

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

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CD4⁺ helper T 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 helper T cells. Decreasing PU.1 expression either by conditional deletion in mouse T cells or the use of small interfering RNA in human T cells impaired IL-9 production, whereas ectopic PU.1 expression promoted IL-9 production. Mice with PU.1-deficient T cells developed normal T helper type 2 (T_H2) responses *in vivo* but showed attenuated allergic pulmonary inflammation that corresponded to lower expression of *Il9* and chemokines in peripheral T cells and in lungs than that of wild-type mice. Together our data suggest a critical role for PU.1 in generating the IL-9-producing (T_H9) phenotype and in the development of allergic inflammation.

Helper T cells promote the development of specific types of inflammation after differentiating into various effector subsets. The cytokine microenvironment present when a naive helper T cell is activated determines the developmental pathway. Interleukin 4 (IL-4; A001262) promotes the differentiation of T helper type 2 (T_H2) cells and allergic inflammation. IL-12 promotes T_H1 differentiation, whereas inflammatory cytokines, including transforming growth factor- β (TGF- β 1), IL-6, IL-1 and IL-23, stimulate the development of IL-17-producing T helper cells (T_H17 cells); both of these cell types contribute to autoimmune inflammation^{1–3}. Helper T cell 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 IL-4-induced activation of the transcription factor STAT6, followed by subsequent induction of the transcription factor GATA-3, a T_H2 lineage-promoting factor. The ability of T_H2 cells to respond to subsequent T cell antigen receptor stimulation with rapid induction of T_H2-specific cytokines further requires members of the transcription factor NFAT and Jun families and the transcription factors c-Maf and IRF4, probably among other factors, for transactivation of loci encoding cytokines^{4–6}.

The transcription factor PU.1 (A001950) also contributes to the T_H2 phenotype by promoting heterogeneity of T_H2 cytokine secretion^{7,8}. PU.1 is expressed specifically in subpopulations of T_H2 cells with low IL-4 expression. Ectopic expression of PU.1 results in lower production of most T_H2 cytokines, whereas short hairpin RNA

that decreases PU.1 expression or PU.1 deficiency results in greater overall cytokine production specifically in populations of cells that secrete 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 target loci encoding cytokines^{7–9}.

IL-9 (A001559) has long been thought to be a T_H2 cytokine, as it promotes allergic inflammation and is associated with various T_H2 responses¹⁰. Transgenic expression of *Il9* results in allergic inflammation^{11–13}. IL-9 promotes mucus production from lung epithelial cells and pulmonary mastocytosis^{14–17}. Although allergic inflammation can develop in *Il9*^{–/–} mice in a standard sensitization-challenge model, blockade of IL-9 in a similar model results in less allergic inflammation^{18,19}. IL-9 expression is higher in lungs of asthmatic patients^{20,21}, and blocking antibodies are being developed as a therapy for atopic disease²². IL-9 may contribute to extracellular parasite immunity^{23,24}. IL-9 has also been shown to promote the development of T_H17 cells while increasing the activity of regulatory T cells (T_{reg} cells)^{25,26}, which suggests that IL-9 biology may be more complex.

In addition to the established helper T cell subsets, reports have described an IL-9-secreting population of T cells²⁷. T_H2 cells and T_{reg} cells can produce IL-9 (ref. 28), and culture with TGF- β 1 may result in more IL-9 production by many cell types²⁶. A helper T cell subset called 'T_H9 cells' is derived in culture with a combination of TGF- β 1 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 they have lower expression

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of T_H2 cytokines^{24,29}. Transcription factors that specifically control the IL-9-secreting T cell phenotype have not yet been identified.

In this report we identify PU.1 as a regulator of IL-9 production in T cells. PU.1-deficient T cells had less IL-9 production, and ectopic expression of PU.1 resulted in more IL-9 production by T_H2 and T_H9 cell cultures. Diminishing PU.1 expression in human IL-9-secreting T cell cultures also resulted in less IL-9 production. In mice that lacked PU.1 only in T cells, allergic airway inflammation was attenuated, and this observation correlated with less production of IL-9 and PU.1-induced production of chemokines 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 helper T cells (called ' T_H9 cells' here) did not identify a transcription factor that distinguished these cells as a distinct lineage^{24,29}. PU.1 limits T_H2 cytokine production in T cells^{7,8}, which suggests that it could be involved in the T_H9 phenotype. To test that hypothesis, we differentiated naive T cells from wild-type mice and PU.1-deficient mice (*Sfp1*^{fl/fl} *Lck*-Cre mice, in which loxP-flanked alleles of the gene encoding PU.1 are deleted by Cre recombinase driven by the promoter of the gene encoding the kinase *Lck*, which results in deletion mainly in T cells) under T_H9 conditions (TGF- β 1 plus IL-4). We observed that IL-9 secretion, mRNA expression and IL-9 intracellular staining were lower in T_H9 cultures that lacked PU.1 expression (Fig. 1a–c).

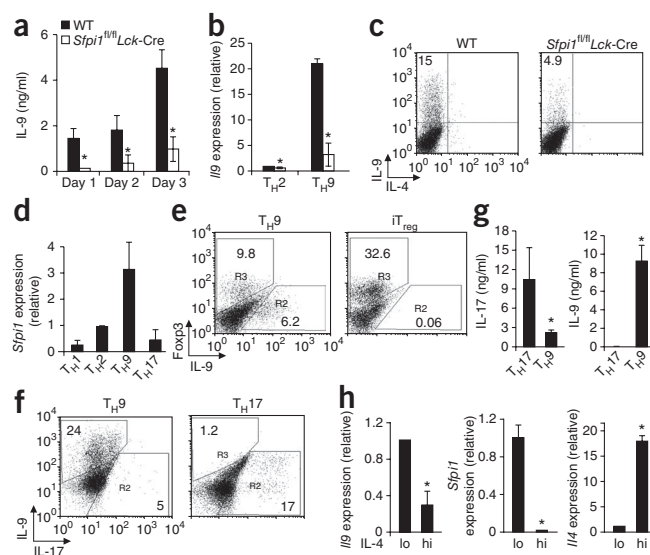
PU.1 expression was low in T_H1 cells, and in T_H2 populations PU.1 was expressed in IL-4^{lo} cells but not IL-4^{hi} cells⁸. To compare the expression of PU.1 in additional helper T cell subsets, we generated T_H1 , T_H2 , T_H17 and T_H9 cells and analyzed expression of RNA encoding PU.1. We observed that T_H1 and T_H17 cells had the lowest expression of *Sfp1* mRNA, T_H2 cells had three- to fivefold more abundant *Sfp1* mRNA expression, and T_H9 cells expressed approximately threefold more *Sfp1* than did T_H2 cells (Fig. 1d). Inducible T_{reg} (iT_{reg}) cultures expressed even more *Sfp1* mRNA than did T_H9 cell cultures (Supplementary Fig. 1a), which suggested that *Sfp1* might be

a TGF- β -induced gene. *Sfp1* expression was not STAT6 dependent, as determined by comparison of wild-type and *Stat6*^{-/-} T_H9 cell cultures (data not shown). As both iT_{reg} and T_H17 cell cultures have been suggested to produce IL-9 (refs. 25,26,28), we also directly compared cells cultured under those conditions with T_H9 cell cultures. By intracellular staining and enzyme-linked immunosorbent assay (ELISA), we observed low or undetectable IL-9 production by iT_{reg} cell cultures (Fig. 1e and data not shown). In comparing T_H9 and T_H17 cell cultures, we observed little IL-9 production in T_H17 cell culture assays by intracellular staining or ELISA, although there was a small amount of IL-17 production by T_H9 cell cultures (Fig. 1f,g). Because we detected IL-9 in T_H2 cell cultures, we separated T_H2 populations into IL-4^{hi} and IL-4^{lo} populations⁸ and observed that *Il9* expression segregated with *Sfp1* 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 with a bicistronic retrovirus in T_H2 , T_H9 and iT_{reg} cell cultures and examined cytokine secretion and gene expression in sorted cells positive for enhanced green fluorescent protein (eGFP). Higher PU.1 expression in T_H2 or T_H9 cell cultures further induced IL-9 production, as assessed by both ELISA and intracellular cytokine staining and measurement of *Il9* mRNA in these cultures (Fig. 2). Ectopic PU.1 expression in T_H2 cells, as described before⁸, resulted in lower expression of T_H2 cytokines (Fig. 2c). Although ectopic PU.1 expression resulted in lower expression of the gene encoding the transcription factor Foxp3 in iT_{reg} cell cultures (Supplementary Fig. 1b), it did not induce *Il9* expression in these cells (data not shown). The generation of iT_{reg} cells by PU.1-deficient cells was normal, and the ability of IL-4 to repress Foxp3 was not substantially impaired in the absence of PU.1 expression (Supplementary Fig. 1c,d). Moreover, there was not a greater number of CD4⁺Foxp3⁺ cells in a variety of *Sfp1*^{fl/fl} *Lck*-Cre lymphoid organs than in those of wild-type mice (Supplementary Fig. 1e,f), which suggested 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 for the generation of T_H9 cells.

