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Th9 cells: differentiation and disease

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Summary

CD4⁺ T-helper cells regulate immunity and inflammation through the acquisition of potential to secrete specific cytokines. The acquisition of cytokine-secreting potential, in a process termed T-helper cell differentiation, is a response to multiple environmental signals including the cytokine milieu. The most recently defined subset of T-helper cells are termed Th9 and are identified by the potent production of interleukin-9 (IL-9). Given the pleiotropic functions of IL-9, Th9 cells might be involved in pathogen immunity and immune-mediated disease. In this review, I focus on recent developments in understanding the signals that promote Th9 differentiation, the transcription factors that regulate IL-9 expression, and finally the potential roles for Th9 cells in immunity *in vivo*.

Keywords

T cells; T-helper cells; transcription factor; differentiation; inflammation

Th9 cells

CD4⁺ T-helper cells differentiate into effector subsets in response to environmental cues including cytokines and ligand–receptor interactions from cell–cell contact. These environmental signals promote the development of T-helper (Th) subsets that secrete specific cytokines and perform distinct functions in regulating immunity and inflammation. Th9 cells are one of the more recently described subsets of effector T cells. They develop from naive T cells in the presence of transforming growth factor β (TGF β) and interleukin-4 (IL-4). As the sobriquet would suggest, cells cultured under these conditions are primed for the production of IL-9 and require transcription factors that include STAT6 (signal transducer and activator of transcription 6), PU.1, IRF4 (interferon response factor 4), and GATA3 (1–4). In addition to IL-9, Th9 cells have been shown to produce IL-10 and IL-21, although these are not regulated coordinately with IL-9, and their role in Th9 cell function is unclear (1, 2, 4–7). Th9 function has been associated with a wide variety of inflammatory disease, and as discussed below, their function does not segregate as neatly as some of the other Th effector subsets. The pleiotropic functions of IL-9 have recently been reviewed extensively (8, 9), and I do not review those again here. Instead, I focus on the development and function of Th9 cells, with a particular focus on some of the recent developments in understanding how *Il9* is regulated and how IL-9-secreting T cells fit into the spectrum of T-

helper effector subsets by addressing some of the outstanding questions regarding Th9 cell biology.

How do Th9 cells develop?

Cytokines

The development of IL-9-secreting T cells is promoted by several cytokines. Foremost among them is IL-4. Several transcription factors downstream of IL-4, including STAT6, GATA3, and IRF4, are required for the differentiation of Th9 cells (2–4, 10). However, IL-4 signaling alone leads to Th2 differentiation. The conversion of the Th2-inducing signals into Th9-inducing signals requires signaling from the TGF β receptor superfamily. The TGF β signal, in the absence of IL-4, results in the development of inducible Treg (T-regulatory) cells (11–13). TGF β signaling in Th9 cells induces Smad activation and expression of PU.1 (1, 10, 14). However, the requirement for TGF β signaling may not be absolute. At least one report has shown that IL-9 production during a parasite infection is unaltered by expression of a dominant negative TGF β receptor (15). One explanation for this may be redundancy with other superfamily members. Indeed, Jones et al. recently showed that Activin A can replace TGF β as a Th9-inducing factor, and in a model of allergic inflammation, both TGF β and Activin needed to be neutralized to see decreases in IL-9 *in vivo* (16). Importantly, the development of Th9 cells requires a balance of signals from cytokines that would otherwise generate distinct T-helper subsets.

Various other cytokines also impact T-cell IL-9 production. IL-2, and presumably the downstream factor STAT5, promotes T-cell IL-9 production (17, 18), although it has not yet been determined how important this pathway is for Th9 development *in vitro* and *in vivo*. IFN γ (interferon- γ) inhibits IL-9 production from T cells (18). A recent report described that CD4⁺ T cells cultured with TGF β , IL-4, and IFN γ develop into a cell type more closely associated with the Th1 phenotype than with Th9 cells. This differentiation was dependent on T-bet, and the IL-4/STAT6 pathway induced expression of Eomesodermin and resulted in IFN γ -producing cells that displayed surface expression of CD103 (19). Although a population of CD103⁺IFN γ ⁺ T cells is observed among intraepithelial lymphocytes, whether this population has a functional niche *in vivo* is still not clear.

IL-23 also represses IL-9 production, a conclusion supported both by *in vitro* culture effects and increased production of IL-9 from *Il23r*^{-/-} T cells (20, 21). Given the ability of IL-23 to promote and/or maintain Th17 development and to specifically induce IL-22, it may be an important component of sculpting the cytokine-secreting potential of developing Th17 cells (22–26). As Th17 cells have been shown to make some IL-9, albeit not at the levels observed from polarized Th9 cultures, IL-23 might refine cytokine production, directing Th17 cells to more specific functions.

IL-1 family members may also contribute to IL-9 production. IL-1 may induce IL-9 under some conditions, and IL-33 is capable of inducing IL-9 in T cells (27–29). IL-1 family members induce NF- κ B (nuclear factor- κ B) activation, which as described later in the review is an important activator of the *Il9* gene. IL-1 and IL-1 family members collaborate with other cytokines in cytokine induction. IL-1 synergizes with IL-23 in the induction of IL-17, and IL-18 synergizes with IL-12 in the induction of IFN γ (30). Whether any IL-1 family member synergizes with other cytokines in *Il9* induction has not yet been tested.

