but abundant H3K27 methylation at the *Il9* locus, *Il9* induction by PU.1 was not detected. The ability of PU.1 to induce IL-9 in T_H2 but not iTreg cultures suggests that additional IL-4-induced factors exist that are required for acquisition of the IL-9-secreting phenotype. While GATA-3 is required for T_H9 development[29], it seems an unlikely candidate since it is expressed only at low

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amounts in T_H9 cultures[24,29] and since we have shown PU.1 antagonizes GATA-3 activity in T_H cells[7,8]. IRF4 also seems unlikely as it represses *Il9* expression in T_H2 cells, and PU.1 antagonizes its function[9]. It will be interesting to determine if other transcription factors, in the presence of PU.1, promote *Il9* expression.

The relatedness between T_H2 and T_H9 phenotypes, and the requirement for STAT6 and GATA3 in the development of both subsets[24,29], raises some interesting questions about phenotypes that have been described in mice deficient in either STAT6 or GATA3, specifically whether phenotypes in allergic inflammation and parasite immunity are a result of defects in the development of one or both subsets. A previous report demonstrated that mice with defective TGF-β1 signaling had decreased parasite clearance that correlated with decreased IL-9 (ref. [24]). Similarly, in this report we show defects in the development of allergic inflammation in mice that have decreased IL-9-secreting T cells, but have normal development of T_H2 immunity. These findings suggest that T_H9 cells might be required for what has classically been termed T_H2 immunity and there is ample data supporting a role for IL-9 in allergic disease. Transgenic models have demonstrated that IL-9 can promote[12,13] and blockade of IL-9 inhibits[18] the development of allergic inflammation. Moreover, increased IL-9 is found in lungs of patients with asthma[20,21], and T cells from patients with atopic disease secreted more IL-9 than non-atopic patient T cells. Importantly, this observation does not diminish the role of T_H2 cytokines in the development of allergic inflammation, rather suggesting that these two T_H subsets work in concert to initiate allergic inflammation.

IL-9 can promote the development of T_H17 cells and was reported to also be produced by T_H17 cells[25,26]. Thus the decreased allergic inflammation in mice with PU.1-deficient T cells might be interpreted to result from the decreased generation of T_H17, which are required for the development of inflammation in this model[37]. However, we did not see decreases in the ability of PU.1-deficient T cells to acquire the T_H17 phenotype *in vitro* and ectopic expression of PU.1 did not induce IL-17 production (data not shown). Moreover, IL-17 production from mice that are sensitized and challenged was higher in Ova-stimulated splenocyte cultures from *Sfpi1*$^{lck-/-}$ mice than from wild-type mice. This finding suggests that PU.1 is not required for T_H17 development. TGF-β1 appears to be an important factor in promoting IL-9 production from cells cultured with various cytokines[26,27]. It is possible that while TGF-β1 promotes IL-9 production, the addition of IL-4 alters the phenotype such that T_H17 cytokine production is decreased and resulting cells are predominantly producers of IL-9 alone. In our experiments, T_H9 cultures produce IL-9, which was barely detectable in T_H17 cultures, and T_H9 cultures produced only low amounts of IL-17 compared to T_H17 cells. Thus, while there may be overlap in the cytokine signatures of these subsets, highly polarized cells might still have distinct functional properties.

The precise contribution of PU.1-expressing T cells to allergic inflammation remains undefined, and is likely a combination of altered expression of IL-9 and chemokines. Our data confirm that blocking IL-9 with antibodies decreases allergic inflammation in the OVA-induced model. As the previous report used BALB/c mice[18], and our study utilized C57BL/6 mice, our data demonstrate that the effects of blocking IL-9 are not strain-specific. The chemokines CCL17, CCL22 and their receptor CCR4, are each important in the

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development of allergic inflammation[38–40]. One possibility is that PU.1-dependent chemokine production from T cells is responsible for the diminished allergic inflammation in *Sfpi1*$^{lck-/-}$ mice. This possibility represents an addition to the current paradigm wherein T cells in the lung secrete cytokines, such as IL-13, that promote chemokine production from resident lung cells including epithelial cells, smooth muscle cells and fibroblasts[41]. Our results suggest that T cell chemokine production also contributes to the overall extent of inflammation. PU.1-dependent IL-9 production, or the expression of other PU.1-dependent genes, may also contribute to inflammation in this model, and the phenotype we observe may indeed be caused by several different factors. As the PU.1-dependent genetic program in T cells becomes better defined, the role of these factors can be assessed more directly.