Figure 1 PU.1 is required for optimal IL-9 production in mouse T cells.

(a) ELISA of IL-9 in supernatants of naive wild-type (WT) and *Sfp1*^{fl/fl} *Lck*-Cre CD4⁺ T cells cultured for 5 d under T_H9 conditions and stimulated for 1, 2 or 3 d with anti-CD3. (b) Quantitative PCR analysis of *Il9* mRNA among RNA isolated from T_H2 or T_H9 cultures stimulated for 24 h with anti-CD3. (c) Intracellular staining for IL-4 and IL-9 in wild-type and *Sfp1*^{fl/fl} *Lck*-Cre CD4⁺ T cells cultured for 5 d under T_H9 conditions and stimulated for 5 h with the phorbol ester PMA and ionomycin. Numbers in top left quadrants indicate percent IL-9⁺IL-4⁻ cells. (d) Quantitative PCR analysis of *Sfp1* expression in naive CD4⁺ T cells cultured under T_H1 , T_H2 , T_H9 or T_H17 conditions. (e) Intracellular staining for IL-9 and Foxp3 in naive CD4⁺ T cells cultured for 5 d under T_H9 or iT_{reg} conditions and stimulated for 6 h with anti-CD3. Numbers in outlined areas indicate percent Foxp3⁺IL-9⁻ cells (top left) or Foxp3⁺IL-9⁺ cells (bottom right). (f) Intracellular staining for IL-9 and IL-17 in naive CD4⁺ T cells cultured for 5 d under T_H9 or T_H17 conditions and stimulated for 6 h with PMA and ionomycin. Numbers in outlined areas indicate percent IL-9⁺IL-17⁻ cells (top left) or IL-9⁺IL-17⁺ cells (bottom right). (g) ELISA of IL-9 and IL-17 in supernatants of naive CD4⁺ T cells cultured for 5 d under T_H9 or T_H17 conditions and stimulated for 24 h with anti-CD3. (h) Expression of *Il4*, *Il9* and *Sfp1* assessed in RNA from T_H2 cultures separated into IL-4^{lo} and IL-4^{hi} populations by magnetic selection. Quantitative PCR results are presented relative to the expression in IL-4^{lo} cells (normalized to β_2 -microglobulin expression), calculated by the change-in-threshold method. **P* < 0.05 (two-tailed Student's *t*-test). Data are representative of more than four experiments (a–c; average and s.d. of three mice), are an average of three to five (d) or three (h) experiments (error bars, s.d.), or are representative of at least three experiments (e–g; mean and s.d.).



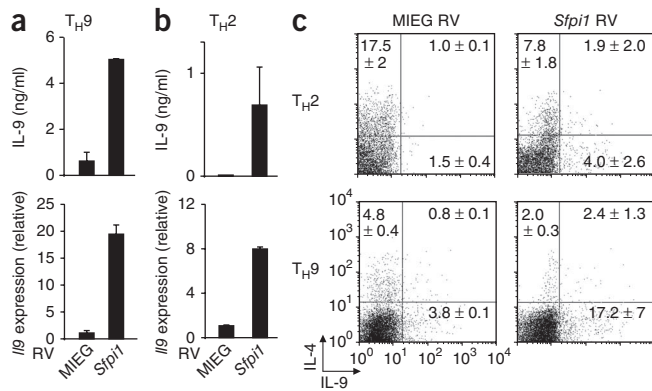


Figure 2 PU.1 promotes IL-9 production. (a,b) IL-9 secretion (top) and *I/9* expression (bottom) by eGFP⁺ cells sorted from TH9 cultures (a) or TH2 cultures (b) transduced with control retrovirus (MIEG) or PU.1 (*Sfp1*)-expressing retrovirus (RV) and stimulated for 24 h with anti-CD3. Expression results (bottom) were normalized to β_2 -microglobulin and are presented relative to expression in cells transduced with control virus. (c) Intracellular staining of IL-4 and IL-9 in eGFP⁺ populations from TH9 or TH2 cultures transduced with control or PU.1-expressing retrovirus. Numbers in quadrants indicate percent cells in each (average $\pm$ s.e.m. of two to three mice). Data are representative of two to three experiments (error bars (a,b), s.e.m.).

IL-9 and IL-10 are not coordinately regulated

Published reports of IL-9-secreting T cells have suggested that they also express IL-10 (refs. 24,29). In contrast, another report has demonstrated that expression of IL-9 and IL-10 in TH2 cells is reciprocally regulated⁹. To further examine this issue, we differentiated TH2 and TH9 cells from cultures of naive CD4⁺ T cells with IL-4 or a combination of IL-4 and TGF- β 1, respectively. Although we detected IL-10 in TH9 cells, production was lower than that in TH2 cell cultures (Fig. 3a,b). Moreover, additional treatment of TH9 cell cultures had a reciprocal effect on the production of IL-9 and IL-10. We observed more IL-9 production by TH9 cell cultures when they were differentiated in the presence of antibody to IL-10 (anti-IL-10), and this was concomitant with less IL-10 production (Fig. 3c). The addition of IL-9 to TH2 cell cultures did not affect IL-10 production (data not shown). The addition of TGF- β 1 to differentiated TH9 cell cultures, during anti-CD3 restimulation, resulted in more IL-9 production but less IL-10 production (Fig. 3d). Although PU.1 deficiency resulted in

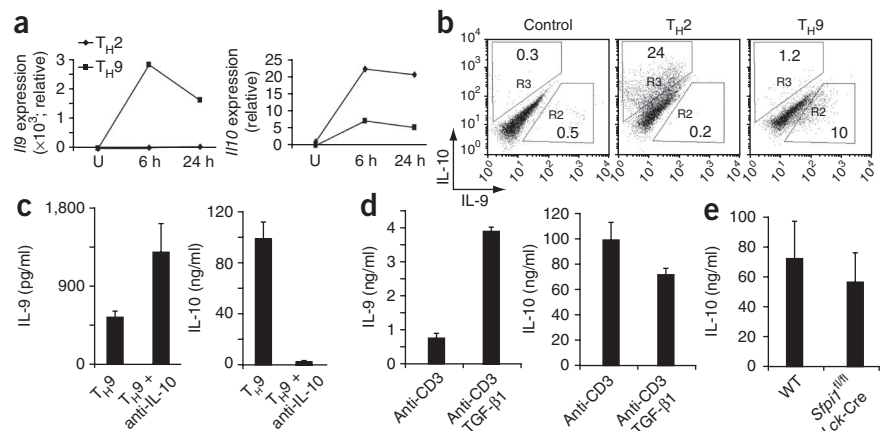
less IL-9 production (Fig. 1), it did not affect IL-10 production by TH9 cell cultures (Fig. 3e). Thus, although TH9 cell cultures may produce IL-10, IL-9 and IL-10 are not coordinately regulated.

