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The Development and in vivo function of T_H9 cells

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Abstract

The specialized cytokine secretion profiles of T helper (T_H) cells are the basis for a focused and efficient immune response. On the 20th anniversary of the first descriptions of cytokine signals that act to differentiate interleukin-9 (IL-9)-secreting T cells, this review focuses on the extracellular signals and transcription factors that promote the development of what we now term T_H9 cells, which are characterized by the production of this cytokine. We summarize our current understanding of the contribution of T_H9 cells to both effective immunity and immunopathological disease and propose that T_H9 cells could be targeted for the treatment of allergic and autoimmune disease.

T helper (T_H) cell subsets differentiate from $CD4^+$ T cells that have been exposed to a specialized cytokine environment (Box 1, Figure 1). Among the T_H cell subsets that have been characterized, the development and function of T_H9 cells, which are characterized by their secretion of interleukin-9 (IL-9), are only beginning to be understood. Schmitt et al first described IL-9 production by activated murine T cells, and subsequently defined the cytokines that promote the differentiation of IL-9-producing T_H cells in culture [1, 2]. IL-9-producing T cells were first thought to be associated with T_H2-type responses *in vivo* and because initial surveys of IL-9 function suggested that this cytokine had restricted effects during immune responses, it was not as extensively studied as many other cytokines that are associated with T_H cells [3-5]. Moreover, the lack of an understanding of how to derive highly polarized T_H cells producing IL-9 hampered further investigation of the molecular control of *Il9* gene expression.

The initial description of polarized IL-9-secreting T_H cells was made from experiments in which T cells were cultured in the presence of IL-2, IL-4 and transforming growth factor β (TGFβ) [1]. Although IL-4 stimulation of T cells alone was not sufficient for IL-9 production, it primed cells to produce IL-9 when they were stimulated with additional cytokines. Veldhoen et al. [6] revisited this paradigm by showing that fully differentiated IL-4-producing T_H2 cells that are cultured in the presence of TGFβ subsequently produce IL-9. Dardalhon et al. [7] observed that the same combination of cytokines can prime IL-9 production by T cells and they demonstrated that IL-4 signalling promoted T_H9 cell differentiation, in part, by suppressing the ability of TGFβ to induce the expression of the T regulatory (T_{Reg}) cell-

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associated transcription factor forkhead box P3 (FOXP3). Thus, the balance of signals from these stimuli determines the extent of T_H9 cell generation.

In this Review, we integrate our current understanding of the factors that control the development and function of T_H9 cells with the role of T_H9 cells in immune responses and disease. We focus on some of the more recent functions described for T_H9 cells in atopic disease, inflammatory bowel disease and in promoting effective anti-tumor immunity.

Regulation of T_H9 cell development

Downstream of T cell receptor (TCR) signalling, co-stimulatory molecule signalling (Table 1, Figure 2), and cytokine signals (Table 2) that promote T_H9 cell differentiation, are a network of transcription factors that regulate the expression of T_H9 cell signature genes, including IL-9 (Figure 2). In contrast to co-stimulatory surface proteins and cytokines that induce the transient activation of transcription factors such as NF-κB and NFAT to acutely stimulate IL-9 production, cytokines including IL-4 and TGFβ regulate multiple genes in T_H9 cells leading to the generation of a *Il9* locus poised to be activated in response to subsequent stimulation (Box 2). This section of the review will focus on how cytokine-induced signals regulate T_H9 cell generation, including through the activation of signal transducer and activator of transcription (STAT) proteins, which is a first step in T_H cell subset differentiation (Box 1), and through TGFβ-induced activation of SMAD proteins, which can alter the outcome of STAT signalling in T_H9 cell differentiation.

STAT6 in Th9 cell development

STAT6 is the major signalling component of the IL-4 receptor, and is required for the *in vitro* generation of T_H9 cells [6-8]. Although STAT6 might bind the *Il9* locus directly [8, 9], it is clear that STAT6 also has a much more profound role in governing the T_H9 cell phenotype. STAT6 suppresses TGFβ-induced FOXP3 expression, and the expression of the T_H1 cell-associated transcription factor T-bet, both of which repress IL-9 production [6-8]. IL-4 and STAT6 also play a critical role in the induction of the T_H9 cell developmental programme. Recent work defined a T_H9-specific gene signature that distinguishes T_H9 cells from their closely related T_H2 and in vitro-derived Treg (iTreg) cell counterparts. Interestingly, the majority of the genes that were enriched in T_H9 cells were STAT6-dependent [10], including the genes encoding the transcription factors interferon regulatory factor 4 (IRF4) and B cell activating transcription factor-like (BATF).

IRF4 expression is critical for T_H9 cell differentiation, in addition to a previously documented requirement in the differentiation of T_H2 and T_H17 cells [11-13]. IL-9-secreting T cells were decreased in T_H9 cell cultures derived from naive IRF4-deficient T cells, or cells treated with *Irf4*-specific siRNA [13]. Moreover, ectopic expression of IRF4 increases IL-9 production in T_H9 cell cultures [10]. IRF4 bound directly to the *Il9* promoter and increased *Il9* transcription in a reporter assay, suggesting that a major role of IRF4 in T_H9 cells is to directly induce transcription of the *Il9* gene [10, 13]. Despite its requirement for *Il9* expression, IRF4 only regulates a subset of other T_H9 cell-associated genes [10]. Interestingly, deletion of STAT6 or IRF4 from T cells results in increased expression of T-bet and IFNγ, which suppress T_H9 cell development [10, 13]. Therefore, it is likely that IRF4 supports T_H9 cell

development by directly inducing *Il9* transcription as well as by blocking the expression of Th1 cell-associated transcription factors that may inhibit T_H9 differentiation.

In addition to IRF4, BATF is required for T_H9 cell development [10] and this parallels a similar requirement seen in the generation of T_H17 cells [14], where BATF and IRF4 operate as a transcriptional module [15-17]. Naïve CD4+ T cells isolated from mice lacking BATF exhibited a marked impairment in T_H9 cell differentiation, and ectopic expression of BATF in naïve CD4+ T cells or in T cells from BATF-transgenic animals exhibited an enhanced propensity to produce IL-9 when cultured under T_H9 cell-inducing conditions [10]. Similar to IRF4, BATF bound the *Il9* promoter and activated *Il9* transcription in reporter assays [10]. However, in contrast to IRF4, which regulates a relatively small number of T_H9 cell-associated genes, BATF-deficiency resulted in a profound defect in the expression of nearly all of the T_H9 cell-associated genes that were examined, which mirrored observations in STAT6-deficient T cells [10]. The difference in impact of BATF and IRF4 in the overall scope of altering the T_H9 cell-specific gene signature highlights that although BATF and IRF4 can function as a transcriptional module [15-17], it is likely that IRF4 and BATF also provide unique contributions to establishing the T_H9 cell phenotype.

Although GATA3 expression is lower in T_H9 cells than in T_H2 cells [6, 8], it is an important STAT6 target gene involved in T_H9 cell differentiation. Dardalhon et al [7] demonstrated that GATA3-null cells failed to produce IL-9 when cultured under T_H9 skewing conditions concomitant with increased FOXP3 expression linked to a possible physical interaction with GATA3. However, the function of GATA3 in T_H9 cell differentiation remains unclear as some studies detected little GATA3 mRNA in T_H9 cells, compared with in T_H2 cells, and ectopic expression of GATA3 in T_H9 cells increased IL-9 production in some studies but not in others [6, 8, 18]. The differing results in these studies may reflect the amount of GATA3 within the cell. Despite GATA3 expression in all T cells, induced expression of GATA3 might repress FOXP3 induction without activating T_H2 genes. In higher amounts, GATA3 might activate T_H2 gene expression and repress the ability of PU.1 (a T_H9-inducing factor discussed below) to activate IL-9 [19-21]. Thus a gradient of GATA3 expression might be instrumental in establishing the identity of multiple T helper subsets.

