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The Transcription factor network in Th9 cells

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Abstract

The development of T helper cell subsets requires activated T cells that respond to a polarizing cytokine environment resulting in the activation and expression of transcription factors. The subset-specific transcription factors bind and induce the production of specific effector cytokines. Th9 cells express IL-9 and develop in the presence of TGFβ, IL-4, and IL-2. Each of these cytokines activates signaling pathways that are required for Th9 differentiation and IL-9 production. In this review I summarize what is currently understood about the signaling pathways and transcription factors that promote the Th9 genetic program, providing some perspective for the integration of the signals in regulating the *Il9* gene and dictating the expression of other Th9-associated genes. I highlight how experiments in mouse cells have established a transcriptional network that is conserved in human T cells, and set the stage towards defining the next important questions for a more detailed understanding of Th9 cell development and function.

As described in this issue, the IL-9-secreting Th9 cell subset is still being defined at the molecular and functional level. In culture, Th9 cells develop in the presence of TGFβ, IL-4, and IL-2 (Fig. 1). Each of these cytokines provides indispensible signals for the differentiation of the Th9 subset, although parallel pathways can provide substitute differentiation signals. Th9 cells participate in a variety of immune responses including parasite and tumor immunity, and allergic and autoimmune inflammation as has been reviewed previously and is described in other articles in this issue [1]. In this review, I will summarize our current understanding of the transcription factors that promote IL-9 production and the differentiation of the Th9 phenotype.

The process of differentiation

The differentiation of any T helper (Th) subset is not a simple matter of expressing a cytokine gene. By definition the differentiated subset is distinguished by preferential or enriched expression of a large number of genes. These genes, including the signature cytokine gene, are programmed by epigenetic changes that occur at the target loci and impact the ability of those genes to be induced following subsequent antigen receptor stimulation. This was first demonstrated in Th1 and Th2 cells where changes in DNase hypersensitivity, DNA methylation, and histone modifications occurred over 5–14 days of culture [2–5]. That length of time is required for lasting epigenetic modifications to occur. If

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epigenetic changes are irreversible, the Th cell is thought to be committed to a particular phenotype. Conversely, if the epigenetic changes are reversible, the T cell is thought to be plastic and can acquire new Th subset phenotypes. The programming of loci in the differentiating T helper cell does not only positively affect loci that are specific for a Th subset; differentiation also involves active repression of cytokine, transcription factor, and surface receptor genes that are linked to other Th subsets.

During the first five days of culture, a period when in vitro derived Th subsets are most frequently analyzed, there are transient changes in gene expression that can be indicative of ongoing programming. There is an increase in cytokine expression during 48–72 hours of culture. This is seen in Th1 and Th2 cells as a transient induction of IFNγ and IL-4, respectively, that is modest compared to the antigen receptor stimulation after five days of culture [6]. In Th17 cells, the distinction is not as apparent, and in some studies the amount of IL-17 production at day three of culture is as great or greater than the amount of IL-17 detected on day 5 or later [7–9]. However, this still represents transient expression and not programming, which requires epigenetic changes that take place over weeks.

In Th9 cells, the kinetics of *Il9* expression are still not as clear. There is an initial peak of expression around 72 hours [10], and several reports have limited studies to this time point. However, amounts of IL-9 produced upon re-stimulation of differentiated cells are still greater than in this initial peak [10]. When 72-hour cultures are examined, it is not clear if results represent acute activation of the *Il9* gene, or whether it truly represents genetic programming. Factors that mediate acute gene activation responses that occur within minutes to hours (such as NF-κB and STAT proteins) can transiently change histone modifications, such as acetylation. This may be indicative of gene activation, but not necessarily programming. Changes in DNA methylation and histone methylation are more indicative of stable and heritable changes in gene expression.

These concepts are important for keeping the work summarized in this review in perspective. Some transcription factors might be mediating acute or transient increases in IL-9 production. Others might be true programming factors and mediate the heritable changes at the cytokine loci that are required for establishing a differentiated state. A thorough kinetic analysis of the function of transcription factors that impact IL-9 production still needs to be performed, but the work described in this review have clearly delineated a transcription factor network that regulates gene expression in Th9 cells.