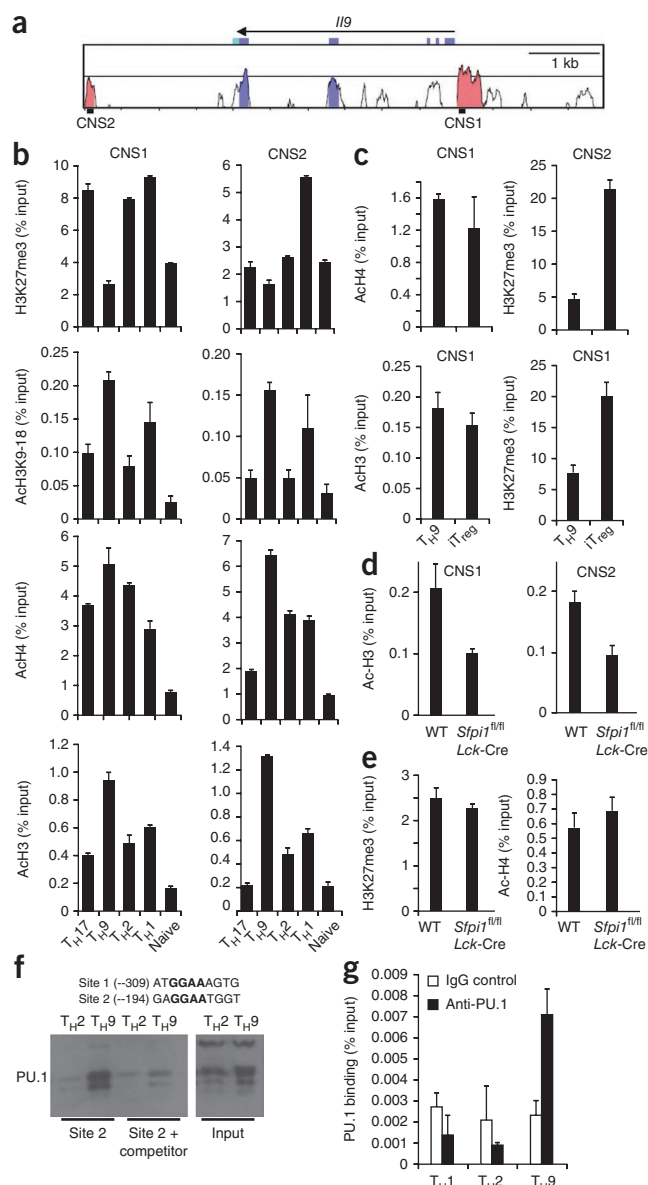
PU.1 required for chromatin modifications of *I/9*

As helper T cell specification is associated with specific chromatin structure, we examined by chromatin-immunoprecipitation (ChIP) assay chromatin modifications at the *I/9* locus in naive T cells and in naive T cells cultured for 5 d under TH1, TH2, TH9 or TH17 conditions. Total acetylation of both histone H3 and H4 and acetylation of histone 3 Lys9 (H3K9) and H3K18 at two *I/9* conserved noncoding sequences (CNSs; Fig. 4a) were highest in TH9 cell cultures compared with that of all other subsets (Fig. 4b), although all subsets demonstrated more acetylation than did naive cells. TH9 cells also had the least trimethylated H3K27, a modification associated with repressed genes (Fig. 4b). Cultures of iTreg cells had histone acetylation similar to that of TH9 cells (Fig. 4c), in agreement with data on the *I/9* locus derived from genome-wide studies³⁰. However, the *I/9* locus had more trimethylated H3K27 in iTreg cell cultures than in TH9 cell cultures (Fig. 4c). Thus, active *I/9* expression was associated with a combination of high H3 acetylation and low H3K27 methylation. To determine whether PU.1 was required for the establishment of these chromatin modifications, we differentiated wild-type and PU.1-deficient naive T cells under TH9 conditions and analyzed chromatin modifications. We observed that H3 acetylation, the modification that showed the greatest difference between TH9 and other helper T cell cultures, was lower in the absence of PU.1 at both CNS sites (Fig. 4d) and that this effect was specific for TH9 cell cultures, as PU.1 deficiency did not affect H3 acetylation of *I/9* in TH2 or TH17 cells (Supplementary Fig. 2). Moreover, the presence of the Cre transgene did not result in less *I/9* H3 acetylation (Supplementary Fig. 2). In contrast, H4 acetylation or H3K27 methylation was not affected by the absence of PU.1 (Fig. 4e). These results suggest that PU.1 is required for 'programming' of the *I/9* locus but has a restricted role in the histone modifications that are affected.

To determine if PU.1 was acting directly on *I/9*, we examined CNS1 for consensus PU.1-binding sites (5'-GGAA-3' core sequence). We found two potential sites in sequences conserved among human, mouse and dog *I/9* promoters (Fig. 4f). Using DNA-affinity-precipitation assay of extracts of TH2 or TH9 cells, followed by immunoblot analysis, we observed binding of PU.1 to both sites, with stronger binding to site 2, and binding was decreased by incubation with a nonbiotinylated

Figure 3 IL-9 and IL-10 are not coordinately regulated in TH9 cells. (a) Quantitative PCR analysis of *I/9* and *I/10* among RNA isolated from naive CD4⁺ T cells cultured for 5 d under TH2 or TH9 conditions, then left unstimulated (U) or stimulated for 6 h or 24 h with plate-bound anti-CD3; results were normalized to β_2 -microglobulin and are presented relative to expression in unstimulated cells. (b) Intracellular staining of IL-10 and IL-9 in TH2 and TH9 cultures derived as in a and left unstimulated (Control) or stimulated for 5 h with anti-CD3. Numbers in outlined areas indicate percent IL-10⁺IL-9⁻ cells (top left) or IL-10⁻IL-9⁺ cells (bottom right). (c) ELISA of IL-9 and IL-10 in supernatants of naive CD4⁺ T cells cultured for 5 d under TH9 conditions in the presence or absence of anti-IL-10, then restimulated for 24 h with anti-CD3. (d) ELISA of IL-9 and IL-10 in supernatants of naive CD4⁺ T cells cultured for 5 d under TH9 conditions, then restimulated for 24 h with anti-CD3 in the presence or absence of TGF- β 1. (e) ELISA of IL-10 in supernatants of wild-type and *Sfp1*^{fl/fl} *Lck*-Cre naive CD4⁺ T cells cultured for 5 d under TH9 conditions, then stimulated for 24 h with anti-CD3. Data are representative of at least three experiments (error bars (c-e), s.e.m.).





competitor oligonucleotide (Fig. 4f and data not shown). We observed more PU.1 binding with extracts of T_H9 cells, parallel to higher PU.1 protein expression in extracts of T_H9 cells (Fig. 4f). We then assessed by ChIP assay whether PU.1 bound to the *IL9* promoter. After differentiation of T_H1, T_H2 and T_H9 cells, PU.1 ChIP assays detected binding of PU.1 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 mouse T_H9 cells to promote a human T_H9 cell 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 mouse cells, the addition of TGF-β1 to T_H2 cultures resulted in less T_H2 cytokine production and more production of IL-9 (Fig. 5a–c). Human T_H9 cell cultures also expressed more *SPI1* than did T_H2 cell cultures (Fig. 5a). We then determined if PU.1 was required for IL-9 expression in human T_H9 cells by transfecting

Figure 4 Histone modifications at the *IL9* locus. (a) VISTA plot of CNSs adjacent to the *IL9* locus. (b,c) ChIP analysis of histone modifications in naive CD4⁺ T cells isolated and analyzed immediately (Naive) or differentiated for 5 d under T_H1, T_H2, T_H9, T_H17 or iT_{reg} culture conditions. H3K27me3, trimethylation of H3K27; AcH3K9-18, acetylation of H3K9 and H3K18; AcH4, acetylation of histone H4; AcH3, acetylation of histone H3. (d,e) ChIP analysis of histone modifications in naive wild-type and *Sfpi1^{fl/fl}* Lck-Cre CD4⁺ T cells cultured under T_H9 conditions. (f) Consensus PU.1-binding sites in *IL9* CNS1 (above) and DNA-affinity-precipitation assay of the binding of PU.1 to site 2 in T_H2 or T_H9 extracts (below), with (right) or without (left) competition of extracts incubated with a fivefold excess of unlabeled PU.1 consensus double-stranded oligonucleotide. Input (far right), expression of PU.1 in cell extracts. (g) ChIP analysis of PU.1 binding in naive CD4⁺ T cells differentiated for 5 d under T_H1, T_H2 or T_H9 culture conditions. Data are representative of two to four experiments (average and s.d. of percent input with subtraction of control IgG (a–e) or with anti-PU.1 and IgG control ChIP values shown separately (g)) or are representative of three experiments (f).

differentiated human T_H9 cell cultures with control or *SPI1*-specific small interfering RNA (siRNA) before restimulating the cells. Inhibiting *SPI1* expression resulted in less IL-9 production (Fig. 5d,e). These results suggested 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 by anti-CD3-stimulated peripheral blood mononuclear cells (PBMCs) from atopic children (positive for at least one allergen-specific immunoglobulin E (IgE) of ten examined) and nonatopic children (no allergen-specific IgE) with ages ranging from 18 months to 30 months. This cohort has been described in greater detail³¹. Notably, whereas these children show signs of developing atopy, they have not had episodes of wheezing and are not considered asthmatic. IL-9 production was significantly greater in atopic patients than in nonatopic patients (Fig. 5f).