STAT5 in T_H9 cell development

STAT5 impacts the development of T_H9 cells downstream of several extracellular factors. IL-2 activates STAT5 in T cells and promotes the development of T_H9 cells. IL-2-deficient $CD4^+$ T cells exhibit a complete loss of IL-9-secreting cells *in vitro* [1], a finding that was revisited 20 years later [22]. In both reports, the addition of exogenous IL-2 rescued IL-9 production from IL-2-deficient T cells that were cultured in the presence of TGFβ alone or with both TGFβ and IL-4 [1, 22]. The role of STAT5 in IL-9 production was directly demonstrated by studies showing that the deletion of STAT5 in cultured T_H9 cells resulted in a dramatic decrease in IL-9 production [1, 22]. These findings were corroborated by the use of STAT5 inhibitors and *Stat5*-targetting siRNAs to block IL-9 production by naïve $CD4^+$ T cells cultured under T_H9 cell-skewing conditions [23]. These studies demonstrated significant STAT5 binding within the *Il9* promoter using chromatin immunoprecipitation, and further showed that a constitutively active STAT5 could directly activate the *Il9* promoter in

reporter assays. In addition to direct effects on *Il9*, STAT5 likely impacts other components of T_H9 cell differentiation. Blockade of IL-2 or inhibition of STAT5 resulted in a decrease in IRF4 and PU.1 expression, both of which are transcription factors that are required for T_H9 cell differentiation [10, 13, 20]. Further the IL-2—STAT5 signalling pathway is intimately linked with cellular proliferation and metabolism, processes that also impact T_H cell differentiation, and approaches to distinguish direct and indirect effects of this pathway will be complex.

Although IL-2 is likely a primary activator of STAT5 in T cells, thymic stromal lymphopoietin (TSLP) also promotes IL-9 production by cells cultured under T_H9 cell-inducing, but not T_H2 cell-inducing conditions [24]. T_H9 cells express the TSLP receptor, which is further upregulated after TSLP stimulation [24]. TSLP also augmented levels of phosphorylated STAT5 throughout the course of T_H9 cell differentiation and resulted in increased STAT5 binding to the *Il9* locus [24]. Importantly, inhibition of STAT5 via siRNA abrogated the capacity of TSLP to augment IL-9 production by T_H9 cells [24]. Interestingly, TSLP was still capable of augmenting IL-9 production when IL-2 was blocked by monoclonal antibodies in these cultures. These data suggest that IL-2 and TSLP may work together to promote IL-9 production in T cells, particularly *in vivo*, and that TSLP might substitute for IL-2 under some conditions.

Additional factors also induce IL-9 production by augmenting IL-2 production. The TNF superfamily member TL1A enhanced IL-9 production independently of STAT6 and PU.1, factors important for in vitro and in vivo T_H9 generation [25]. Ligation of the TL1A receptor, DR3, resulted in enhanced IL-2 production and STAT5 activation that was required for the induction of IL-9 [25]. Nitric oxide also functions by increasing endogenous IL-2 production and STAT5 activation [26]. Thus, in some environments TL1A or nitric oxide might enhance IL-9 production by activating the IL-2—STAT5 pathway.

STAT5 also has broader effects in T_H9 cell programming. Studies found that blockade of IL-2 or inhibition of STAT5 resulted in an increase in the mRNA and protein expression of the transcriptional repressor BCL6. BCL6 was originally identified as a proto-oncogene in B cell lymphomas and was later identified as a primary transcriptional regulator in $CD4^+$ T follicular helper (T_{FH}) cells [27, 28]. T_H9 cells exhibited the lowest levels of BCL6 expression as compared with other *in vitro*-generated T_H cells, and BCL6 expression inversely correlates with *Il9* and *Il2* mRNA levels in T_H9 cells [22, 23]. Moreover, retroviral-mediated expression of BCL6 in T_H9 cells inhibited both T_H9 cell differentiation and IL-2-induced *Il9* transcription, although only one report demonstrated increased IL-9 production from T cells that had diminished BCL6 expression [22, 23]. However, both studies demonstrated that BCL6 bound the *Il9* promoter at overlapping or adjacent regions to STAT5, and repressed the ability of STAT5 to promote *Il9* transcription when co-transfected in reporter assays [22, 23]. These data suggest a molecular circuit whereby early IL-2 production activates STAT5 binding to the *Il9* promoter that outcompetes BCL6 binding to the promoter, while at the same time repressing BCL6 expression. As IL-2 exposure and STAT5 activation wane, the STAT5-mediated suppression of BCL6 diminishes and allows greater access of the *Il9* promoter to BCL6 that subsequently limits IL-9 production.

STAT1 in T_H9 cell development

STAT1 is activated in T cells by a number of cytokines, including IFNγ, type I IFNs, IL-6, IL-10, IL-21, and IL-27 that have varying influences on T_H9 cell development. Activation of STAT1 by treatment of naïve mouse T cells cultured under T_H9 cell-inducing conditions with IFNγ dramatically suppressed IL-9 production by reducing the sensitivity of these cells to IL-4 [1]. In an asthma model, deficiency in Tyk2, the Janus kinase required for type I IFN signalling and STAT1 activation, resulted in increased IL-9 production [29]. In an experimental autoimmune encephalomyelitis (EAE) model system, IFNγ induced IL-27 production by dendritic cells (DCs) that suppressed T_H9 cell differentiation both *in vivo* and *in vitro* in a STAT1 and T-bet-dependent manner [30]. Although treatment of T_H9 cells with IL-27 induced the production of IFNγ and IL-10, which are cytokines that suppress T_H9 cell differentiation, the inhibition of IL-9 production by IL-27 was independent of these cytokines [30]. Further work must be done to understand how STAT1 acts downstream of IFNγ and IL-27 to suppress IL-9 production in T_H9 cells.

In contrast to the inhibitory effect of STAT1 on T_H9 cell differentiation following IFNγ or IL-27 exposure, other reports suggest that a distinct subset of STAT1-activating cytokines may promote IL-9 production in T cells. Naïve human CD4+ T cells that were cultured under T_H9 cell-inducing conditions with the STAT1-activating cytokines IFNα, IFNβ, IL-6 or IL-21 showed a marked increase in their capacity to produce IL-9 [31]. Similarly, the treatment of mouse T_H9 cell cultures with IL-1β resulted in the indirect activation of STAT1, which induced expression of IRF1 [32]; IRF1 subsequently bound the *Il9* promoter and, in combination with IRF4 and PU.1, directly enhanced *Il9* transcription in a reporter assay. It has also been shown that IL-21 can have either positive or negative effects on IL-9 production depending on the culture conditions [22, 31, 32]. Thus, the effects of STAT1-activating cytokines on T_H9 cell development are complex and are shaped by additional cytokine signals present in the environment.