Finally, strong evidence supports a role for IL-25 in IL-9 production by T cells. Th9 cells express greater amounts of the IL-25 receptor chain IL-17RB than other Th cell subsets suggesting that they are uniquely sensitive to this cytokine (31). Transgenic IL-25 induces IL-9 production *in vivo*, and the inflammation that develops in IL-25 transgenic mice is IL-9

dependent (31). Blocking IL-25 *in vivo* reduces IL-9 production and IL-9-dependent inflammation (31). Thus, IL-25 plays a critical role in promoting IL-9-dependent immune responses.

The development of Th9 cells requires the integration of multiple signals, and clearly a complex cytokine milieu is required for optimal IL-9 production. At this point, it seems unlikely that all of the cytokine cues that impact Th9 development have been identified, and further work will undoubtedly reveal additional factors that both positively and negatively affect Th9 differentiation.

Costimulation

One of the emerging themes in the development of Th9 cells is the requirement for potent costimulatory signals. In our own unpublished studies, we have observed that TCR (T-cell receptor) transgenic T cells stimulated with peptide on antigen-presenting cells develop very poorly into IL-9-secreting cells in the absence of additional costimulatory signals. Experiments comparing the apoptosis inducing effects of anti-CD28 found that plate-bound anti-CD28 resulted in a greater induction of IL-9 production than cultures stimulated with soluble anti-CD28 (32). This was at least partly dependent on the induction of endogenous IL-4 and associated with increased expression and phosphorylation of FoxO3a. Whether FoxO3a might directly affect *Il9* expression or if this pathway affects survival of cytokine-secreting cells is not clear.

The Notch pathway is required for efficient Th9 development. Conditional deletion of Notch1 and Notch2 decreased IL-9 production in Th9 cultures (14). Although both are Notch ligands, Jagged2 but not Delta-like 1 was able to induce IL-9 from cells cultured with TGF β alone. Similarly, Jagged2 induced IL-9-dependent immunity in an experimental autoimmune encephalitis (EAE) model, and EAE was attenuated in mice with conditional Notch1 and Notch2 deletion in T cells (14). The mechanism of Notch-dependent induction of IL-9 is not entirely clear but may involve increased expression of GATA3 because higher concentrations of exogenous IL-4 could overcome the effects of Notch1/2 deficiency. There are also direct effects of the Notch signaling pathway on *Il9* as discussed below (14).

The requirement for additional costimulation in the development of IL-9-secreting T cells was also reported during the process of identifying additional Th9-inducing pathways (33). Xiao *et al.* (33) demonstrated a potent ability of the TNFRSF (tumor necrosis factor receptor superfamily) member OX40 to induce IL-9 production, further emphasizing a role for costimulation. The greatest effects of OX40 activation were observed when OX40L was overexpressed on the surface of antigen-presenting cells, resulting in a striking increase in IL-9 production. Induction of IL-9 was specific, as there was no induction of IL-4, and under the respective culture conditions for Treg and Th17 cells, there was OX40-induced repression of Foxp3 (forkhead box protein-3) and IL-17. Importantly, the effects of OX40 were only observed in Th9 culture conditions. The OX40-induced IL-9 effect was also observed *in vivo*, where OX40L transgene expression and injection of antibody to OX40 resulted in allergic airway inflammation characterized by eosinophil inflammation and goblet cell metaplasia (33). Although it is not yet known if other TNFSF members can induce IL-9, there are data suggesting that GITR (glucocorticoid-induced TNFR) engagement induces IL-9 from Treg cells (34), and these results raise the possibility that developing IL-9-secreting T cells will be sensitive to the engagement of a wide array of cytokines and surface receptors.

How is IL-9 regulated?

The *Il9* locus is linked to Th2 cytokine loci, about 3.2 Mb telomeric from the *Il5* and *Il4-Il13* loci. Chromatin at the *Il9* promoter is most acetylated in Th9 cells, compared with other T-helper cells, and tri-methylation of H3K27, a repressive chromatin modification, is minimal at the *Il9* promoter in Th9 cells, compared with other T-helper subsets (1). Three conserved non-coding sequences (CNS) have been identified near the *Il9* locus. CNS1 is the promoter, and where most factor binding related to gene regulation has been established (1, 35) (Fig. 1). CNS2 is downstream of *Il9* (+5 kb from the transcriptional start), and although the sequence was conserved between murine and human genomes (1), it was not between murine and canine genomes (35). CNS0 at -6.3 kb from the transcriptional start site is conserved among multiple species. The *Il9* promoter (CNS1) has been shown to function in reporter assays and is responsive to multiple factors including IRF4, NF- κ B, and Smad/Notch complexes (3, 14, 33, 36). The other putative elements have not been tested for regulatory function, although they do reflect changes in chromatin modifications (1, 6, 10).