The recent description of an IL-9-secreting T cell population added further complexity to effector T_H phenotypes. One factor that prevented this population from initially being termed a lineage was the absence of an identified transcription factor that conferred lineage-specific expression. Our work has identified PU.1 as a factor that promotes the T_H9 phenotype by both repressing T_H2 cytokine production and increasing IL-9 production. PU.1 is likely not the only factor that promotes this lineage, but will be an important component of continuing investigations into T_H heterogeneity.

METHODS

Methods and any associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Research was supported by Public Health Service Grants RO1 AI57459 and U19 AI070448 (MHK), RO1 CA118118 (MJR), and RO1 HL080071 (RST). GLS was supported by T32 AI060519.

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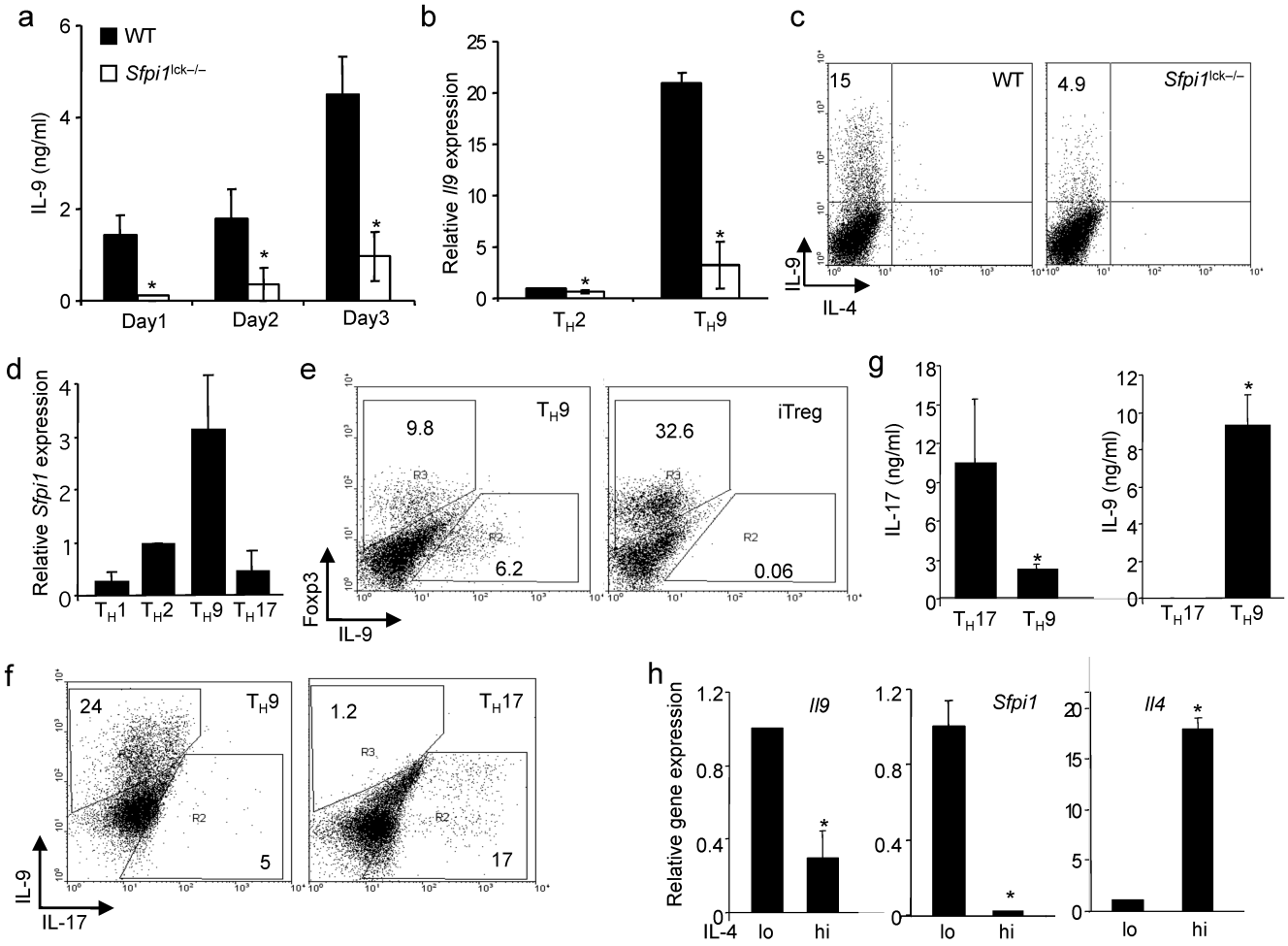

Figure 1.