TGFβ and SMAD signaling in T_H9 cell development

Studies have shown that TGFβ can ‘re-programme’ Th2 cells into IL-9-secreting T_H9 cells [6, 7]. Both SMAD-dependent and SMAD-independent pathways are required for T_{Reg} cell and T_H17 cell differentiation [33-35]. Wang et al. [36] and Tamiya et al. [18] demonstrated that SMAD2, SMAD3 and SMAD4 are required for optimal T_H9 cell differentiation. SMAD2 and SMAD4 deficiencies in T cells resulted in increased expression of genes encoding Th2 cell-associated cytokines and a loss of IL-9 expression [36]. SMAD2 and SMAD3 bind multiple conserved non-coding regions within the *Il9* promoter [18], and SMAD2, SMAD3 and SMAD4 were also demonstrated to alter the chromatin structure of the *Il9* locus. However, these reports described distinct mechanisms for SMAD activity. Wang et al. [36] observed an increase in repressive histone marks in the *Il9* promoter in the absence of either SMAD2 or SMAD4, due to enhanced polycomb protein (EZH2) recruitment to the *Il9* promoter. In contrast, Tamiya et al. [18] showed a loss of permissive histone marks in the absence of SMAD2 and SMAD3 and no change in repressive marks. Interestingly, the SMAD pathway might also provide a link to other T_H9 cell-inducing pathways. Notch proteins were required for the binding of SMAD3 to the *Il9* gene [37]. Moreover, SMAD2 or SMAD3 can form a physical complex with IRF4, and IRF4 and SMADs were reciprocally

required for optimal binding to the *Il9* promoter. IRF4 was also required for SMAD-mediated *Il9* transcription in a reporter assay [18]. These data provide the beginning of a model to explain how the IL-4—STAT6-induced activation of IRF4 and the TGFβ-driven activation of SMADs work synergistically to transactivate the *Il9* gene.

PU.1 is an ETS family transcription factor that is also induced by TGFβ, in conjunction with antigen receptor stimulation, and it is critical for T_H9 cell differentiation [8, 19-21, 38]. PU.1-deficient $CD4^+$ T cells have a diminished capacity to produce IL-9 and an increased propensity to produce T_H2 cell-related cytokines, whereas ectopic PU.1 expression in either T_H2 or T_H9 cells increases IL-9 production [20]. Therefore, to date, PU.1 is the only transcription factor that induces IL-9 production in other T helper cell subsets. Similarly to the other factors described above, PU.1 binds the *Il9* promoter and likely acts to enhance gene transcription by recruiting histone acetyl transferases (HATs) and enhancing permissive chromatin structure [20, 38]. Interestingly, PU.1 is one of the few genes that is not induced by IL-4–STAT6 signalling in T_H9 cells, but is instead induced downstream of TGFβ signalling [8, 10]. Reports that compared SMAD-deficient and wild-type T cells that were cultured under T_H9 cell-inducing conditions failed to observe differential expression of the PU.1-encoding *Sfpi1* gene, suggesting that PU.1 may be regulated by SMAD-independent TGFβ signalling mechanisms.

In summary, the development of T_H9 cells involves the coordinated function of key transcription factors downstream of IL-4-STAT6 and IL-2-STAT5 signalling including IRF4 and BATF. There is a requirement as well for TGFβ-induced SMAD proteins and the SMAD-independent induction of PU.1 in the generation of IL-9-secreting T cells. These pathways converge in the ability of IRF4 to interact with BATF and SMAD proteins. How these factors contribute to the stability of the T_H9 phenotype is still unclear (Box 2). The outcome of the activity of these transcription factors in T_H cell function is discussed in the following sections focusing on the role of T_H9 cells in inflammatory disease and immunity.

T_H9 cells in immunity and disease

As IL-9 is a pleiotropic cytokine (Box 3), T_H9 cells might contribute to both protective immunity and immunopathological disease through a myriad of pathways. However, it is important to remember that T_H9 cells are not the only source of IL-9 during an immune response and, accordingly, the relative importance of T_H9 cells *in vivo* has been hard to define. The remainder of this review will emphasize our current understanding of T_H9 cell immune activity in specific diseases using human patient data. We also describe the complementary experimental research in mouse models of disease that has supported a role for T_H9 cell-mediated inflammation in human diseases (Figure 3).

T_H9 cells in atopic disease

Atopic diseases, including atopic dermatitis and asthma, are associated with T_H2-type cytokine responses and IgE-mediated immediate hypersensitivity. More recently, reports have identified T_H9 cells as major contributors to human atopic disease. Genes related to T_H9 cell development and function (for example, *IL4RA*, *STAT6*, *IL9*, *IL9R*, *SMAD3*, *IL33*, *IL1RL1*) have been linked to the development of asthma and food allergies in humans [39-45].

Allergic patients have elevated numbers of circulating T cells that are capable of producing IL-9 in response to pollen, cat dander, peanuts and house dust mite (HDM) extract [39, 46-50], and DCs isolated from these patients were more likely to stimulate IL-9 production from T cells[51]. Furthermore, IL-9-producing T cells from atopic patients expressed the T_H9 cell-related proteins IL-17RB, IRF4, and PU.1 but did not express the T_H2-type cytokine IL-5 (Table 2) [51, 52]. The numbers of T_H9 cells and the serum levels of IL-9 in atopic individuals directly correlate with allergen-specific IgE titers [24, 46, 47, 49]. Additionally, T_H9 cell numbers and T cell IL-9 production are significantly elevated, with respect to control patients, in atopic children [20, 49] and sufferers of atopic dermatitis and psoriasis [48].

In mouse models of asthma (in which HDM, *Aspergillus* and ovalbumin (OVA) are used as sensitizers), T_H9 cells are detectable in the respiratory tract and draining lymph nodes, particularly during early stages of the disease [47, 53, 54]. In the OVA sensitization model, T_H9 cells are a principal *in vivo* source of IL-9 during allergic airway disease as determined by an IL-9 fate reporter mouse [55]. The presence of T_H9 cells and IL-9 in these models is elevated when the pro-allergic cytokines IL-25 and TSLP (Table 2) are ectopically or exogenously expressed, and IL-9 is inhibited by the activity of cyclooxygenase-2, which may decrease IL-25R expression [24, 56, 57]. Furthermore, inhibiting T_H9 cell differentiation *in vivo* by neutralizing TGFβ or Activin A**,** a TGFβ-family member that also induces T_H9 differentiation, impairs development of allergic disease [47]. Moreover, inflammation is attenuated in mice with conditional deficiencies in PU.1 and IRF4, or in mice with germ line deficiencies in BATF [10, 13, 18, 20, 24, 47, 58, 59]. Importantly, PU.1-deficient mice, which have more specific defects in T_H9 cell development, develop normal T_H2 cell responses but still do not develop severe inflammation in an OVA sensitization model [20].

Mouse atopic disease models indicate that T_H9 cells mediate disease through the production of IL-9. The adoptive transfer of T_H9 cells promotes mast cell and eosinophil accumulation, mucus production, T_H2-type cytokine production and bronchial hyperresponsiveness [10, 13, 24, 26, 60]; however, these effects are negated upon transfer of *Il9*$^{-/-}$ T_H9 cells or following IL-9 neutralization [10, 13, 24, 26]. Furthermore, adoptive transfer of T_H9 cells promotes significantly more mast cell accumulation compared to Th2 cells, a function that is dependent on IL-9 but not IL-13 [59]. In an HDM-induced asthma model requiring an endogenous T cell response, IL-9 neutralization or PU.1-deficiency within the T cell compartment significantly reduced mucus hyperplasia, mast cell accumulation, lung remodelling, and airway hyperreactivity [59, 61]. IL-9 stimulates mast cell proliferation and activity *in vivo* [62-64] and can promote cytokine production by type 2 innate lymphoid cells (ILC2s)[55, 65]. Furthermore, transgenic expression of IL-9 is sufficient in itself to cause bronchial hyperresponsiveness via its effects on the respiratory epithelium and the enhancement of T_H2-type cytokine release [66-68]. Collectively, these models indicate that although other cells can produce IL-9, T_H9 cells are critical in a diverse set of pulmonary atopic disease models, serve a distinct role in atopic disease development from that of T_H2 cell immune responses, and in transfer models are sufficient to generate allergic inflammation.