PU.1

PU.1, encoded in mice by the *Sfp1* locus, is required for optimal Th9 development (1). PU.1 is expressed in Th9 cells at greater levels than Th1, Th2, or Th17 cells and is induced by the TGF β signaling pathway, independently of IL-4 and STAT6 (1, 10). PU.1-deficient Th9 cultures have diminished production of IL-9, and decreased *Il9* mRNA, compared with wildtype cultures. Transduction of PU.1 into Th9 and Th2 cultures induces IL-9 production, as it decreases Th2 cytokine production (1, 37, 38). PU.1 function in T cells was first identified by its expression in the IL-4-low subpopulation of Th2 cells (37, 38). When transduced into Th2 cells, PU.1 diminished expression of most Th2 cytokines, while leaving intact other markers of Th2 cells including expression of CCR4 and GATA3 (38). Conversely, reducing PU.1 expression, either by shRNA or conditional mutation, resulted in increased Th2 cytokine production, particularly in cells that were positive for more than one cytokine (37, 38). Based on its ability to inhibit the Th2 phenotype and promote Th9 development, PU.1 is the only factor identified thus far that can convert another T-helper lineage into a Th9 phenotype.

In Th2 cells, PU.1 functions by interfering with GATA3 and IRF4 activity (38, 39). PU.1 interacts with each of these factors and interferes with their ability to bind DNA and activate transcription. In PU.1-deficient Th2 cells, there is increased binding of GATA3 to the *Il4* locus, and IRF4 to the *Il10* locus, coincident with increased expression of each cytokine (37, 39). Thus, although PU.1 is expressed in lower amounts in Th2 cells, compared with Th9 cells, it contributes to the heterogeneity of cytokine expression in the population by modifying the function of Th2 cytokine-regulating transcription factors.

In Th9 cells, PU.1 functions by directly binding to the *Il9* promoter and recruiting chromatin-modifying enzymes (1, 6) (Fig. 1). PU.1-deficient Th9 cultures have decreased total histone H3 acetylation and decreased acetylation of several specific histone residues including H3K9/18, H3K14, H4K5, H4K8, and H4K16 at the *Il9* locus (1, 6). However, acetylation of H3K27 and H3K36, and H3K27 methylation are not affected by PU.1 deficiency. Decreased histone acetylation at the *Il9* locus is coincident with decreased association of the histone acetyltransferase (HAT) proteins Gcn5 and PCAF, and increased association of histone deacetylase (HDAC) proteins HDAC1 and HDAC2 observed in the absence of PU.1 (6). IL-9 production can be increased in Th9 cells by culture with the HDAC inhibitor trichostatin A, although importantly, PU.1 is required for this activity. PU.1 interacts directly with Gcn5 and treatment of Th9 cells with siRNA for Gcn5 results in decreased amounts of IL-9 production. However, other cytokines produced by Th9 cells, including IL-10 and IL-21, are not affected either by trichostatin A treatment or by Gcn5

short interfering RNA (siRNA) (6). This observation suggests that there is a specificity in the function of this pathway and that a PU.1-Gcn5 complex is critical in the regulation of IL-9 (6).

In human T cells, PU.1 similarly promotes IL-9 production. Human Th9 cells have increased expression of *SPI1* (encoding PU.1) compared with Th2 cells (1). Decreasing PU.1 expression in Th9 cultures by siRNA also decreases *IL9* expression (1, 27). CD4⁺IL-9⁺ and PU.1⁺ cells can be found in inflamed skin (40). In peripheral blood samples from patients with atopic asthma, T cells that had the highest IL-9 production were also positive for expression of PU.1 (16). Moreover, in Th9 cultures from atopic infants that demonstrated an increased propensity for the development of Th9 cells, there was coincident increased expression of *SPI1* (41). Together, these studies support a significant function for PU.1 in IL-9 production from human T cells.

The expression of PU.1 and its ability to regulate IL-9 are also regulated by histone modifications. Human naive cord blood T cells are defective in their ability to differentiate into Th9 cells (27). The Th9 deficiency correlates with diminished H3K4 methylation and increased H3K27 methylation of the *SPI1* locus. Modulation of histone methylation at the PU.1 locus by cytokine treatment or using inhibitors of histone methyltransferases results in increased *SPI1* and *IL9* expression (27). Modifying histone acetylation also affects PU.1 expression. Treatment of human Th9 cultures with curcumin, a histone acetyltransferase inhibitor, decreases expression of PU.1 (27). In parallel, treatment of mouse Th9 cultures with trichostatin A (TSA), a histone deacetylase inhibitor, results in increases of both PU.1 and IL-9 expression (6). Importantly, TSA-induced IL-9 expression is dependent on the expression of PU.1, as TSA had no effect in PU.1-deficient Th9 cultures (6). Together, these data support a central and conserved role for PU.1 in the regulation of IL-9 in both human and mouse Th9 cells (Fig. 1).

STAT6, GATA3, and IRF4

STAT6 is phosphorylated throughout Th9 development *in vitro*, at levels very similar to those observed in Th2 development (10). Not surprisingly, considering the importance of IL-4 in the development of Th9 cells, STAT6 is absolutely required for IL-9 production (2, 4, 10). Consistent with this, the STAT6 cofactor PARP-14, required for optimal Th2 development (42), is also required for optimal IL-9 production (10). In contrast with the requirement for STAT6, STAT3, which is activated during Th9 development and is required for normal Th2 development, is not required for Th9 development (10, 43).