PU.1 is required for optimal IL-9 production in murine T cells. Wild-type and *Sfpi1*$^{lck-/-}$ naïve CD4$^+$ T cells were cultured under T_H9 conditions. (**a**) After five days of culture, cells were stimulated with anti-CD3 and supernatants after 1, 2 or 3 days of stimulation were tested for IL-9 using ELISA. (**b**) RNA was isolated from wild-type and *Sfpi1*$^{lck-/-}$ T_H2 and T_H9 cultures after stimulation with anti-CD3. *Il9* mRNA was assessed using qPCR. (**c**) Wild-type and *Sfpi1*$^{lck-/-}$ T_H9 cultures were stimulated with PMA-ionomycin for 5 h before intracellular staining for IL-9. Results in (**a-c**) are the average of 3 mice and representative of more than four experiments. (**d**) Naïve CD4$^+$ T cells cultured under T_H1, T_H2, T_H9 or T_H17 conditions were analyzed for *Sfpi1* expression using qPCR. Results are an average of 3-5 experiments. (**e**) Naïve CD4$^+$ T cells were cultured under T_H9 or iTreg conditions for five days and stimulated with anti-CD3 for 6 h before intracellular staining for IL-9 and Foxp3. (**f,g**) Naïve CD4$^+$ T cells were cultured under T_H9 or T_H17 conditions for five days and stimulated with (f) PMA-ionomycin for 6 h before intracellular staining for IL-9 and IL-17 or (**g**) anti-CD3 for 24 h before supernatants were collected for analysis using ELISA. Results in (**e-g**) are representative of at least three experiments. (**h**) T_H2 cultures were separated into IL-4^{lo} and IL-4^{hi} populations using magnetic selection. RNA was isolated

from separated cells to examine expression of *Il4*, *Il9* and *Sfpi1*. Results are an average of three experiments. *, $P < 0.05$ using two-tailed student *t* test.

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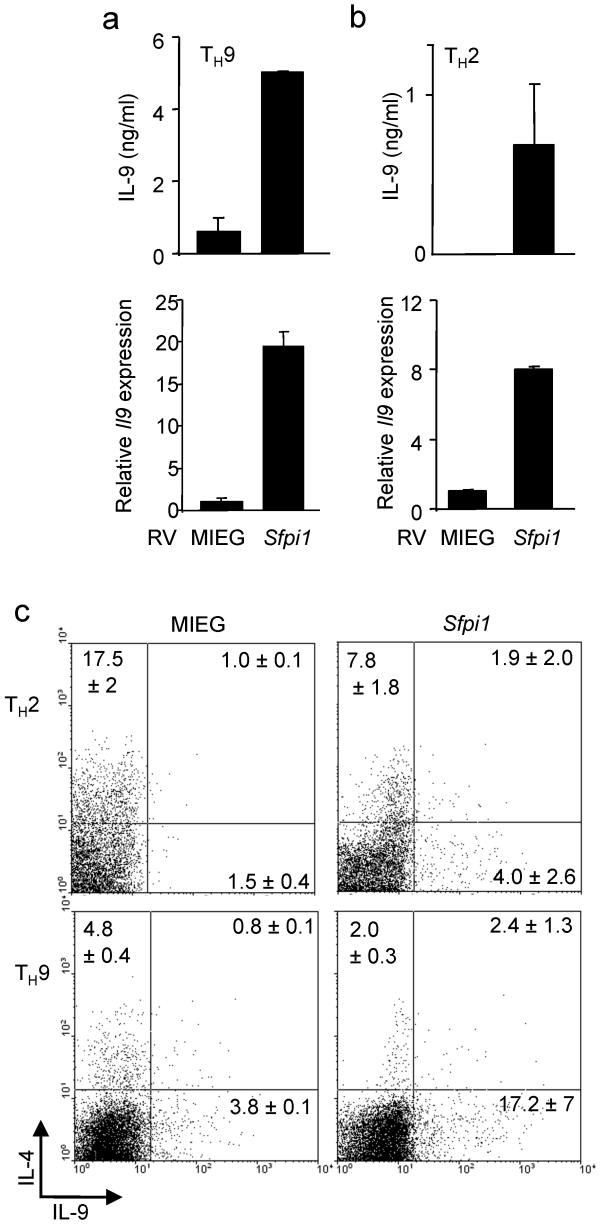

Figure 2.