T_H9 cells in inflammatory bowel disease

Crohn's disease and ulcerative colitis are the principal forms of inflammatory bowel disease (IBD), which is defined as a chronic relapsing inflammation of the gastrointestinal tract that is independent of specific pathogen infection. Patients with Crohn's disease exhibit a pronounced T_H1-type immune response (associated with IFNγ and TNF production), whereas patients with ulcerative colitis have elevated levels of T_H2 cell-derived cytokines. In both diseases, patients have been shown to have increased numbers of T_H17 cells and diminished T_{Reg} cell numbers [69].

Recently, T_H9 cells were also found to have a critical role in the pathogenesis of IBD. Patients with Crohn's disease or ulcerative colitis that show ongoing inflammation have elevated numbers of $CD4^+PU.1^+$ and $CD4^+IRF4^+$ T cells within the gastrointestinal tract compared to control patients or patients in remission [70-72]. Interestingly, IL-9 production by these cells was only observed in patients with ulcerative colitis, and there was an association of *IL-9* expression and IL-9^+ T cells with the severity of pathology [70, 72].

In mouse models of IBD, the co-transfer of $CD4^+CD45RB^{hi}$ T cells and *in vitro*-derived T_H9 cells into recombination-activating gene (*Rag)*-deficient hosts resulted in increased development of colitis compared to control animals receiving $CD4^+CD45RB^{hi}$ T cells alone [7], in a mechanism dependent on IL-9 [70]. In an oxazolone-induced colitis model [73, 74], IRF4-deficient and IL-9-deficient mice had significantly reduced colitis scores [70, 75]. The impact of IRF4 deficiency on colitis is at least partially due to reduced T_H17 cell responses [71].However, the fact that attenuated colitis is seen in mice that lack PU.1 expression in T cells indicates that T_H9 cells are also important in the development of colitis [70]. In these experiments, the ability of T_H9 cells to mediate colitis was IL-9-dependent as IL-9-deficient T cells and IL-9 neutralization attenuated colitis [70]. IL-9R expression is also elevated in gastrointestinal epithelial cells in patients with ulcerative colitis [70, 72]. Treatment of Caco-2 cells, a human epithelial cell line, with IL-9 was shown to inhibit cellular proliferation [72]. Gerlach *et al* not only substantiated the negative impact of IL-9 on epithelial cell proliferation but also noted that topical administration of recombinant IL-9 attenuated epithelial cell tissue repair mechanisms in vivo. Furthermore, IL-9 increased intestinal permeability, which may be a result of IL-9 altering tight junction protein composition in the gastrointestinal epithelial layer [70]. Collectively, these results indicate Th9 cells and IL-9 directly alter epithelial cell biology within the gastrointestinal tract, potentially contributing to the pathology of IBD. The relative impact of T_H9 cells on colitis via epithelia versus the immune system is not defined, although some effects of IL-9 on epithelial cells could be indirect, as noted by mast cell-dependent effects in a model of antigen-induced anaphylaxis [76].

It is interesting to note that in a T_H1 cell-mediated colitis model that shares some of the hallmarks of Crohn's disease [74], IL-9 attenuates disease development[77]. The role of T_H9 cells in this disease model remains to be explored although the investigators did demonstrate that NKT cells were a significant source of IL-9. Although NKT cell-derived IL-9 production appears to be limited in human UC disease [72], it may be that the role of IL-9, and

T_H9 cells themselves, can be protective or inflammatory in the gastrointestinal tract depending on the inflammatory milieu (Crohn's disease versus ulcerative colitis).

T_H9 cells in EAE

EAE is an experimental animal model of multiple sclerosis that is characterized by T cell-dependent demyelination. EAE is associated with T_H1 and T_H17 cell responses, and T_H17 cells have been identified in the central nervous system (CNS) of patients with multiple sclerosis [78]. T_H9 cells are present in the draining lymph nodes and CNS of mice administered myelin proteolipid protein $(PLP)_{180\text{-}199}$ peptide to induce EAE [53], and the adoptive transfer of myelin oligodendrocyte glycoprotein (MOG)-specific T cells cultured under T_H9 cell-inducing conditions into RAG-deficient mice promoted EAE disease [23, 30, 79]. Although T_H9 cell-mediated EAE is not as severe as the EAE disease induced by T_H17 cells [79], adoptive transfer of T_H9 cells can trigger peripheral neuropathy that is not as apparent in recipients of T_H1 or T_H17 cells [7, 79].

T_H9 cell-mediated EAE is dependent on IL-9 [30], which has a well-documented role in EAE pathogenesis in its ability to enhance T_H1 and T_H17 cell immune responses during disease progression [37, 80, 81]. In contrast, additional studies have suggested a protective effect of IL-9 when activity is blocked prior to the induction of EAE, possibly through affects on enhancing T_{Reg} activity [37, 82]. Further complicating the interpretation of these studies, subsequent to adoptive transfer in an EAE model, many T_H9 cells acquire the capacity to produce IFNγ and IL-17 [23, 79], and the acquisition of IFNγ production, in part, promotes EAE severity [23]. This parallels observations from a model of autoimmune uveitis [83]. Furthermore, it is unclear in non-adoptive T cell transfer models whether the cellular source of IL-9 is T_H9 cells or another cell type such as T_H17 cells [81]. Further use of non-adoptive transfer models will be required to validate the role of T_H9 cells in EAE and ultimately define their role in multiple sclerosis.

Th9 cells in helminth infections

Parasitic helminth infections affect over one billion people worldwide, with a documented contribution of T_H cells in exacerbating or limiting pathology [84, 85]. Circulating antigen-specific T_H9 cells have been detected in individuals with lymphatic filariasis, a chronic helminth infection [86]. In a *Trichuris muris* murine infection model, mice with a dominant negative TGFβRII receptor in CD4+ T cells have reduced IL-9 levels, decreased mast cell numbers, and a significantly increased worm burden, indicating that T_H9 cells may be important in immunity to helminths[6]. T_H9 cells, but not T_H2 cells, were able to reduce *Nippostrongylus brasiliensis* worm burdens upon adoptive transfer into *Rag*-deficient hosts [62]. In this model, T_H9 cell transfer enhanced numbers of infiltrating eosinophils, basophils, and mast cells. Furthermore, T_H9 cells increased ILC2 numbers and activity [62], which may be due to the impact of IL-9 in promoting ILC2 survival *in vivo* [55, 65].

In the current paradigm, T_H9 cells mediate anti-helminth immunity though the local or systemic production of IL-9. During *T. muris* and *N. brasiliensis* infections, IL-9 is required for worm expulsion [62, 63, 87], and transgenic expression of the cytokine enhances resistance to *T. muris infections* [88]. During *Trichinella spiralis* infection, IL-9 facilitates, although is

not essential, for overall worm expulsion. In this model, IL-9 modulates intestinal muscle contraction, epithelial cell mucus production, and mast cell activity, which, in particular, is required for IL-9 mediated worm expulsion [87, 89, 90].

The relative contributions of T_H9 cells and ILC2s to systemic IL-9 production in helminth infections are still not clear. In pulmonary *N. brasiliensis* infection where an IL-9 fate reporter was used, IL-9 is derived from ILC2s that were previously reported as an IL-9 source in a T cell-independent asthma model [55, 65]. In contrast, Licona-Limon *et al.*, using a transcriptional reporter mouse, noted that $CD4^+$ T cells were the principal producers of IL-9 within the gastrointestinal tract in a similar *N. brasiliensis* infection model [62]. Furthermore, transfer of T_H9 cells into *Il9*$^{-/-}$ mice rescued any defect associated with worm expulsion. Thus, T_H9 cells are likely a significant source of protective IL-9 in certain helminth infections. However, IL-9 may not be essential for protection in all helminth infections and may even be detrimental for host health as parasite-specific T_H9 cell numbers positively correlated with disease severity in individuals suffering with lymphatic filariasis [86].