To further examine the role of PU.1-dependent T cell function in allergic inflammation, we subjected wild-type and *Sfpi1^{fl/fl}* Lck-Cre mice to a standard protocol for the development of ovalbumin (OVA)-induced allergic airway inflammation. We sensitized mice with OVA in aluminum hydroxide (alum) and challenged them intranasally with OVA in PBS. We first tested the generation of helper T cell responses in the periphery by stimulating splenic cells with OVA or anti-CD3. Consistent with published *in vitro* analyses^{7,8}, there was no impairment of T_H2 cytokine responses in *Sfpi1^{fl/fl}* Lck-Cre mice compared with those of wild-type mice (Fig. 6a). The production of T_H17 cytokines was also unimpaired by the absence of PU.1 (Supplementary Fig. 3). The generation of antigen-specific IgE and IgG1 was not significantly different in wild-type versus *Sfpi1^{fl/fl}* Lck-Cre mice ($P > 0.15$; data not shown).

In contrast to the normal development of peripheral responses, inflammation in the lung was lower in mice with PU.1-deficient T cells than in wild-type mice (Fig. 6b). *Sfpi1^{fl/fl}* Lck-Cre mice also demonstrated significantly less airway hyper-responsiveness in response to methacholine challenge than did wild-type mice (Fig. 6c). Analysis of the cellular composition of bronchoalveolar lavage (BAL) fluid demonstrated greater infiltration in challenged wild-type and *Sfpi1^{fl/fl}* Lck-Cre mice than in unchallenged mice, but total cell numbers and the cell numbers of specific cell populations, including eosinophils, were lower in *Sfpi1^{fl/fl}* Lck-Cre mice than in wild-type mice (Fig. 6d). To confirm that Lck-Cre-mediated deletion of the *Sfpi1* allele did not affect the PU.1-dependent development of dendritic cells important for the development of inflammation, we examined

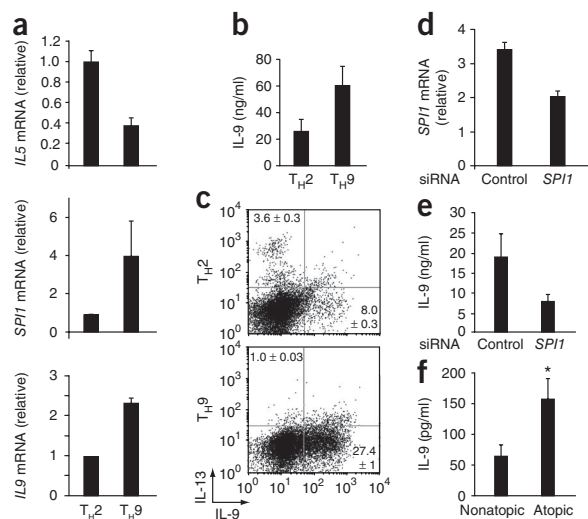


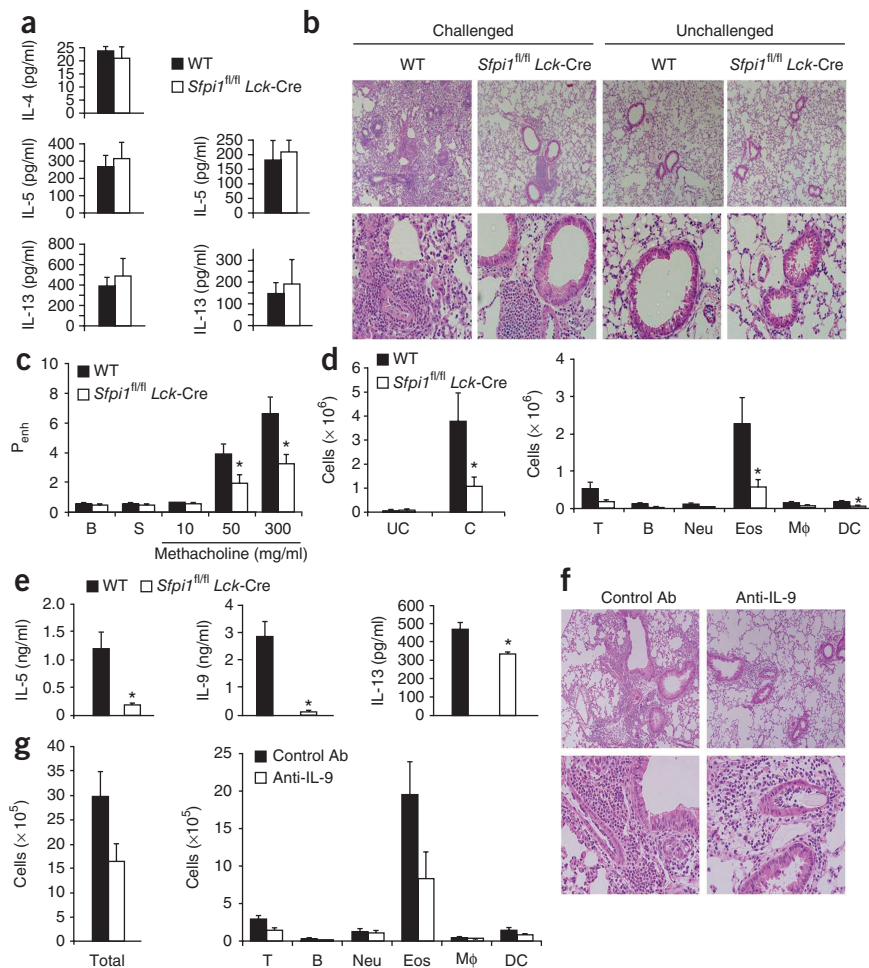
Figure 5 PU.1 promotes IL-9 production in human T cells. (a) Quantitative PCR analysis of gene expression by naive human CD4⁺ T cells cultured for 5 d under T_H2 or T_H9 conditions and then restimulated for 24 h with anti-CD3; results were normalized to β_2 -microglobulin and are presented relative to expression in T_H2 cells. (b) ELISA of IL-9 in supernatants of T_H2 and T_H9 cultures derived and restimulated as in a. (c) Intracellular staining for IL-9 and IL-13 in cells from T_H2 and T_H9 cultures derived as in a and stimulated for 6 h with anti-CD3. (d,e) Quantitative PCR analysis of *SPI1* expression (d) and ELISA of IL-9 production (e) by T_H9 cultures derived as in a and transfected for 24 h with control or *SPI1*-specific siRNA. (f) Multiplex analysis of IL-9 in supernatants of PBMCs from infants (18–30 months of age) classified as atopic ($n = 49$) or nonatopic ($n = 33$) on the basis of positive allergen-specific serum IgE; cells were stimulated for 48 h with anti-CD3. * $P < 0.04$ (unpaired Student's *t*-test). Data are representative of three to five experiments with different donors (a–e) or one study (f).

To demonstrate that blocking 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 less inflammation and lower numbers of allergic inflammatory cells in mice treated with anti-IL-9 than in mice treated with control antibody (Fig. 6f,g). This result confirmed a role for IL-9 in this model established before with a similar protocol and BALB/c mice¹⁸.