PU.1 promotes IL-9 production. T_H9 (**a,c**) or T_H2 (**b,c**) cultures were transduced with control (MIEG) or PU.1-expressing retroviruses. (**a,b**) EGFP-positive cells were sorted and stimulated to determine secretion of IL-9 (top) and *Il9* expression (bottom). (**c**) IL-4 and IL-9 production was determined by intracellular staining in the EGFP+ populations of control and PU.1-transduced cells. Percentages in each quadrant are the average of results from 2-3 mice. Data are representative of 2-3 experiments.

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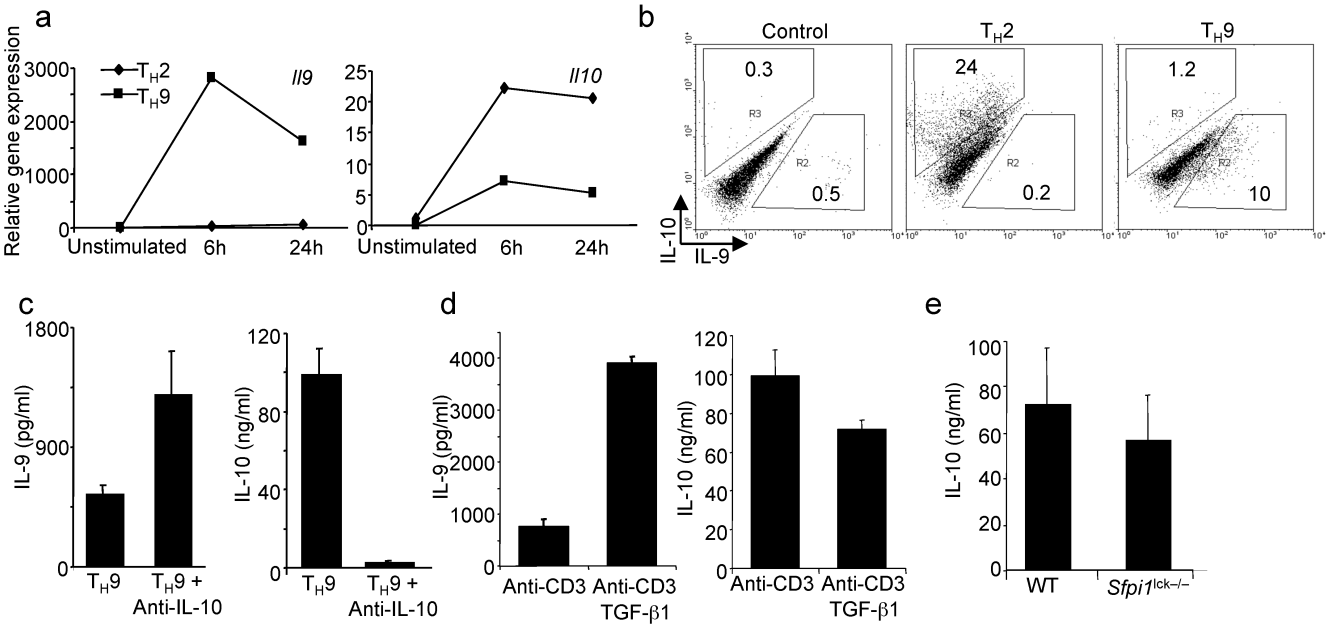

Figure 3.

IL-9 and IL-10 are not coordinately regulated in T_H9 cells. (**a**) Naïve $CD4^+$ T cells were cultured under T_H2 or T_H9 conditions for five days before cells were left unstimulated or stimulated with plate-bound anti-CD3 for 6 or 24 hours and RNA was isolated for qPCR analysis of *Il9* and *Il10*. (**b**) T_H2 and T_H9 cultures derived as in **a** were stimulated for 5 hours before intracellular cytokine analysis of IL-10 and IL-9. (**c**) Naïve $CD4^+$ T cells were cultured under T_H9 conditions in the presence or absence of anti-IL-10. After five days in culture cells were restimulated with anti-CD3 and supernatants were analyzed for cytokines using ELISA. (**d**) Naïve $CD4^+$ T cells were cultured under T_H9 conditions. After five days in culture, cells were restimulated with anti-CD3 in the presence or absence of TGF-β1 and supernatants were analyzed for cytokines using ELISA. (**e**) Wild-type and *Sfpi1*$^{lck-/-}$ naïve $CD4^+$ T cells were cultured under T_H9 conditions for five days, and stimulated with anti-CD3 for 24 hours before supernatants were tested for IL-10 using ELISA. Results are representative of at least three experiments.