Th9 cells in tumour immunity

An intriguing function of T_H9 cells is their highly potent antitumour activity, particularly in melanoma. T_H9 cells in the blood and skin are significantly reduced in patients with melanoma [91], and an *IL9* SNP is associated with an increased risk of cutaneous malignant melanoma [92]. In the B16-F10 melanoma models, several labs have demonstrated that adoptive transfer of T_H9 cells dramatically reduced tumour masses and disease severity compared to controls [32, 91, 93]. Through the administration of neutralizing antibody or recombinant protein, researchers have demonstrated that IL-9 has potent antitumour effects in the melanoma murine model. Furthermore, T_H9 cells and IL-9 are protective in a lung adenocarcinoma model [32, 91], indicating T_H9 cells may have anti-tumour properties within a number of tissues.

Although there are conflicting reports indicating that T_H9 cells can directly induce tumour cell death [91, 93], the capacity of T_H9 cells to limit tumour growth appears to be largely dependent on the production of cytokines including IL-9, IL-3 and IL-21 to enhance developing and existing tumor immunity. IL-9 can induce expression of CC-chemokine ligand 20 (CCL20) within the tumour site and CCR6 expression on leukocytes to facilitate tumour infiltration and rejection [93]. Furthermore, IL-9 antitumour activity is mast cell dependent [91]. T_H9 cells are also potent producers of IL-3 upon TCR stimulation [10]. T_H9 cell-derived IL-3 has been shown to promote DC survival, which could potentially enhance antitumour immunity [94]. Végran *et al.* recently showed that IL-21 derived from T_H9 cells promotes tumour inhibition even in the absence of IL-9 production [32]. In the *in vitro* setting, T_H9 cells can produce amounts of IL-21 comparable to T_H17 cells upon TCR stimulation [10, 95], and Végran *et al.* [32] showed that IL-1β enhances T_H9 cell production of IL-21 to inhibit tumour growth, in part, by enhancing $CD8^+$ T cell responses and IFNγ production. Because IL-1β may also augment IL-9 production [31, 32, 60, 96, 97], the antitumour microenvironment might further amplify T_H9 cell immune responses and thus antitumour immunity.

The protective effects of T_H9 cells in tumour immunity might be restricted to solid tumours, such as melanoma and lung adenocarcinoma. In contrast, IL-9 has been linked with the promotion of certain cancers, particularly lymphomas. *IL9* gene expression is strongly expressed in a subset of patients with anaplastic lymphoma and Hodgkin's disease [98]. Transgenic IL-9 expression promotes the development of spontaneous lymphomas in mice, and in a T-lymphoblastic lymphoma mouse model, IL-9 can enhance tumour development [99]. *In vitro* studies indicate IL-9 promotes tumour growth by both enhancing proliferation and inhibiting apoptosis of tumours [100, 101]. It is unclear whether T_H9 cells are a relevant source of IL-9 during lymphoma development, but it may indicate that T_H9 cells can be both pro- and anti-tumorigenic depending on the cancer type. The basis for IL-9, and potentially T_H9 cells, to have discriminatory effects on different cancers is unexplored but could be linked to the differential expression of IL-9R on the cancer cell type.

Concluding remarks

Signals from TGFβ and IL-4 promote T_H9 cell differentiation, but neither cytokine is sufficient by itself to generate the T_H9 cell transcriptional profile or to induce high relative amounts of IL-9 expression in T cells [10]. Among the transcription factors that respond to these cytokine signals, none can be regarded as 'master regulators' in the same way that T-bet, GATA3 or FOXP3 can be associated with their respective T_H cell lineages. However, it is still possible that there is an unidentified T_H9 cell lineage-defining transcription factor. Another possibility is that these cytokine signals work cooperatively in promoting *Il9* gene expression. The TGFβ-induced SMADs and PU.1 remodel chromatin and might function to allow the IL-4-induced transcription factors BATF and IRF4 to transactivate gene expression. As noted in the description above, there is cooperation at the level of inducing transcription and factors activated by both signals are likely critical in recruiting RNA polymerase to target loci to cooperate in generating the phenotype and function of the T_H9 cell subset.

Our understanding of the biology of T_H9 cells is rapidly developing in multiple human diseases and mouse disease models. It is becoming clear that as is the case with many other T_H cell subsets, T_H9 cells can perform both beneficial and detrimental functions in the body. Where they mediate these effects is not entirely clear, but some emerging paradigms suggest that T_H9 cells are found in the skin and express receptors that would facilitate their recruitment to mucosal surfaces [10, 48, 53, 91]. Among the beneficial effects T_H9 cells mediate is their ability to initiate immunity to helminth parasites and effective antitumour immunity. In contrast, T_H9 cells promote allergic inflammation and mediate some types of autoimmunity. These latter functions raise the questions of where T_H9 or IL-9-targeted therapies might be useful. MEDI-528, a humanized IL-9 neutralizing antibody, has an acceptable safety profile and exhibits some promise in minimizing exacerbations in adult patients with mild to moderate asthma[102, 103] although no positive effects were noted in severe adult asthmatics [104]. The discrepancy in the effects of treatment might be in the subtypes of asthma studied; it is possible that the mild-moderate group had disease that might be more dependent on the function of IL-9 such as a requirement for mast cells in disease exacerbations. Furthermore, targeting IL-9 in other allergic diseases has not been reported. Because increases of IL-9 are seen early in life in an atopic population of infants

and increases are associated with early changes in airway responsiveness [24, 105], an acceptable safety profile might provide a basis for early intervention in high-risk infants to limit the development of atopic diseases. The recent descriptions of T_H9 cells in IBD suggest that targeting IL-9 might be a potential treatment in this disease. Thus, even in our infancy of understanding their development and function, T_H9 cells are revealed to be important mediators in multiple diseases and the cells, and the cytokines they produce, remain intriguing targets for therapy.

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Box 1

STAT proteins and T helper cell subsets

The differentiation of $CD4^+$ T cells into T helper cells is initiated when naive $CD4^+$ T cells are stimulated by antigen in the context of MHC class II molecules and acquire the ability to respond to a cytokine environment that promotes the development of specialized effector cell phenotypes. The specialized or polarized effector cell, termed a T helper cell subset, is identified by the production of signature cytokines and expresses a characteristic transcriptional signature. Polarized T helper subsets develop largely in response to a specific cytokine environment, although activation signal strength and non-cytokine signals also impact differentiation. The cytokine environment activates signal transducer and activator of transcription (STAT) family members that induce the expression of lineage-specific genes, modify local chromatin architecture, and establish a genome-wide enhancer profile [106-111]. Among the genes activated by STAT proteins are cytokines that confer specific functions to the T helper cell subset, chemokine receptors and adhesion molecules that allow the T cell to migrate into sites of inflammation, and additional transcription factors that function to further establish and maintain the characteristic pattern of gene expression. STAT proteins, although transiently activated, are gateways to the process of T helper cell differentiation and are the first step on the road to commitment to a specific T helper cell phenotype.