To determine if *Il9* expression was lower in T cells from sensitized *Sfp1^{fl/fl}* *Lck-Cre* mice than in those from wild-type mice, we used quantitative PCR to assess mRNA abundance in antigen-stimulated splenocyte cultures. We observed less *Il9* mRNA in *Sfp1^{fl/fl}* *Lck-Cre* cultures (Fig. 7a). It was possible that PU.1 was regulating other genes

CD11b⁺ and CD103⁺ subpopulations of CD11c⁺ major histocompatibility complex class II–positive cells in BAL fluid and lung-cell suspensions of nonsensitized and unchallenged mice and observed no significant differences between wild-type and *Sfp1^{fl/fl}* *Lck-Cre* mice ($P > 0.2$; data not shown). There were smaller amounts of IL-9 and T_H2 cytokines present in BAL fluid (Fig. 6e). In contrast, the amount of interferon- γ and IL-17 present in the BAL fluid was not affected (data not shown).

Figure 6 PU.1 expression in T cells is required for the development of allergic inflammation. (a) ELISA of T_H2 cytokines in supernatants of splenocytes from wild-type and *Sfp1^{fl/fl}* *Lck-Cre* mice sensitized and challenged intranasally with OVA; cells collected 48 h after the final intranasal challenge were stimulated for 72 h with anti-CD3 (left) or OVA (right). (b) Hematoxylin and eosin staining of paraffin-embedded sections of lungs from mice challenged as in a (left) or left unchallenged (right). Original magnification, $\times 100$ (top row) or $\times 400$ (bottom row). (c) Whole-body plethysmography of airway hyper-reactivity in wild-type and *Sfp1^{fl/fl}* *Lck-Cre* mice at 24 h after the final intranasal challenge as in a, tested at baseline (B), after saline inhalation (S) and after the inhalation of various doses (horizontal axis) of methacholine. P_{enh} , enhanced pause. (d) Cells recovered by BAL in mice challenged as in a (C) or left unchallenged (UC; left), and specific cell types in BAL from mice challenged as in a, assessed by flow cytometry (right). T, T cell; B, B cell; Neu, neutrophil; Eos, eosinophil; M ϕ , macrophage; DC, dendritic cell. (e) ELISA of IL-9 and T_H2 cytokines present in BAL fluid of mice challenged as in a. (f,g) Histological analysis of lungs, assessed as in b (f), and BAL fluid cells, analyzed as in d (g), from wild-type mice sensitized and challenged intranasally daily for 5 d with ovalbumin and injected intravenously with control immunoglobulin (control Ab) or anti-IL-9 30 min before the first, third and fifth intranasal challenge; lungs and BAL fluid were obtained 48 h after the final intranasal challenge. Original magnification (f), $\times 100$ (top row) or $\times 400$ (bottom row). * $P < 0.05$ (two-tailed Student's *t*-test). Data are representative of three experiments (average and s.e.m. of five to seven mice in a,c–e,g).



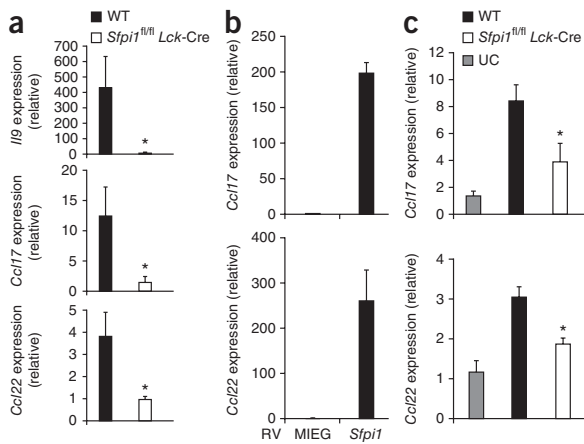


Figure 7 PU.1 is required for the expression of IL-9 and chemokines in allergen-sensitized mice. **(a)** Quantitative PCR analysis of the expression of *Il9* and chemokines associated with allergic inflammation among RNA isolated from splenocytes stimulated with OVA as described in **Figure 6a**, presented relative to expression in PU.1-deficient cultures. **(b)** Quantitative PCR analysis of the expression of *Ccl17* and *Ccl22* in eGFP⁺ cells sorted from T_H9 cultures transduced with control (MIEG) retrovirus or PU.1-expressing retrovirus and stimulated for 6 h with anti-CD3, presented relative to expression in PU.1-deficient cultures. **(c)** Quantitative PCR analysis of *Ccl17* and *Ccl22* mRNA among total lung RNA isolated from sensitized and challenged wild-type and *Sfpi1^{fl/fl}* Lck-Cre mice or unchallenged wild-type mice (UC), presented relative to expression in unchallenged wild-type cultures. **P* < 0.05 (two-tailed Student's *t*-test). Data are representative of three **(a,c)** or two **(b)** experiments.

associated with a T_H9 phenotype. PU.1 expression segregates with expression of the gene encoding the chemokine CCL22 in IL-4^{lo} cells, and ectopic PU.1 expression results in more CCL22 production⁸. Consistent with those observations, we observed that chemokines associated with allergic inflammation, CCL17 and CCL22, were produced less abundantly by antigen-stimulated *Sfpi1^{fl/fl}* Lck-Cre cultures than by wild-type cultures (**Fig. 7a**). Conversely, ectopic expression of PU.1 induced the expression of both chemokines in T_H9 cell cultures (**Fig. 7b**). To determine if the deficiency in chemokine production by T cells was reflected in the allergic inflammation model, we analyzed whole-lung homogenates for *Ccl17* mRNA and *Ccl22* mRNA. Although mRNA for both chemokines was present in tissue from sensitized and challenged mice, both were less abundant in *Sfpi1^{fl/fl}* Lck-Cre lung tissue than in wild-type lung tissue (**Fig. 7c**). These results suggest that PU.1 in T cells regulates allergic inflammation by inducing the expression of IL-9 and proallergic chemokines.

DISCUSSION

The defined phenotypes of effector helper T cell subsets, once classified simply as T_H1 and T_H2, have been expanded to include T_H17 cells, follicular helper T cells and iT_{reg} cells. An IL-9-secreting T cell, the 'T_H9 cell', has been identified as arising after treatment with a cytokine combination of TGF-β1 and IL-4 (refs. 24,29). Distinguishing these subsets has become more difficult as the plasticity of the phenotypes has become apparent³². However, each lineage expresses 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 the T_H2 lineage by the developmental requirement for IL-4 and the downstream transcription factors STAT6 and GATA-3 (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 proallergic chemokines.

In published analyses of PU.1 in T_H2 cells, PU.1 expression has been found to segregate with a subpopulation of T_H2 cells with low expression of IL-4 and other T_H2 cytokines⁸. That observation corresponds with the ability of ectopically expressed PU.1 to inhibit the production of T_H2 cytokines and the ability of decreasing PU.1 expression, either by siRNA or gene deletion, to increase T_H2 cytokine production^{7,8}. This function is at least in part due to interference with factors that promote the T_H2 phenotype, including GATA-3 and IRF4 (refs. 8,9). The data presented in our report here suggest that the IL-4^{lo} T_H2 subset characterized before might contain some T_H9 cells. Given the apparent plasticity of T_H2 cells in acquiring a T_H9 phenotype²⁴, these two cell types might exist in a continuum in which 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 have indicated that PU.1 can bind the *Il9* promoter directly in T_H9 cells and that conserved PU.1-binding sites exist in CNS1. Although ectopic expression of PU.1 enhanced the T_H9 characteristics of cells and converted T_H2 cells to a T_H9 phenotype, it had more modest effects on the induction of *Il9* in iT_{reg}, T_H1 or T_H17 cell cultures (data not shown). These results might suggest that whereas PU.1 promotes the T_H9 phenotype, it may be most active in cells in which additional factors are present to mediate these effects. This scenario is not uncommon in the helper T cell differentiation paradigm. As described above, many factors contribute to the T_H2 phenotype and many factors, including T-bet, STAT4, Hlx and Runx3, contribute to the T_H1 genetic program^{33–35}. One possible context requirement might be lower expression of IRF4, which we have demonstrated repressed *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 lower 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 more histone acetylation and less H3K27 methylation. However, in iT_{reg} cell cultures that had a similar extent of histone acetylation but abundant H3K27 methylation at the *Il9* locus, we did not detect induction of *Il9* by PU.1. The ability of PU.1 to induce IL-9 in T_H2 but not iT_{reg} cell cultures suggests that additional IL-4-induced factors exist that are required for acquisition of the IL-9-secreting phenotype. Although GATA-3 is required for T_H9 development²⁹, it seems an unlikely candidate, as T_H9 cultures have only low GATA-3 expression^{24,29} and PU.1 antagonizes GATA-3 activity in helper T cells^{7,8}. IRF4 also seems unlikely, as it represses *Il9* expression in T_H2 cells and PU.1 antagonizes its function⁹. It will be useful to determine if other transcription factors, in the presence of PU.1, promote *Il9* expression.