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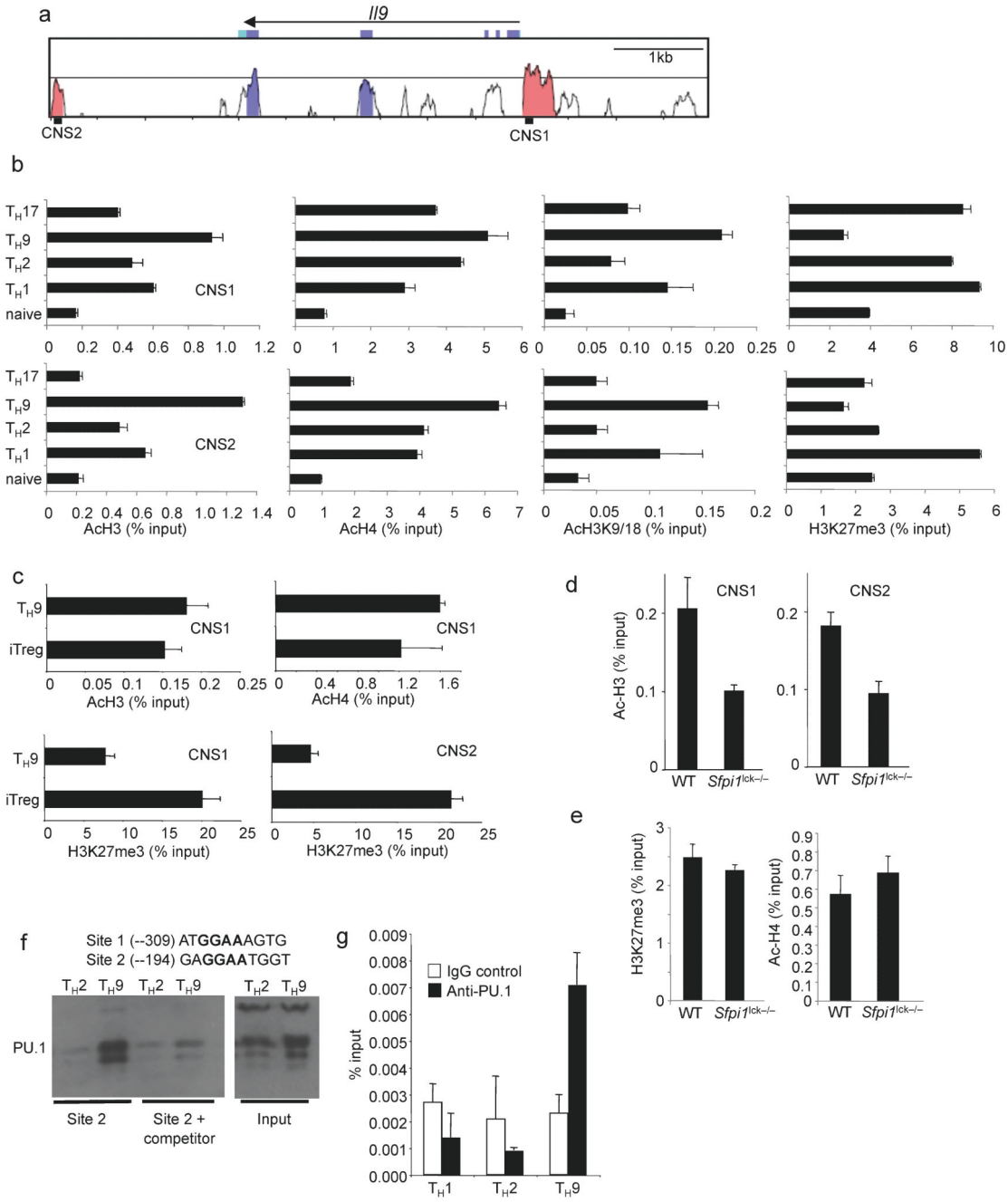

Figure 4.