Box 2

Distinguishing acute expression of a cytokine and commitment to a Th subset

T helper subsets are often identified solely by the expression of the hallmark cytokine, typically assessed following stimulation of the cell with T cell receptor ligation or surrogate pharmacological activators such as PMA and ionomycin. This stimulation results in acute activation of a particular cytokine locus (or loci) that is programmed or poised to be expressed upon stimulation. Among the factors activated by these pathways are NFAT and NF-κB family members that, in the case of T_H9 cells, can bind directly to the *Il9* locus (see Figure 2 and Tables 1 and 2). In addition to T cell receptor ligation, several other ligands can induce IL-9 production through mechanisms that have been proven or are likely to require NF-κB family members including OX40 and other TNF superfamily members, IL-1 and related family members, and IL-25 (see details and references in Table 2). The details of NF-κB family members in these IL-9 co-stimulatory pathways have recently been reviewed [112].

The effects of the ligands that acutely induce IL-9 production are likely distinct from those responsible for the programming of the *Il9* locus that requires many of the transcription factors described in detail in this review, including STAT5, STAT6, PU.1, BATF, IRF4, and Smad proteins. These subset-promoting transcription factors induce genes other than *Il9* and initiate changes in chromatin structure characteristic of differentiated cells. It has been difficult, however, to examine the commitment of T cells to the IL-9-secreting phenotype because the T_H9 phenotype appears to be transient in vitro and in some autoimmune disease models [79, 83]. Despite these observations, IL-9-producing T cells have been identified in both healthy and diseased human skin [48, 91] and in a number of mouse models of disease [62, 70]. Moreover, IL-9+ T cells, that are negative for other cytokines (including IL-13 and IFNγ) are observed in vivo from patient samples [47]. Adoptive transfer of in vitro-derived T_H9 cells display IL-9-dependent effector function in allergic lung inflammation [10, 13, 24]. Thus, there is considerable evidence for the in vivo stability of the T_H9 phenotype, and it is possible that the inability to examine the stability of the T_H9 subset in vitro is a result of the technical limitations of the current culture systems.

Box 3

IL-9: Sources and functions

Although this review focuses on T_H9 cells, there are other sources of interleukin-9 (IL-9) within the immune system (see Box Figure). Type 2 innate lymphoid cells (ILC2) potently produce IL-9 in mice challenged with papain, helminthic parasites, and *in vitro* following stimulation with IL-2 [55, 62]. Mast cells also produce IL-9 in response to TLR and cytokine signals [113-115]. CD8+ T cells can acquire an IL-9-producing phenotype and these cells have been referred to as 'T_C9' cells [116, 117]. Natural killer T (NKT) cells express IL-9 in models of allergic and autoimmune inflammation [77, 118]. Finally, other $CD4^+$ T cells, including T_H2, T_H17, and regulatory T cells have been reported to produce IL-9, although the amounts of IL-9 produced by each of these cell types is generally less than that produced by a T_H9 cell [20, 81, 119]. The relative abundance of IL-9 from each of these cell types, and the contribution of each cell type during various immune responses, is only beginning to be defined (see Box Figure).

IL-9, like many cytokines, is pleiotropic in function. On hematopoietic cells, IL-9 signals through a specific IL-9 receptor chain (IL-9Rα) and the gamma common chain shared with the receptors for related cytokines include IL-2, IL-4, and IL-21 [120, 121]. IL-9 promotes mast cell and T cell growth, stimulates mast cell accumulation in tissues, promotes ILC survival, enhances class switching to IgE in B cells, and alters hematopoietic progenitor cell activity [4, 5, 65]. Although some in vivo IL-9 functions require T_H2 cytokine production, IL-9 can promote the differentiation of IL-17-secreting cells in vitro [24, 68, 82]. IL-9Rα is also expressed on non-hematopoietic cells including airway and intestinal epithelial cells, smooth muscle cells, and keratinocytes [4, 5]. If there are additional signaling requirements for IL-9 function in non-hematopoietic cells, they are not yet defined. IL-9 stimulates chemokine production from all of these cell types, enhances mucus production from airway epithelial cells and alters barrier function in the intestine [4, 5, 70]. Thus, IL-9 is instrumental in the ability of many cells to orchestrate inflammation and immunity by affecting many cell types.

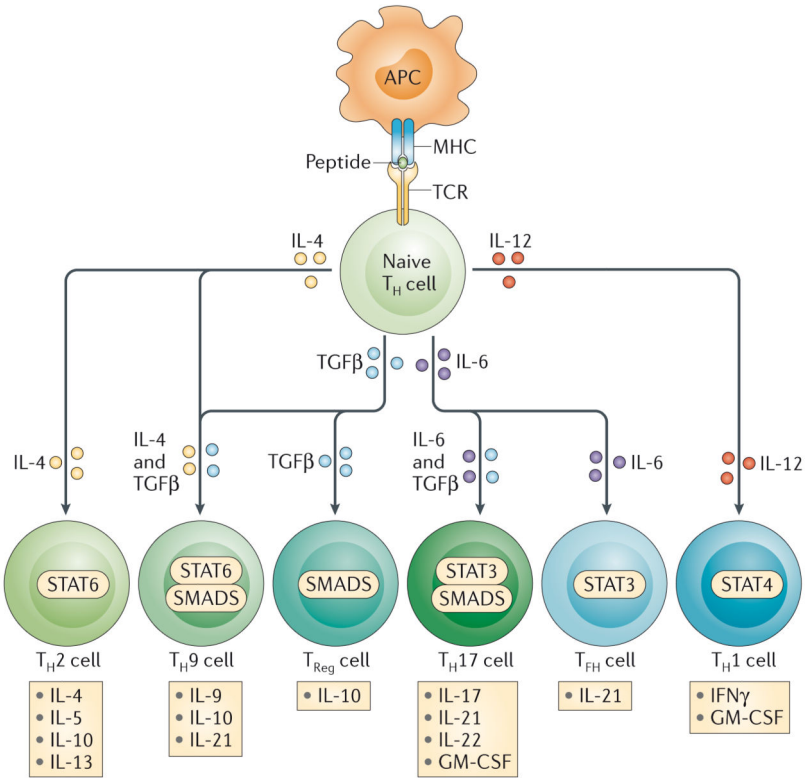


Figure 1. The influence of cytokine environment on T_H cell differentiation

While the strength of TCR signaling and the quality of co-stimulation have been demonstrated to influence naïve CD4+ T cell polarization, the cytokine environment largely dictates T_H differentiation. The prototypical cytokines and their corresponding signaling pathways (STATs, Smads) regulating each T_H fate are depicted. Additional cytokine and signaling pathways influencing T_H differentiation exist but have effects on the development of multiple T_H subsets. For example, IL-2, through the STAT5 signaling pathway, is important during T_H1, T_H2, T_H9, and T_{reg} development but inhibits T_{FH} and T_H17 differentiation.