The relatedness of the T_H2 and T_H9 phenotypes and the requirement for STAT6 and GATA-3 in the development of both subsets^{24,29} raise some questions about phenotypes that have been described for mice deficient in either STAT6 or GATA-3, specifically whether phenotypes in allergic inflammation and parasite immunity are a result of defects in the development of one or both subsets. A published report has demonstrated that mice with defective TGF-β1 signaling have less parasite clearance that correlates with less IL-9 (ref. 24). Similarly, in this report we have shown defects in the development of allergic inflammation in mice with fewer IL-9-secreting T cells but normal development of T_H2 immunity. These findings suggest that T_H9 cells might be required for what has classically been called T_H2 immunity, and there are ample data that support the idea of a role for IL-9 in allergic disease. Transgenic models have demonstrated that IL-9 can promote the development of allergic inflammation^{12,13} and

that blockade of IL-9 inhibits this development¹⁸. Moreover, more IL-9 is found in lungs of patients with asthma^{20,21}, and we found that T cells from patients with atopic disease secreted more IL-9 than did T cells from nonatopic patients. Notably, this observation does not diminish the role of T_H2 cytokines in the development of allergic inflammation and instead suggests that these two helper T cell subsets work in concert to initiate allergic inflammation.

IL-9 can promote the development of T_H17 cells and is also reported to be produced by T_H17 cells^{25,26}. Thus, the lower allergic inflammation in mice with PU.1-deficient T cells might be interpreted to result from the diminished generation of T_H17 cells, which are required for the development of inflammation in this model³⁷. However, we did not see a lower 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 by mice that were sensitized and challenged was higher in OVA-stimulated splenocyte cultures from *Sfp1^{fl/fl} Lck-Cre* mice than in those from wild-type mice. This finding suggests that PU.1 is not required for T_H17 development. TGF- β 1 seems to be an important factor in promoting IL-9 production from cells cultured with various cytokines^{26,27}. It is possible that whereas TGF- β 1 promotes IL-9 production, the addition of IL-4 alters the phenotype such that T_H17 cytokine production is lower and resulting cells mainly produce IL-9 alone. In our experiments, T_H9 cell cultures produced IL-9, which was barely detectable in T_H17 cell cultures, and T_H9 cell cultures produced less IL-17 than did T_H17 cells. Thus, whereas 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 probably a combination of altered expression of IL-9 and chemokines. Our data have confirmed that blocking IL-9 with antibodies resulted in less allergic inflammation in the OVA-induced model. As the previously published report noted above used BALB/c mice¹⁸ and our study used C57BL/6 mice, our data have demonstrated that the effects of blocking IL-9 are not strain specific. The chemokines CCL17 and CCL22 and their receptor, CCR4, are each important in the development of allergic inflammation^{38–40}. One possibility is that PU.1-dependent chemokine production by T cells is responsible for the diminished allergic inflammation of *Sfp1^{fl/fl} Lck-Cre* mice. This possibility represents an addition to the present paradigm in which T cells in the lung secrete cytokines, such as IL-13, that promote chemokine production by resident lung cells, including epithelial cells, smooth muscle cells and fibroblasts⁴¹. Our results suggest that chemokine production by T cells also contributes to the overall extent of inflammation. PU.1-dependent production of IL-9 or the expression of other PU.1-dependent genes may also contribute to inflammation in this model, and the phenotype we observed 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 description of an IL-9-secreting T cell population has added further complexity to the understanding of effector helper T cell phenotypes. One factor that prevented this population from initially being called a 'lineage' was the absence of an identified transcription factor that confers 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 probably not the only factor that promotes this lineage but will be an important component of continuing investigations into helper T cell heterogeneity.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org/>): A001262, A001950 and A001559.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

M.H.K. designed and supervised the study and wrote the manuscript; H.-C.C., R.G., R.J. and L.H. did experiments in **Figures 1–3** and **Supplementary Figure 1**; Q.Y., R.G. and G.L.S. did experiments in **Figure 4** and **Supplementary Figure 2**; W.Y., M.J.R. and R.S.T. obtained human samples; W.Y. did all experiments in **Figure 5**; S.S., E.T.N., C.M. and A.-N.A. did experiments in **Figures 6** and **7** and **Supplementary Figure 3**; N.B.P. provided bioinformatics analysis; and S.L.N. provided mice.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Wild-type C57BL/6 female mice were from Harlan Bioscience. Mice with conditional deletion of the gene encoding PU.1 (*Sfp1^{fl/fl}*) on the C57BL/6 background have been described⁴² and were mated to mice carrying a transgene encoding Cre recombinase under control of the *Lck* promoter (B6(CBA)-Tg(Lck-cre)1540Jxm/J). Mice were maintained in pathogen-free conditions and all studies were approved by the Animal Care and Use Committee of the Indiana University School of Medicine.

Mouse helper T cell differentiation. Naive CD4⁺CD62L⁺ T cells were purified from spleens and lymph nodes by magnetic isolation (Miltenyi Biotec). Naive CD4⁺ T cells (1×10^6 cells per ml complete RPMI-1640 medium (supplemented with 10% (vol/vol) FBS (Atlanta Biologicals), 1 mM glutamine (BioWhittaker), 100 U/ml of penicillin (BioWhittaker), 100 µg/ml of streptomycin (BioWhittaker), 10 mM HEPES, pH 7.3 (BioWhittaker), 1 mM sodium pyruvate (BioWhittaker) and 50 µM 2-mercaptoethanol)) were cultured with plate-bound anti-CD3 (2 µg/ml; 145-2C11; BD Biosciences) and soluble anti-CD28 (0.5 µg/ml; 37.51; BD Biosciences) under T_H2 conditions (IL-4 (10 ng/ml; PeproTech) and anti-interferon- γ (10 µg/ml; XMG: hybridoma, American Type Culture Collection; antibody, prepared in-house)) or T_H9 conditions (IL-4 (10 ng/ml; R&D Systems), TGF- β (2 ng/ml; R&D Systems) and anti-interferon- γ (10 µg/ml)). T_H1 and T_H17 conditions have been described⁴³. After 3 d, cultures were supplemented with complete RPMI-1640 medium. After 5–6 d of culture, differentiated cells were restimulated for 1, 2 or 3 d with plate-bound anti-CD3 (1 µg/ml), and cell-free supernatants collected after centrifugation were stored at –20 °C until use. The amount of IL-9 produced was determined by ELISA with anti-IL-9 (D8402E8; BD Biosciences) as the capture antibody and biotin-labeled anti-IL-9 (D9302C12; BioLegend) as the secondary antibody. Where noted, cultures were supplemented with anti-IL-10 (10 µg/ml; JES5-2A5; BioLegend) during differentiation or TGF- β 1 (2 ng/ml) during restimulation with anti-CD3. In some experiments, cell pellets collected after 1 d of restimulation were used for RNA extraction. ChIP and DNA-affinity-precipitation assays were done as described^{7–9}. Biotinylated oligonucleotides were incubated with cell extracts and bound proteins were precipitated with streptavidin-agarose before immunoblot analysis. Nonbiotinylated oligonucleotides were used for competition. Human and mouse genomic sequences were used for VISTA analysis. Primers for ChIP assay were *Il9*CNS1a (5′-CAGTCTACCAGCATCTTCCAGTCTAGC-3′ and 5′-GTGGGCACTGGGTATCAGTTTGATGTC-3′), *Il9*CNS1b (5′-GTCACCTTGACAAAGGCTGTCTTATGCC-3′ and 5′-CAGAACCCGACTATTGAAGAGCATC-3′) and *Il9*CNS2 (5′-AATTACAGAAATTTGCCCCAGGTCCTG-3′ and 5′-GTTAATGCACAATTCATGTGCCAATCC-3′).