Histone modifications at the *Il9* locus. (**a**) VISTA plot of conserved non-coding sequences adjacent to the *Il9* locus. (**b,c**) Naïve CD4$^+$ T cells were isolated and analyzed directly or differentiated under T_H1, T_H2, T_H9, T_H17 or iTreg culture conditions for five days. Cells were analyzed for histone modifications using chromatin immunoprecipitation for the indicated histone modifications and primers for *Il9* CNS. (**d,e**) Wild-type and *Sfpi1*$^{lck-/-}$ naïve CD4$^+$ T cells were cultured under T_H9 conditions and used for ChIP analysis as described in **b** performed for AcH3 at CNS1 and CNS2 (**d**), or for AcH4 and me3H3K27 at

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CNS1 (**e**). (**f**) Top, consensus PU.1 binding sites in the *Il9* CNS1. Bottom, DAPA analysis of PU.1 binding from T_H2 or T_H9 extracts to site 2. For competition, extracts were incubated with a 5-fold excess of unlabelled PU.1 consensus double-stranded oligonucleotide. Expression of PU.1 in each cell extract is shown as input. Results are representative of three experiments. (**g**) Naïve $CD4^+$ T cells were differentiated under T_H1, T_H2, or T_H9 culture conditions for five days. Cells were analyzed for PU.1 binding by ChIP with primers for *Il9*CNS1. Results of ChIP experiments are shown as average ± SD of percent input with control IgG subtracted (a-e) or with anti-PU.1 and IgG control ChIP values shown separately (g) and are representative of 2-4 experiments.

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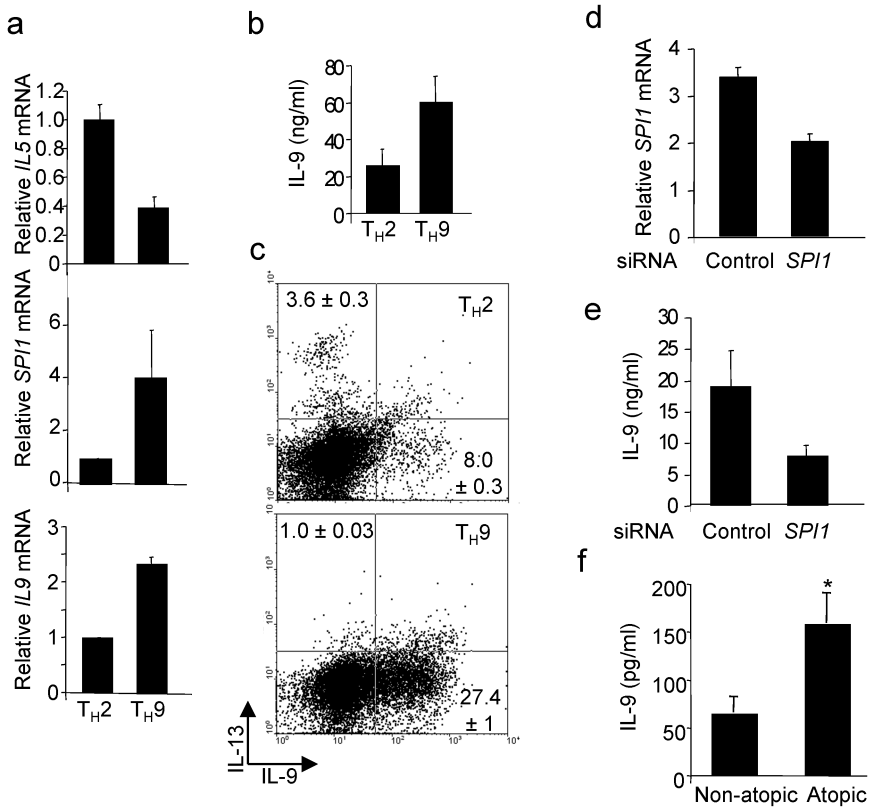

Figure 5.

PU.1 promotes IL-9 production in human T cells. (**a**) Naïve human CD4$^+$ T cells were cultured under T_H2 or T_H9 conditions and after five days in culture restimulated to analyze gene expression by qPCR. (**b,c**) T_H2 and T_H9 cultures were derived as in **a** and **b** supernatants from cells stimulated for 24 hours were tested for IL-9 production using ELISA or (c) cells were stimulated with anti-CD3 for 6 hours for analysis of IL-9 and IL-13 by intracellular cytokine staining. (**d,e**) Th9 cultures derived as in **a** were transfected with control or *SPI1*-specific siRNA. Twenty-four hours after transfection cells were analyzed for *SPI1* expression by qPCR (**d**) and IL-9 production using ELISA (**e**). Results in (a-e) are representative data from 3-5 experiments with different donors. (**f**) PBMCs from infants (age 18-30 months) classified as atopic (n= 49) or non-atopic (n= 33) based on positive allergen-specific serum IgE, were stimulated with anti-CD3 for 48 hours and supernatants were tested for production of IL-9 using multiplex analysis. *, $p<0.04$ using an unpaired student t test.