The identification of factors downstream of STAT6 seems the next critical question. One obvious candidate would be GATA3. In initial reports of the Th9 subset, it was shown that Th9 development was lost in GATA3-deficient cells, a phenotype similar to the requirement for GATA3 in Th2 development (2, 44, 45). *Gata3* mRNA rises through the differentiation period, although there is more of an increase in Th2 cultures toward the end of the 5-day differentiation period, and expression is STAT6 dependent (10). Protein expression of GATA3 was slightly higher in Th9 cells than in Th2 cells, but GATA3 protein was not induced by anti-CD3 in Th9 cells as it was in Th2 cells (10), suggesting that differences in GATA3 regulation between the subsets might be critical. In support of GATA3 expression being tightly regulated in Th9 cells, retroviral expression of GATA3 did not induce but rather repressed IL-9 production in wildtype Th9 cells, and although GATA3 induces Th2 cytokines in *Stat6*^{-/-} Th2 cultures, ectopic GATA3 expression did not rescue IL-9 expression in *Stat6*^{-/-} Th9 cultures (10, 38, 46). Thus, although GATA3 contributes to the Th9 phenotype, it is not the primary STAT6 target.

Another potential candidate is IRF4. IRF4 is required for Th9 development (3). IRF4-deficient T cells have defective Th9 development, and IRF4-deficient mice have diminished development of allergic inflammation, although this could be attributed to multilineage effects of IRF4 deficiency on Th2 and Th17 as well as Th9 cells (3, 47, 48). IRF4 is also induced in human Th9 cultures and binds to the *Il9* promoter (3) (Fig. 1). Expression of IRF4 and binding of IRF4 to the *Il9* promoter in Th9 cells is dependent on STAT6 (10). Furthermore, IRF4 transduction into wildtype Th9 cultures results in increased IL-9. However, transduction of IRF4 into *Stat6*^{-/-} cultures did not rescue IL-9 production. Importantly, double transduction of IRF4 and GATA3 into *Stat6*^{-/-} Th9 cultures also does not rescue production of IL-9. Thus, although IRF4 and GATA3 clearly contribute to the STAT6-dependent development of Th9 cells, and the expression of IL-9, they are not the only relevant targets of STAT6 in Th9 differentiation.

NF- κ B and NFAT

NF- κ B is involved in cytokine regulation from a broad range of stimuli. Two different stimuli have been shown to require NF- κ B for the induction of IL-9.

In its ability to induce IL-9 production, OX40 induces both the classical and alternative pathways of NF- κ B activation (33). However, the classical pathway, culminating in the activation of RelA-p50 heterodimers, is not required for OX40-induced IL-9, and ectopic expression of RelA-p50 does not induce IL-9. In contrast, ectopic expression of RelB-p52 induces endogenous IL-9 production, and activated an *Il9* reporter. OX40-induced IL-9 was absent in p52-deficient Th9 cultures, and in Th9 cultures deficient in TRAF6, which was required for activation of the alternative pathway (33). The specificity for the alternative NF- κ B pathway may reflect the ability of a restricted set of NF- κ B proteins to interact with other transcription factors at the *Il9* promoter.

NF- κ B, in cooperation with NFAT1 (nuclear factor of activated T cells), is required for TCR-induced IL-9 production from Th9 cells (36). RelA (p65) was unique in the ability to induce an *Il9* promoter reporter, and siRNA for RelA diminished production of IL-9. NFAT1 regulated access for RelA by altering histone modifications, and chromatin structure, at least partly through recruitment of the HAT p300 to the *Il9* promoter. NFAT1 function was also required as cells deficient in NFAT1 demonstrated greatly reduced amounts of IL-9 production (36).

It is interesting that two different NF- κ B proteins activate *Il9* in different contexts. This may represent distinct promoter complexes following each stimulus. It will be interesting to see which complex is more important when additional stimuli are considered, including IL-25 and IL-33, which are known to activate NF- κ B and induce IL-9 as mentioned above.

Notch, Smad, and RBP-J κ

Activation of the Notch pathway results in the release of the Notch intracellular domain (NICD), which acts as a cofactor with RBP-J κ in binding to target genes promoters. Elyaman *et al.* (14) demonstrated that Jagged2 activation results in increased levels of Smad3 protein and phospho-Smad3 activated by TGF β . NICD interacts with Smad3, potentially stabilizing expression. RBP-J κ and Smad3 bind directly to the *Il9* promoter, with the greatest amounts in Th9 cultures. Moreover, Smad3 binding is virtually absent in Th9 cultures deficient in Notch signaling (14). The cooperation between RBP-J κ , Smad3, and the NICD was also observed in an *Il9* reporter assay (14). Thus, TGF β and Notch signaling cooperate in the induction of IL-9.