Retroviral transduction. Bicistronic retroviral vectors encoding mouse PU.1 and eGFP or IRF4 and human CD4 have been described^{8,9}. After 2 d of differentiation, helper T cells were transduced with retroviral supernatant containing polybrene according to a published protocol⁸. In transduced cells, eGFP⁺ cells were sorted by flow cytometry before cytokine production and gene-expression analysis.

Quantitative RT-PCR. Total RNA was isolated with TRIzol from unstimulated cells or cells restimulated with anti-CD3 (2 µg/ml), followed by reverse transcription according to the manufacturer's instructions (Invitrogen Life Technologies). Taqman Fast Universal PCR Master Mix and commercially available primers for human and mouse genes and the 7500 Fast Real-Time PCR system (Applied Biosystems) were used for quantitative PCR. RNA expression was normalized to the expression of β_2 -microglobulin and relative expression was calculated by the change-in-threshold ($-\Delta\Delta C_T$) method.

Intracellular cytokine staining. After differentiation, cells were incubated in monensin for the final 2 h of a 5-hour restimulation with anti-CD3 or PMA (phorbol 12-myristate 13-acetate) plus ionomycin. Intracellular cytokines were stained by standard protocols with fluorochrome-conjugated anti-IL-9 (mouse, RM9A4 (BioLegend); human, MH9A4 (BD Pharmingen)), anti-IL-10 (JES5-16E3; BD Pharmingen), anti-IL-4 (11B11; BD Pharmingen), anti-IL-13 (JES10-5A2; BD Pharmingen), anti-IL-17 (eBio17B7; eBioscience) or anti-Foxp3 (FJK-16s; eBioscience), followed by flow cytometry

with a FACSCalibur (Beckton Dickinson). Data were analyzed by Windows Multiple Document Interface for Flow Cytometry.

Human helper T cell differentiation. The use of human cells was approved by the Institutional Review Board of Indiana University. Naive CD4⁺ T cells were isolated from PBMCs by magnetic sorting (Miltenyi Biotec). T_H2- or T_H9-primed cultures were activated with Dynabeads Human T-Activator CD3/CD28 for cell expansion and activation (Invitrogen) supplemented with IL-4 (20 ng/ml; R&D Systems) and anti-interferon- γ (2.5 µg/ml; 25718; R&D Systems) or with IL-4 (20 ng/ml) and human TGF- β 1 (5 ng/ml), respectively. Cells were cultured at 37 °C in a humidified incubator at 5% CO₂. Cell populations were expanded on day 3 by transfer to a six-well plate with 5 ml culture media. Cells on day 5 of culture were replated (0.5×10^6 cells per ml) and were stimulated with plate-bound anti-CD3 (2 µg/ml; HIT3a; BD Pharmingen). After culture for 24 h, supernatants and cells were collected for ELISA and quantitative PCR, respectively. Reagents from BioLegend (MH9A4 and MH9D1) were used for IL-9 ELISA.

Transfection of siRNA. T_H2 or T_H9 cells were transfected with control or *SPII*-specific siRNA (Santa Cruz Biotechnology) with a Human T Cell Nucleofector kit according to the manufacturer's instructions (Amaxa Biosystems). T cells (3×10^6) were suspended in Human T cell Nucleofector Solution and were transfected with 1 µg siRNA by the T-23 transfection program (Amaxa). Transfected cells were plated in prewarmed culture media supplemented with human IL-2 (50 U/ml; R&D Systems) and were cultured at 37 °C in a humidified incubator at 5% CO₂. After culture for 24 h, supernatants and RNA from cells were collected for ELISA and quantitative PCR.

Multiplex analysis of cytokine production by patient PBMCs. Patient sample collection and analysis were approved by the Institutional Review Board of Indiana University and required parental consent for samples from infants. Venous blood (5 ml) was collected by venipuncture. PBMCs were separated on a gradient of Ficoll-Paque PLUS (GE Healthcare). Cells were counted by trypan blue staining and were stored in dimethyl sulfoxide freezing medium (Bioveris). Patients were defined as atopic on the basis of positive allergen-specific serum IgE³¹. After being thawed, isolated PBMCs were activated with plate-bound anti-CD3 (2 µg/ml; HIT3a; BD Pharmingen) and were cultured for 3 d at 37 °C in a humidified incubator at 5% CO₂. The concentration of IL-9 in the supernatant was measured with the Milliplex MAP Kit according to the manufacturer's protocol (Millipore). Samples and standards were loaded on a filter plate (Millipore) with Multiplex beads after being vortexed and sonicated. Plates were incubated on a shaker (Lab-Line Instruments) for 1 h at 24 °C in the dark. Plates were washed twice with wash buffer before the addition of biotinylated detection antibody to each well, followed by incubation for 30 min at 24 °C. Streptavidin-phycoerythrin was added to each well, followed by incubation for 30 min at 24 °C. After plates were washed twice with wash buffer, sheath fluid was added to each well, followed by shaking for 5 min. Assay plates were then transferred to a Luminex 200 system (Luminex) for data acquisition and analysis. Cytokine concentrations were calculated with Bio-Plex Manager 2.3 software with a five-parameter curve-fitting algorithm applied for standard-curve calculations.

Induction of allergic airway inflammation. Mice were sensitized by intraperitoneal injection on days 0 and 7 of OVA (Sigma) adsorbed with alum (Sigma) at a dose of 20 µg OVA per 2 mg alum. On day 14, mice were exposed for 5–6 consecutive days to intranasal OVA (100 µg per day). Where specified, mice were given 20 µg control antibody or anti-IL-9 (222622; R&D Systems) intravenously 30 min before the first, third and fifth challenges. Mice were killed by intraperitoneal injection of pentobarbital (5 mg per mouse) 48 h after the final intranasal challenge. The trachea was cannulated and lungs were lavaged three times with 1 ml PBS. Cells recovered in BAL fluid were counted with a hemocytometer. Eosinophils, neutrophils, T cells, B cells and mononuclear cells in the BAL fluid were distinguished by cell size and by expression of CD3, B220, CCR3, CD11c and major histocompatibility complex class II, analyzed by flow cytometry as described⁴⁴. Cytokine concentrations in cell-free BAL fluid were measured with Multiplex reagents (Millipore). For quantitative PCR analysis, lung tissues were homogenized in a tissue lyser (Qiagen) and RNA isolated

with an RNeasy kit (Qiagen) was used for synthesis of cDNA for subsequent analysis. Peripheral immune responses in sensitized and challenged wild-type or *Sfp1^{fl/fl} Lck*-Cre mice were assessed by culture of splenocytes for 72 h with anti-CD3 (4 µg/ml) or OVA (100 µg/ml) before cytokines in cell-free supernatants were assessed by ELISA. Paraffin-embedded sections were stained with hematoxylin and eosin for evaluation of the infiltration of inflammatory cells by light microscopy.

Airway hyper-reactivity. Airway hyper-reactivity to methacholine challenge was determined 24 h after the final intranasal challenge as described above. Noninvasive unrestrained whole-body plethysmography (Buxco Research Systems) was used to record airway responsiveness with the dimensionless

parameter 'enhanced pause' for estimation of total pulmonary resistance, an indicator of bronchoconstriction. Mice were placed in whole-body plethysmographs and baseline measurements were recorded. Saline was administered by nebulization for 2 min, followed by increasing doses of methacholine, and the enhanced-pause parameter was recorded over 5 min.