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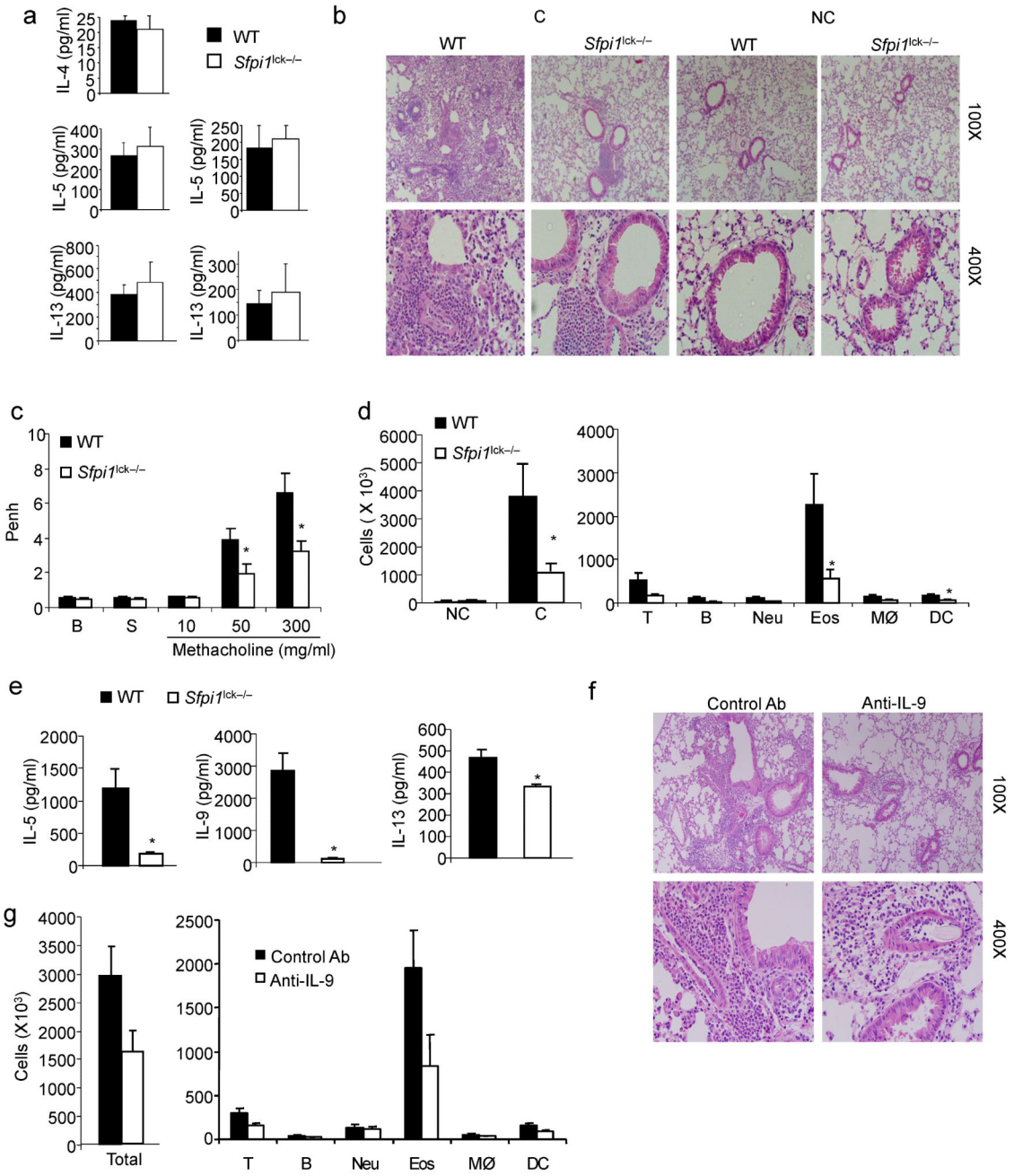

Figure 6.