Transcription factors in opposing T-helper lineages

As transcription factors promote the development of one lineage, they often inhibit cytokine and gene expression associated with other T-helper cell lineages. This paradigm is also true in the Th9 lineage. The Th1-associated transcription factors T-bet and Runx3 decrease expression of *Il9* in Th9 cells (10). The Treg cell lineage factor Foxp3 also decreases IL-9 production in Th9 cells (10). Expression of both T-bet and Foxp3 are inhibited by IL-4/STAT6 signaling in developing Th9 cells (10). IL-9 expression is also increased in Ror γ -deficient mice (21). Although the effects of the T-follicular helper lineage factor Bcl6 on IL-9 production have not been documented, the previously reported ability of Bcl6 to negatively regulate IL-4 signaling and GATA3 (49) suggests that it might be a negative regulator. Retroviral transduction of any of these factors into developing Th9 cells decreases production of IL-9 (10, author's unpublished results).

Are there really Th9 cells *in vivo*?

Given the relatively specific culture conditions that promote Th9 development and the relative difficulty in detecting IL-9-secreting T cells *in vivo*, either because of reagent limitations or because they display transient production of IL-9, it has been natural to question whether Th9 cells are a real *in vivo* subset. But the answer to the question, 'Are there really Th9 cells *in vivo*?' is simple. Yes.

There is considerable evidence of IL-9-secreting T cells from perhaps the most important 'model', humans. Th9 cells (CD4⁺IL-9⁺IL-13⁻IFN γ ⁻) are found in the peripheral blood of allergic patients, and this population is rare in non-allergic individuals (16). Among cutaneous lymphocyte antigen-positive cells in the peripheral blood, there is a significant population of CD4⁺IL-9⁺IL-4⁻IL-17⁻IFN γ ⁻ T cells (21). IL-9-positive T cells are also found in both normal and inflamed skin (21, 40).

IL-9 responses can also be observed to specific antigen stimulation. In a population of atopic and non-atopic infants, stimulation with house dust mite extract or cat allergen resulted in greater production of IL-9, but not other Th cell cytokines, from cells isolated from infants that had been sensitized to the specific allergen (41). Similar responses can be seen in response to pollen and dust mite allergen in peripheral blood mononuclear cell cultures of adult asthmatics and from T-cell clones isolated from sensitized patients (50–52).

In murine systems, the detection of IL-9-secreting T cells has been more challenging, and the detection might depend on the model system used. Using an IL-9 fate reporter system, Th9 cells were detected using an ovalbumin (OVA)/ alum model of airway inflammation but not in a papain model of inflammation, and the reason for this difference could be due to the preferential activation of innate versus acquired immune cells by each stimuli (53). The appearance of IL-9-secreting T cells seemed to be transient. However, using flow cytometry, IL-9-positive CD4⁺ T cells (that are negative for IL-13 and IFN γ) can be identified during the development of house dust mite extract-induced lung inflammation (16). IL-9-secreting T cells are also observed as melanoma-infiltrating lymphocytes (21). Similarly, OVA stimulation induces *Il9* expression from sensitized mice that is dependent on the expression of the Th9 transcription factor PU.1 (1).

The data thus far have focused on identifying Th9 cells in atopic patients and in mouse models of allergic disease. It has not yet been clearly established whether Th9 cells can be identified in other inflammatory diseases. However, the existence of IL-9-secreting T cells in allergic disease leads to the next logical question regarding the role of Th9 cells in disease.

What can Th9 cells do?

One approach for testing the *in vivo* function of T-helper subsets is to adoptively transfer T cells, either polyclonal or TCR transgenic populations, that have been polarized *in vitro* to a specific phenotype, such as Th9, after transfer cells can be challenged *in vivo*. Key to this approach is being able to show that IL-9 is an effector cytokine by coupling adoptive transfer experiments with antibody to block IL-9 function. This approach has been used for Th9 cells and they appear to be able to initiate a broad range of inflammatory diseases (Fig. 2).

Allergic inflammation

Two reports have demonstrated that transferred Th9 cells lead to allergic inflammation in the lung. Transferred DO11.10 Th9 cells result in allergic airway disease following OVA challenge (3). Importantly, treatment of recipient mice with anti-IL-9 was able to block many features of the inflammation including airway hyperresponsiveness, eosino-phil recruitment, and goblet cell metaplasia. Transferred OT-II Th9 cells were able to rescue the generation of allergic airway disease in *Irf4*^{-/-} mice, which, given the defects in Th2 and Th17 generation in the absence of IRF4 (47, 48), further supports the ability of Th9 cells to promote allergic inflammation (3). Transfer of polyclonal Th9 cells, differentiated *in vitro* with either TGF β or Activin A, was also sufficient to induce airway inflammation characterized by increased amounts of eosinophil recruitment, tissue mast cell numbers, and serum IgE (16). Thus, transfer of Th9 cells, into either BALB/c or C57BL/6 mice, results in all of the cardinal features of allergic airway disease.

Autoimmune disease

In the first description of Th9 cells, the overlapping production of the immunosuppressive cytokine IL-10 with IL-9, the latter having been linked with tolerance in some models (34), led to the hypothesis that Th9 cells might be immuno-regulatory (2). However, in the classical colitis model to test for regulatory T-cell function, Th9 cells showed no regulatory function but rather initiated disease on their own, and exacerbated disease when injected with naive cells (2). Thus, Th9 cells can promote intestinal inflammation. It is not clear whether IL-9 itself promotes intestinal inflammation, whether IL-9 impacts the production of additional cytokines from Th1 or Th17 cells that can promote intestinal inflammation, or whether Th9 cells directly acquire the ability to secrete other inflammatory cytokines in this model.