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

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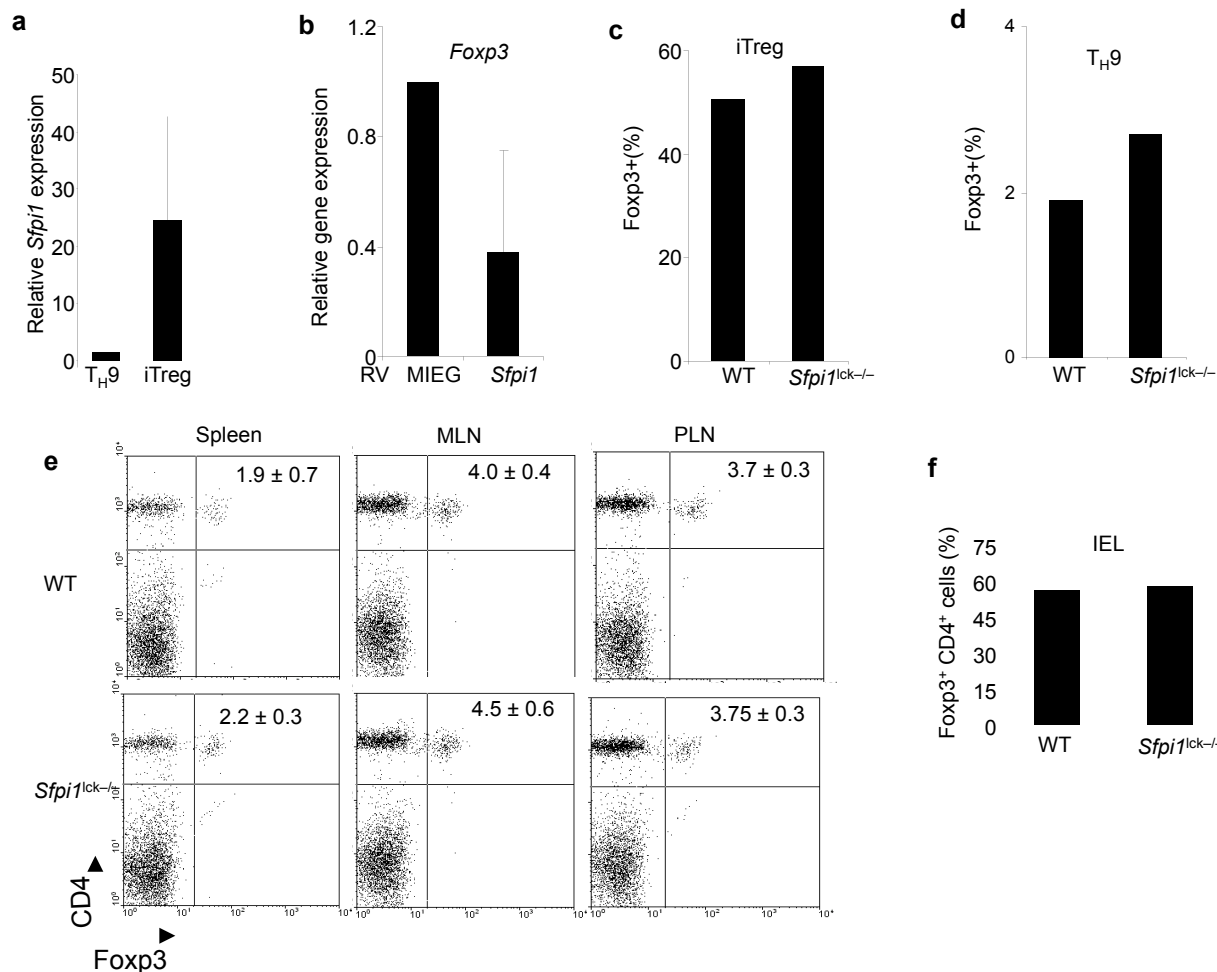
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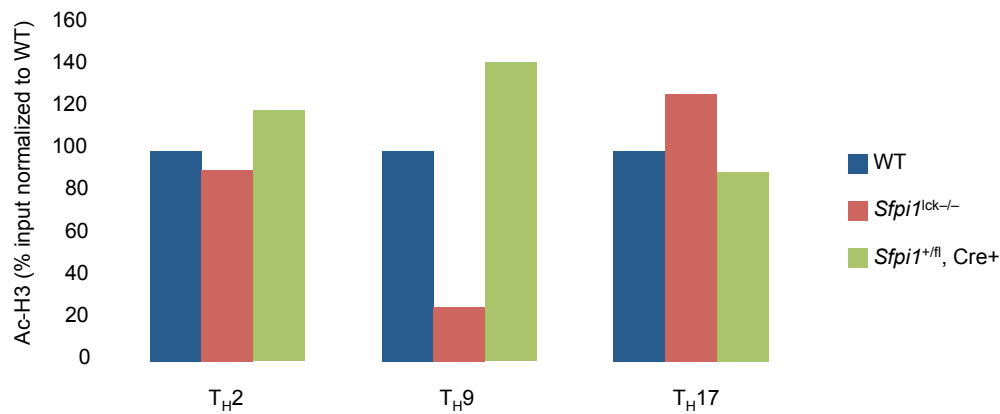
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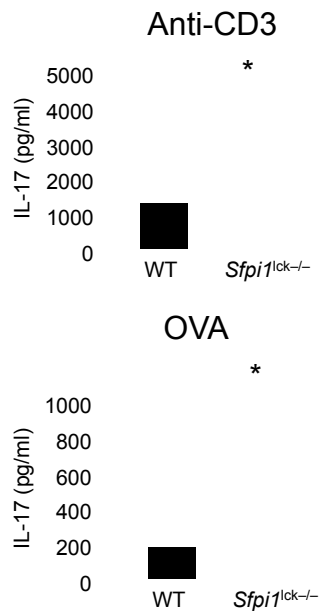
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Supplementary Figure 1. PU.1 is not required for regulation of Foxp3. **(a)** Naïve CD4⁺ T cells cultured with TGF- β 1 or TGF- β 1 + IL-4 to promote iTreg and T_H9 development, respectively. RNA was isolated from cells cultured for five days and expression of *Sfp1* was determined using qPCR. Results are the average $\pm$ SD of 4-6 experiments. **(b)** Naïve CD4⁺ T cells cultured with TGF- β 1 to promote iTreg development were transduced with control or PU.1-expressing RV. After 5 days in culture, EGFP⁺ cells were sorted and analyzed for *Foxp3* expression using qPCR. Results are the average $\pm$ SD of 3 experiments. **(c, d)** Wild-type and PU.1-deficient naïve CD4⁺ T cells were cultured under iTreg, **c**, or T_H9 , **d**, conditions for five days and analyzed for the percentage of Foxp3 positive cells using intracellular staining. Results are the average of three mice and are representative of three experiments. **(e)** Flow cytometric analysis of CD4⁺ Foxp3⁺ cells from the spleen, mesenteric lymph nodes (MLN) and popliteal lymph nodes (PLN). Numbers in the upper right quadrant indicate the average percentage of cells of 2-4 mice. **(f)** Flow cytometric analysis of CD4⁺ Foxp3⁺ cells among intraepithelial lymphocytes. Data are expressed as the % of CD4⁺ cells that are Foxp3⁺ and are an average $\pm$ SD of results from 2-4 mice. Data in **e** and **f** are representative of two experiments.



Supplementary Figure 2. PU.1-dependent H3 acetylation is specific for T_H9 cells. Naïve CD4⁺ T cells were isolated and differentiated under T_H2, T_H9 or T_H17 culture conditions for five days. Cells were analyzed for H3 acetylation using chromatin immunoprecipitation and primers for *Il9* CNS1. Results are shown as average $\pm$ SD of percent input, with IgG control ChIP values subtracted from the specific ChIP values, and are representative of two experiments. Values are normalized to WT Ac-H3 amounts which were set at 100%.



Supplementary Figure 3. Allergen-induced IL-17 production is independent of PU.1. As described in **Fig. 6**, wild-type and *Sfp1*^{lck-/-} mice were sensitized and challenged intranasally with ovalbumin. 48h after the last intranasal challenge mice were sacrificed for analysis. Splenocytes were stimulated with anti-CD3 or OVA for 72 h and supernatants were tested for the amounts of IL-17 by ELISA. Results are the average of 5-7 mice $\pm$ SEM and are representative of three experiments.