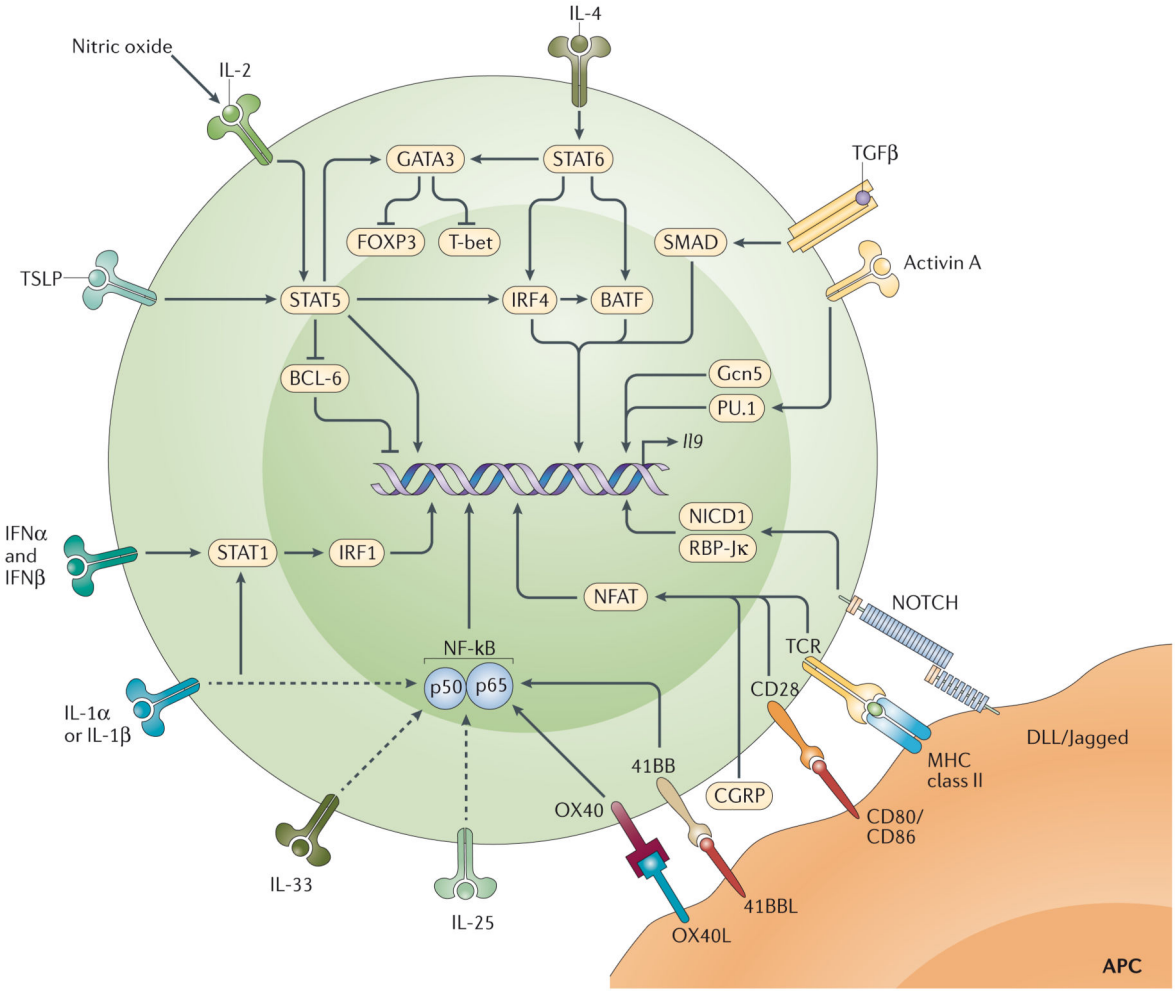


Figure 2. Cytokine and signal transduction pathways that promote IL-9 production and T_H9 differentiation

The differentiation of IL-9-secreting T cells requires IL-2/STAT5 and IL-4/STAT6 signaling and polarized from Th2 differentiation via TGF-β-derived signals. Although this combination of cytokines is sufficient for differentiation of T_H9 cells, a number of other signaling pathways have been identified that further enhance IL-9 production by these cells. Accessory cytokines act to induce NF-κB (IL-1, IL-25 and IL-33) or act through STAT1 to induce IRF1 expression (type I IFNs, IL-1β). TCR and co-stimulatory molecules also play an important role in *Il9* transcription upon interaction with antigen presenting cells, largely by activation of NFAT (TCR, CD28-mediated co-stimulation) and NF-κB (TCR, 41BB and OX40 co-stimulation).

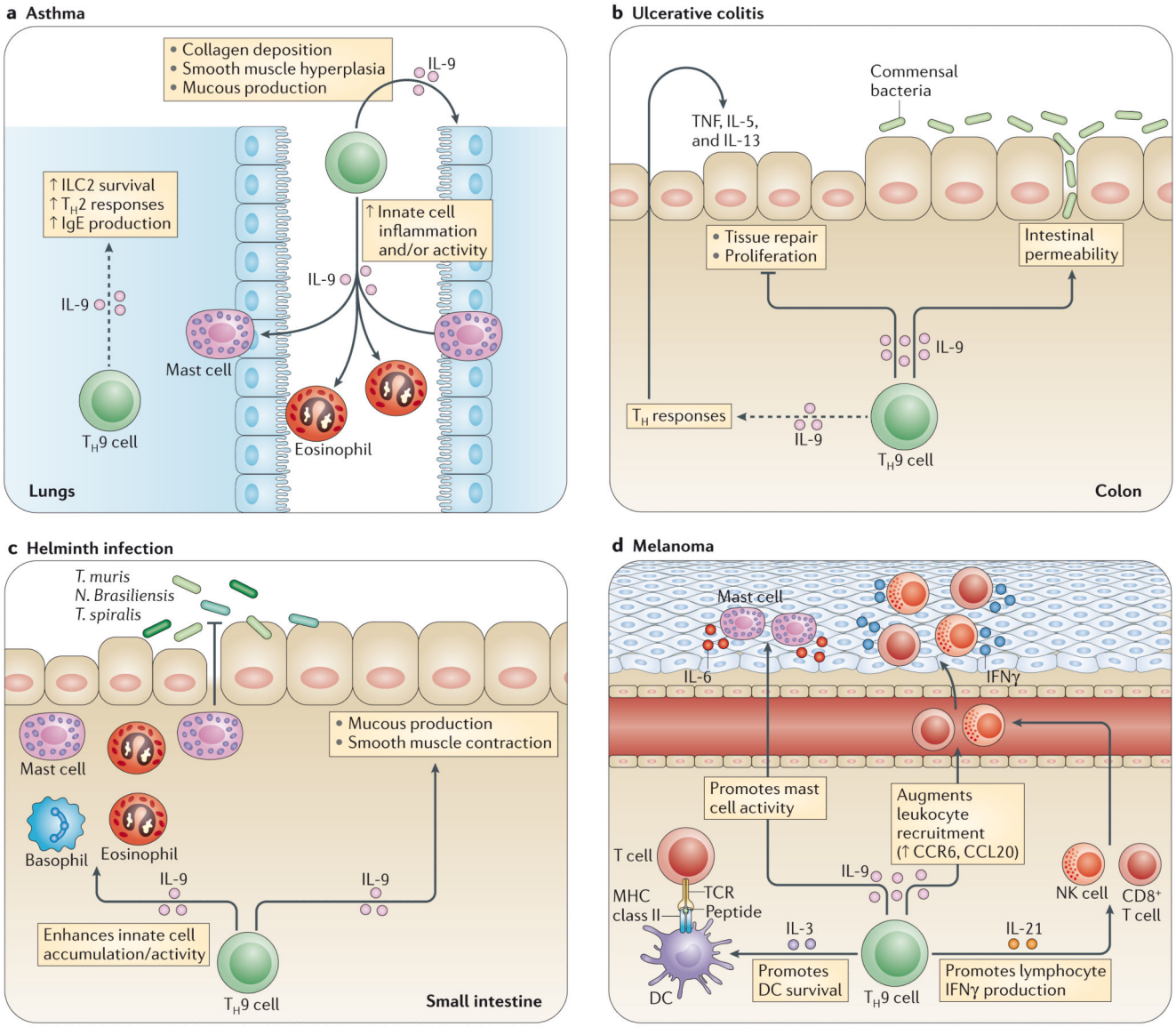
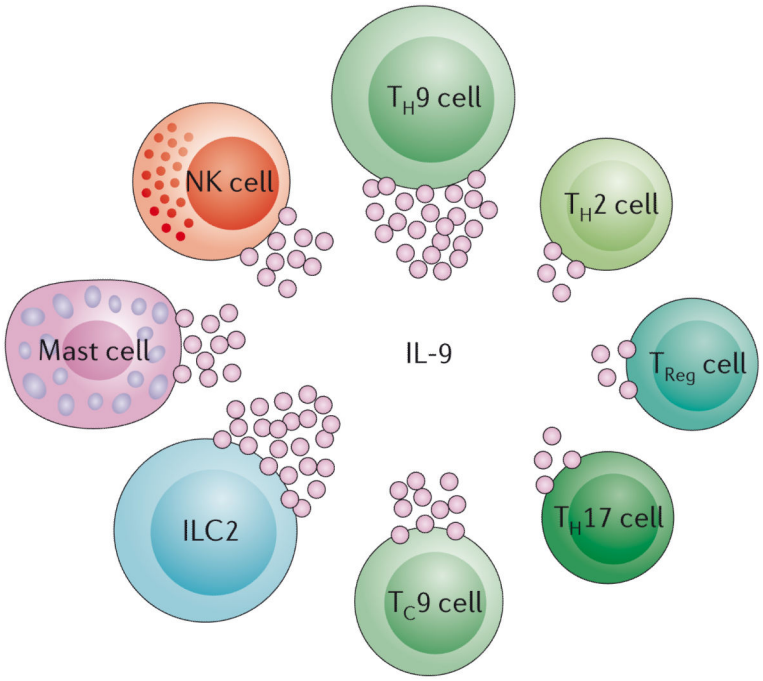