Th9 cells also promote central nervous system inflammation. When transfer of MBP (myelin basic protein)-specific TCR transgenic Th9 cells was compared with the ability of other subsets of T-helper cells to induce EAE, clinical disease started earlier but was less severe in Th9 recipients than in Th17 recipients (20). Moreover, the lesions that develop were distinct in recipients of Th9 cells compared with Th1 or Th17 recipients, suggesting parallel pathways to inflammatory disease resulting from transfer of each subset (20). However, there are conflicting reports regarding the role for IL-9 in EAE. In some reports, blocking IL-9 or IL-9 signaling results in diminished pathology (54–56). In contrast, IL-9 may also promote Treg function and inhibit EAE development (57). A recent report demonstrating that the IL-9-inducing effect of the Notch ligand Jagged2 during EAE has different effects depending on when it is introduced to the model (14) suggests that context-dependent effects of IL-9 might impact the outcome of blocking effector functions on CNS disease.

Hen egg lysozyme (HEL)-specific Th9 cells result in ocular inflammation when HEL is expressed in the lens (7). However, in the experimental autoimmune uveitis (EAU) model,

anti-IL-9 did not block inflammation, and most of the transferred Th9 cells were observed to be secreting IFN γ during disease. The stability of the Th9 subset *in vivo* is still not clear, and in both EAE and EAU models, transferred Th9 cells acquire IFN γ -secreting potential (7, 20). Although this might suggest that Th9 cells do not cause disease and that it is the ability to become Th1 cells that is pathogenic, it is also possible that sequential secretion of IL-9 and IFN γ results in different pathological outcomes. This concept is supported by the distinct pathology in recipients of Th1 and Th9 transferred cells (7, 20).

Tumor immunity

Most recently, Th9 cells have been implicated in tumor immunity (21). In examining tumor immunity in *Rorc*^{-/-} mice, Purwar *et al.* (21) observed decreased tumor development and increased IL-9 production. IL-9-secreting CD4⁺ T cells were found in tumor-infiltrating lymphocytes, and the authors observed that adoptively transferred Th9 cells resulted in superior tumor immunity in models of mela-noma and lung cancer growth that could be inhibited by anti-IL-9 (21). The anti-tumor effects of Th9 cells and IL-9 were dependent on mast cells, although how mast cells mediate the anti-tumor effect is still not clear (21). Another recent study by Lu *et al.* (58) showed a similar function of transferred OVA-specific Th9 cells in several models of OVA-expressing melanoma. Endogenous immunity to mela-noma was dependent on IL-9. The proposed mechanism of IL-9 activity was to induce local CCL20 production, thus enhancing the recruitment of dendritic cells and cytotoxic CD8 T cells to the tumor (58). Additional mechanistic insight into the anti-tumor functions of Th9 cells will require further studies.

Th9 function in patients

Definitively establishing *in vivo* functions of Th9 cells in patients is very difficult. However, several studies have demonstrated strong correlations of *in vivo* IL-9 with serum IgE concentration. In atopic patients, the percentages of IL-9-secreting T cells correlated with serum IgE in adults with asthma (16). Similarly, the amounts of IL-9 secreted by *ex vivo* stimulated T cells correlated with serum IgE in atopic infants (41). In agreement with a functional link to IgE production, some but not all studies have found an association of *IL9* SNPs with serum IgE (59–62). Ongoing studies will hopefully define additional correlations of Th9 function with human physiology.

Function of IL-9 from other T-helper cell subsets

Although Th9 cells are specialized for production of IL-9, other cells may also be able to produce some IL-9 following an appropriate stimulus. TGF β can convert Th2 cells to IL-9-secreting cells (4). The presence of TGF β in a culture also stimulates acute IL-9 production from several T-helper cells subsets, including Th17 cells (56). The role of IL-9 in Th17-mediated disease is still unclear, with reports indicating both pro- and anti-inflammatory effects that may be disease or context dependent (14, 56, 57, 63, 64).

An *in vivo* biological function of IL-9 has also been attributed to T-regulatory cells. A study linking mast cells to allo-geneic skin transplantation tolerance observed IL-9 production from Treg cells (34). IL-9 production by Treg cultures was induced following short-term stimulation with anti-CD3 and either anti-CD28 or anti-GITR, echoing the importance of costimulation in generating IL-9-secreting T cells, as discussed above (34). Anti-IL-9 was able to block the function of transferred Tregs in this model (34). Similar results were seen in a model of nephritis, wherein Tregs-mediated immune suppression that was dependent on IL-9 and mast cells (65). Precisely, how the Treg-IL-9-mast cell circuit promotes immune suppression has not been determined.

The caveat to the studies by Lu *et al.* and Eller *et al.* (34, 65) is that transferred Tregs were isolated based on positivity for CD4 and CD25, raising the issue that non-Tregs (Foxp3-negative cells) might have also been transferred. Subsequent studies have not observed IL-9 production from differentiated inducible Treg cultures (1, 10), although it is possible that differentiating Treg cells, in the context of the proper stimulus, undergo an IL-9-secreting intermediate or that IL-9 production is restricted to natural Tregs. Further studies should be able to define the source of IL-9 in these models.