PU.1 expression in T cells is required for the development of allergic inflammation. Wild-type and *Sfpi1*$^{lck-/-}$ mice were sensitized and challenged intranasally with ovalbumin. Forty-eight hours after the last intranasal challenge mice were sacrificed for analysis. (**a**) Splenocytes were stimulated with anti-CD3 (left) or Ova (right) for 72 h and supernatants were tested for the amounts of T_H2 cytokines by ELISA. (**b**) Lungs of challenged (C) or non-challenged (NC) mice were embedded in paraffin and analyzed by H/E staining. Magnification is indicated in the panel. (**c**) Wild-type and *Sfpi1*$^{lck-/-}$ mice were analyzed for

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airway hyperreactivity using whole body plethysmography 24 h after the last intranasal challenge. Airway function was tested at baseline (B), with saline inhalation (S) and following inhalation of the indicated doses of methacholine. (**d**) Numbers of cells recovered by bronchoalveolar lavage in challenged or non-challenged mice (left). Numbers of specific cell types in BAL from challenged mice, as determined by flow cytometry, are indicated (right). (**e**) Amounts of IL-9 and T_H2 cytokines present in BAL fluid were determined using ELISA. (**f, g**) Wild type mice were sensitized and challenged intranasally with ovalbumin daily for five days. Mice were injected intravenously with control Ig or anti-IL-9, 30 minutes before the first, third and fifth intranasal challenge. Forty-eight hours after the last intranasal challenge, lungs were processed for histological analysis (**f**) as in **b**, and BAL cells were harvested and analyzed (**g**) as in **d**. *, $p<0.05$ using two-tailed student t test. All results are the average ± SEM of 5-7 mice and are representative of three experiments.

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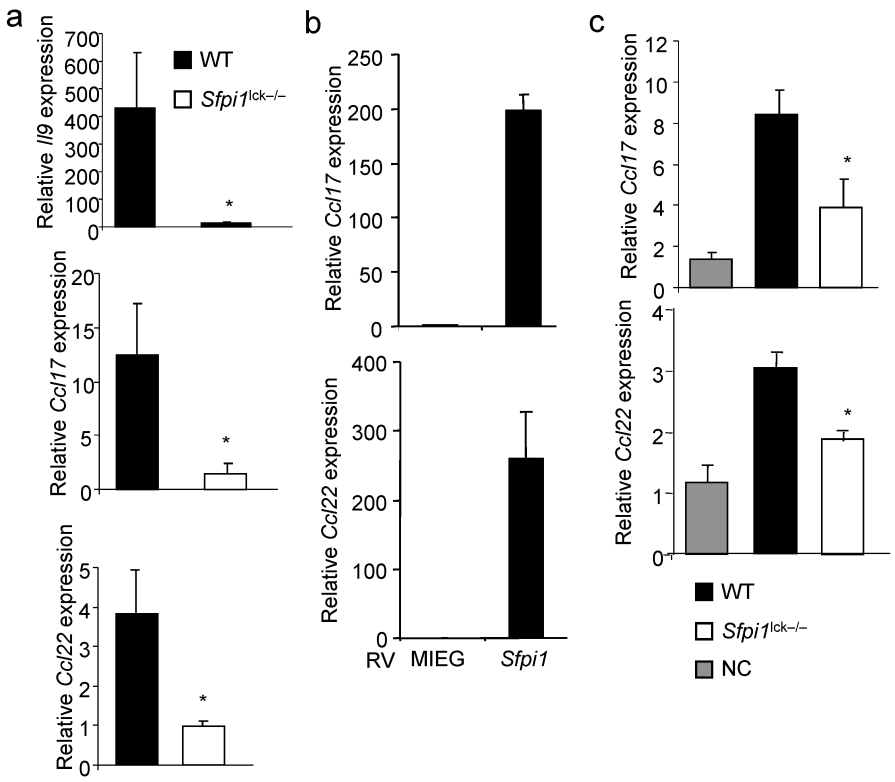

Figure 7.

PU.1 is required for IL-9 and chemokine expression in allergen sensitized mice. (**a**) RNA isolated from Ova-stimulated splenocytes as described in (Fig. 6a) was analyzed for expression of *Il9* and chemokines associated with allergic inflammation. (**b**) T_H9 cultures were transduced with control (MIEG) or PU.1 expressing retroviruses. EGFP-positive cells were sorted and stimulated to determine expression of *Ccl17* and *Ccl22*. (**c**) Total lung RNA was isolated from sensitized and challenged WT and *Sfpi1*$^{lck-/-}$ mice, or WT non-challenged (NC) mice, and analyzed for *Ccl17* and *Ccl22* mRNA by qPCR. *, $p<0.05$ using two-tailed student t test.