Figure 3. T_H9 cells in immunity and disease

Proposed effector activity of T_H9 cells in human disease. (I) Th9 cells, present in the draining lymph nodes and airways, are strongly linked in contributing to asthmatic disease. Th9-derived IL-9 can exacerbate the immune response by enhancing antibody production and increasing immune cell infiltration and activity within the respiratory tract. Furthermore, IL-9 can increase collagen deposition, induce smooth muscle hyperplasia, and alter respiratory epithelial cell function. (II) Th9 cells have been implicated in contributing to IBD particularly ulcerative colitis. Th9 cells, through IL-9 production, can impair tissue repair process, increase intestinal permeability, and may enhance inflammatory Th responses (III) Depending on the parasite, Th9 cells can exhibit potent anti-helminth activity through the production of IL-9. IL-9 mediates anti-parasitic activity by altering epithelial cell function, increasing immune cell infiltration into infected locations, and augmenting leukocyte immune function. (IV) Murine studies have strongly implicated Th9 cells in

exhibiting anti-tumor activity, particularly in melanoma. Th9 cells, through IL-9 production, can increase lymphocyte infiltration into the tumor and enhance the anti-tumor activity of mast cells. Furthermore, Th9-derived IL-21 can limit tumor growth by stimulating lymphocyte anti-tumor activity, and Th9-derived IL-3 can promote DC survival, potentially enhancing the induction of anti-tumor adaptive immune responses. The location of T_H9 effector activity (draining lymphoid tissue and/or inflammatory site) has not been clearly determined.



Box 2.

Table 1

TCR and co-stimulatory molecules that are involved in T_H9 differentiation. NA, data not available

Ligand on APC	Receptor on T cells	Signalling components induced by molecule	Effects on T_H9 cell development	Reference
Peptide-MHC class **II**	TCR	NFAT1/NF-κB	Promotes T_H9 cells	122
CD80 or **CD86**	CD28	PI3K/NF-κB	Promotes T_H9 cells	123, 124
OX40L	OX40	TRAF6/NF-κB/(p52-RelB)	Promotes T_H9 cells	125
4-1BBL	4-1BB	TRAF2 and NF-κB ?	Promotes T_H9 cells	126
Delta-like Ligand Jagged	Notch	NICD1	Promotes T_H9 cells	37
PDL2	PD1	SHP2	Inhibits T_H9 cells	54

APC, Antigen presenting cell. MHC, Major histocompatibility complex. NFAT, Nuclear factor of activated T cells. NF-kB, Nuclear factor kappa B. PDL2, Programmed cell death ligand 2.

Table 2

Soluble factors that influence the differentiation of T_H9 cells.

Cytokine/ Soluble Factor	Receptor	Signaling Component	Effect on T_H9		Reference
IL-1α	IL-1R1 /IL-1RAcP	MyD88 IRAK	***Promotes***		1, 97
			-	NF-κB?	
IL-1β	IL-1R1 /IL-1RAcP	MyD88 IRAK NF-κB STAT1 IRF1	***Promotes***		32, 60, 86, 96, 97
			-	STAT1-dependent induction of IRF1 to transactivate *Il9*	
IL-2	IL-2Rα/ IL-2Rβ/ γc chain	STAT5	***Promotes***		1, 22-24, 127
			-	STAT5 binding to *Il9* promoter	
			-	Represses BCL6	
			-	Enhances IRF4 and GATA3	
IL-4	IL-4Rα/ γc chain	STAT6	***Promotes***		1, 6-8, 10
			-	STAT6 binding to *Il9* promoter	
			-	Inhibition of FOXP3 and T-bet expression	
			-	Required for expression of most T_H9-associated genes	
IL-6	IL-6R/ Gp130	STAT1/STAT3	***Promotes***		6, 31
			-	In human cells, possibly through induction of IL-21	
			Inhibits		
IL-10	IL-10R1/ IL-10R2	STAT1/STAT3	***Promotes*** ***Inhibits***		20, 31, 128
IL-21	IL-21R/ γc chain	STAT1 STAT3	***Promotes***		22, 32
			-	induced by IL-1β	
			Inhibits		
			-	via BCL6 induction	
IL-23	IL-23R IL-12Rβ1	STAT3	***Inhibits***		79, 82
IL-25	IL-17RB	Act1/TRAF6?	***Promotes***		56
			-	NFAT/ NF-κB	
IL-27	IL-27R /gp130	STAT1	***Inhibits***		30
			-	STAT1 and T-bet dependent	
IL-33	ST2/ IL-1RAcP		***Promotes***		129
IFN-γ	IFNγRI/II	STAT1	***Inhibits***		1, 30
			-	Limits IL-4 sensitivity	
IFN-α/β	IFNαRI/II	STAT1	***Promotes***		31

Cytokine/ Soluble Factor	Receptor	Signaling Component	Effect on T_H9	Reference
			- induction of IL-21	
TGF-β	TGFβRII	SMAD/Non-SMAD	***Promotes***	6, 8, 18, 36
			- SMAD binding to the *Il9* locus	
			- Induction of PU.1	
			Inhibits	
			- Induction of FOXP3	
TSLP	TSLPR/ IL-7Rα	STAT5	***Promotes***	24
			- Enhances STAT5 binding to *Il9* promoter	
Activin A	ACTRII/ALK4	SMAD	***Promotes***	47
			- SMADs?	
			- redundant function with TGF-β in vivo	
CGRP	NA	PKA/NFATc2	***Promotes***	130
			- PKA to induce NFATc2, GATA3 and PU.1	
Nitric Oxide	NA	p53	***Promotes***	26
			- Induces IL-2 production, STAT5 activation, IL-4Rα and TGFβRII expression	
TL1A	DR3		***Promotes***	25
			- induces IL-2 production and STAT5 activation	

IL-1RacP, IL-1R Accessory Protein. IRAK, IL-1R-Associated Kinase. TRAF6, tumor necrosis factor receptor-associated factor 6. IRF1, Interferon regulatory factor 1. IFN-γ, Interferon gamma. IFNγR, Interferon gamma receptor. IFNαR, Interferon alpha receptor. TGFβRII, Tumor growth factor beta receptor II. TSLP, Thymic stromal lymphopoietin. ACTRII, Activin receptor II. ALK4, Activin-like kinase 4. CGRP, calcitonin gene-related protein. PKA, Protein kinase A. NA, Not applicable.