Th9 stability

One issue that is still not well explored is the stability of the IL-9-secreting phenotype *in vivo*. Use of an *Il9* reporter model suggested that IL-9 production is transient and that few of the former IL-9-secreting cells maintain IL-9 production (53). Using a HEL-specific TCR transgenic model, studies suggest that antigen stimulation results in diminished IL-9 production *in vitro* and *in vivo* (7). This may depend on the inflammatory environment, since several cytokines, including IFN γ and IL-23, can alter Th9 cell phenotype or reduce IL-9 production (18–21). However, several studies described above have demonstrated that blocking IL-9 in recipients of transferred Th9 cells is able to block the function of the transferred cells (3, 20, 21, 58), which would suggest that in the right environment Th9 cells are capable of maintaining IL-9-secreting potential, at least long enough to carry out biological function *in vivo*. Further studies with adoptive transfer models and reporter mice will be useful in refining our understanding of maintenance of the Th9 phenotype both *in vitro* and *in vivo*.

What are Th9 cells required for?

What Th9 cells are actually required for has been a question more difficult to answer. IL-9- and IL-9R-deficient mice, as well as antibodies to IL-9, have been used to define the role of this cytokine in several model system (reviewed in 8, 9). However, there are clearly cells other than Th9 cells, including mast cells and innate lymphoid cells (53, 66, 67), that produce IL-9. Thus, although studies that block cytokine or cytokine receptor are critical for defining the cytokine function, they are not useful for defining the relevant source of that cytokine. Demonstrating that Th9 cells are the critical source of IL-9 would require lineage-specific deficiency or adoptive transfer experiments that have not yet been performed.

For other T-helper cells subsets, the approach has been to examine mice that are defective in one of the inducer cyto-kines, or in a transcription factor that is specific for that subset of Th cells. This has been a limitation for the Th9 lineage where a gene-deficient model with selective deficiency of only the Th9 lineage has not yet been defined. IL-4, its receptor, STAT6, and GATA3 are required for both Th2 and Th9 cell development (2, 4, 10). IRF4 is required for Th2, Th9, and Th17 cell development (3, 47, 48). Notch signaling affects multiple T-helper lineages (14, 68, 69). Thus, it has been difficult to examine disease models in mice that are only deficient in Th9 cells.

Initial reports utilized mice expressing a dominant negative TGF β RII transgene in CD4⁺ T cells to demonstrate that Th9 cells are required for immunity to *Trichuris muris* (4). However, TGF β RII signaling is also required for Th17 and Treg development suggesting that this is not a lineage-restricted approach. Moreover, a subsequent report suggested that IL-9 production was not dramatically altered in mice expressing this transgene, and that altered immunity was dependent on increased IFN γ production in the absence of TGF β signaling (15).

Perhaps the best ‘Th9-deficient’ model described thus far is using mice that are conditionally deficient in the transcription factor PU.1. Using mice that lack PU.1

expression in T cells, allergic inflammation is decreased, and *in vivo* IL-9 production is diminished (1). There is a modest increase in peripheral Th2 responses in the absence of PU.1 expression in T cells, but production of Th1 or Th17 cytokines in the periphery is not altered (1, 37). Thus, although the effects of PU.1 deficiency in T cells are not entirely restricted to Th9 cells, this model still facilitates the most accurate analysis of the effects of Th9 deficiency on inflammation. The phenotype of allergic inflammation in mice that lack PU.1 expression in T cells closely paralleled that of mice where IL-9 was neutralized (1).

The greatest evidence for a role of Th9 cells *in vivo*, from mouse models and from correlation studies in patients, is in allergic disease. Based on the requirement for various Th2 cytokines (70), Th9 cells likely collaborate with Th2 cells in regulating allergic inflammation. Whether these two subsets promote distinct aspects of allergic disease or whether they have overlapping functions is still not clear. Defining the role of Th9 cells in these and other diseases awaits broader usage of mice with PU.1-deficient T cells or the development of additional mouse models that specifically lack the development of Th9 cells.

How does IL-9 promote inflammation?

Despite the broad ability of IL-9 to impact multiple types of inflammation, it is not clear how it functions. IL-9, like many cytokines, has pleiotropic effects. The earliest defined function may be one of the more important. IL-9 promotes survival of CD4⁺ T cells, mast cells, and other cells (8, 9), and it therefore seems reasonable that this function might be capable of impacting the multiple cell types involved in generating the spectrum of inflammatory immunity. A recent commentary has even proposed that this is the primary function of IL-9 (71). Indeed, there is ample evidence that IL-9 performs this function *in vivo*, including expansion of Th17 and Treg cells under various conditions (14, 34, 57, 65).

However, a simple survival signal does not explain the more specific effects of IL-9 *in vivo* and *in vitro*. Transgenic expression of IL-9 in the lung or intestine leads specifically to allergic and not other types of inflammation (72–75). Moreover, adoptive transfer of Th9 cells has shown that they can have divergent function from other transferred subsets in models of tumor immunity, EAE, and allergic airway disease (16, 20, 21). *In vitro* and *in vivo*, IL-9 stimulation results in the induction of chemokines such as eotaxin in smooth muscle cells or in goblet cell metaplasia, suggesting that there is specific induction of genetic programs (76–78).

Some or perhaps even many of these observations can be linked to growth effects of IL-9 on mast cells *in vivo* (16, 79, 80). IL-9 not only stimulates mast cell growth and expansion but also stimulates changes in mast cell gene expression that might alter responsiveness to other stimuli (81). Thus, IL-9 stimulation of mast cells might specifically result in allergic inflammation or promote mast cell-dependent functions in anti-tumor responses and inflammatory disease. In some models, the *in vivo* effects of IL-9 rely on other cytokines. For example, in the lung-specific IL-9 transgenic models, inflammation depends on IL-13 (82). However, the source of IL-13 is not known and could be mast cells, innate lymphoid cells, or Th2 cells. Thus, defining the relevant IL-9-responding cells will be critical for understanding how IL-9 promotes inflammation.

IL-9 clearly has effects in models of allergic inflammation that are mast cell independent. OVA/alum-induced inflammation develops in the absence of mast cells, and yet inflammation is diminished in mice treated with blocking antibody to IL-9 and in mice lacking PU.1 expression in T cells, which results in diminished Th9 cells (1, 31, 83). In these models, the direct effects of IL-9 on lymphoid or tissue-resident cells may be more important. Defining these functions is likely complicated by some overlap in the functions

of Th2 and Th9 cytokines, and the demonstration that IL-4 can mediate type II inflammation in the absence of IL-9 and other Th2 cytokines (84). Among the various models of inflammation, there is likely specificity of what cells produce IL-9 and which cells respond to IL-9. The central question will be which of these models reflect how Th9 cells function in human disease.

Concluding comments

Despite a recent increase in the number of reports on IL-9-producing cells, IL-9 is still an understudied cytokine. It languished for many years, misrepresented as just another Th2 cytokine, and although a vast number of biological functions were ascribed to it, how it contributed to immune responses was unclear. The more recent description of a specialized subset of T cells that produce IL-9 has resulted in a beachhead to further explore IL-9 function *in vivo*. Th9 cells develop in response to a complex cytokine milieu. Several costimulatory signals provided by antigen-presenting cells also impact the abundance of IL-9 production. Both cytokines and costimulatory signals lead to the activation of transcription factors that directly or indirectly regulate *Il9* transcription. A more detailed analysis of *Il9* regulation will provide further clues about the development of this subset, and how regulation of IL-9 might be therapeutically altered. Although studies on the regulation of *Il9* have provided important insight into the development of the Th9 subset, it is still not clear if there are genes that are essential for Th9 function, aside from the specialization for IL-9 production. Moreover, whether the same transcription factors that promote *Il9* expression also activate a larger Th9 genetic program is unknown.

Th9 cells promote inflammation in a variety of models but seem to be particularly capable of promoting allergic inflammation. Some of the effects of Th9 cells could be mediated through mast cells, but other effects are likely direct effects on tissue-resident cells. Whether Th9 cells are involved in pathogen immunity or whether Th9 function contributes primarily to inflammatory disease is also not clear. There are a number of questions remaining including how Th9 cells cooperate with other T-helper cell lineages in cellular recruitment to sites of inflammation. The established functions of Th9 cells have been divided into type II immunity, associated with Th2 cells, and autoimmunity, associated with Th1 and Th17 cells. It has not been established if there are any immune responses that are strictly dependent on Th9 cells, or if IL-9-secreting T cells function *in vivo* as a helper cell of T-helper cells. As additional models and reagents are established to study their function, these questions will undoubtedly be answered with more questions about the intriguing biology of Th9 cells.

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Fig. 1. Regulation of *Il9* in Th9 cells

Schematic of the *Il9* gene indicating the intron–exon structure and the transcriptional start site from the anti-sense strand, with numbering on mouse chromosome 13 according to GRCm38. Conserved non-coding sequences (CNS) are indicated by red bars, CNS0 is –6.3 kb from the transcriptional start site (TSS), CNS1 is the *Il9* promoter, and CNS2 is +5kb from the TSS (1, 35). The lower schematic indicates the 300 bp, numbered from the TSS, in CNS1. In our previous work, we performed bioinformatics analysis of these sequences; in contrast, this schematic focuses on experimentally determined binding sites for transcription factors that regulate *Il9* expression. NF-κB and NFAT sites (–46; –313) were identified by Jash *et al.* and Xiao *et al.* (33, 36). PU.1-binding sites (–194; –309) were determined by Chang *et al.* (1). Smad and RBP-Jκ sites (–4065 and –4270, respectively) were defined by Elyaman *et al.* (14). IRF4 binding and regulation was determined by Staudt *et al.*, and potential binding sites (–100; –240, sense strand orientation) were defined by Perumal and Kaplan (3, 35).

Fig. 2. Functions of Th9 cells

Although the functions of Th9 cells have not been completely documented, they have been shown to have roles in several types of inflammatory disease. The diagram indicates the ability of Th9 cells to regulate allergic inflammation, autoimmune inflammation, and anti-tumor immunity, and documents some of the features of Th9-mediated function in each inflammatory scenario.