Regulatory elements of the *Il9* gene

The *Il9* gene has not been investigated to the same extent as many of the other Th cytokines. Based on conserved non-coding sequences (CNS), three elements have been identified that are close to the *Il9* gene (Fig. 2); the promoter (*Il9p*, CNS1), a region 6 kb upstream of the transcriptional start site (CNS0, CNS-6), and a region 5.4 kb downstream of the transcriptional start (CNS2) [11, 12]. Although the CNS1 and CNS-6 are conserved across multiple species, this was not true for CNS2. The CNS2 element was clearly conserved between human and mouse genomes, but was more divergent in other species. Despite this

divergence, Th9 transcription factors are found associated with CNS2 as well as the CNS1 and CNS-6 elements. These elements are depicted in Fig. 2.

Cytokine-activated STAT signals

Signal transducer and activator of transcription (STAT) protein activity is the common gateway to the programming of Th cell differentiation [13, 14]. STAT proteins, activated in response to cytokines in the environment, bind DNA, establish an enhancer landscape that is unique to a particular Th subset, and directly activate the expression of genes that are important for the development and function of the differentiated T cell. STAT protein activity includes recruiting histone acetylatransferases such as p300, initiating monomethylation of H3K4 at enhancers, and displacing DNA methyltransferases and histone methylases [4, 13, 15–22].

STAT6 is activated downstream of the Th9-inducing cytokine IL-4, and is critical for IL-9 production [23–25]. In the absence of STAT6 there is increased expression of transcription factors that repress IL-9 including T-bet and Foxp3 [24]. STAT6 can also directly bind the *Il9* CNS1 [24, 26](Fig. 2), although it is not clear that this is a critical function, as IL-4 stimulation does not acutely activate IL-9 production [12]. However, STAT6 is required for the expression of additional activators of IL-9 production including BATF and IRF4 [24, 27]. STAT6 is also required for a large number of additional genes that are preferentially expressed within Th9 cells [27].

STAT5 is a common nexus for multiple signals that induce IL-9 production. IL-2, one of the earliest cytokines identified to induce IL-9 [28], functions through STAT5. The IL-2-STAT5 pathway is modulated by a number of other factors including nitric oxide, the TNF superfamily member TL1A, and Itk activation [29–31]. STAT5 might function through multiple mechanisms in promoting IL-9. It can directly bind to the *Il9* promoter [32–34](Fig. 2). STAT5 also activate other transcription factors that promote IL-9 production. IRF4 is downstream of STAT5 and is a critical regulator in several experimental systems [29, 30, 34]. Additionally, IL-2 and STAT5 regulate cytokine receptors that are critical for differentiation, including IL-4Rα [35].

The IL-2-STAT5 pathway is also a target of negative regulators. BCL6 directly competes with STAT5 in binding to the *Il9* promoter, and might also repress the *Il9* gene directly [32, 33]. In the absence of STAT3, Th9 cells have greater endogenous IL-2 production and STAT5 activation [36]. IL-6 and to a lesser extent IL-21 activate STAT3 to interfere with IL-2 production. Yet, other STAT3-activating cytokines do not have the same effect [36]. The exact mechanism of STAT3-mediated IL-2 repression is not yet clear, but it is independent of BCL6 [36].

As mentioned above, STAT5 seems to be a common transcription factor for multiple ligands to induce IL-9 production. TSLP activates STAT5 to promote IL-9 production and increased STAT5 bound to the *Il9* promoter, as well as IRF4 expression [34]. DR3, the receptor for TL1A, also induces IL-9 production [31] that is independent of TRAF6 or STAT6, although induction was slightly less efficient in the absence of STAT6 [31]. TL1A activity did not

require PU.1, although stimulation with TL1A resulted in increased binding of PU.1 and IRF4 to *Il9* regulatory elements [31]. TL1A activity did require IL-2 and STAT5, and TL1A stimulation increased intracellular phospho-STAT5 and STAT5 bound to the *Il9* promoter [31].

Finally, STAT1 appears to have very context-dependent effects on IL-9 production. Previous reports have indicated that STAT1 impairs IL-9 production downstream of IFNγ and IL-27 [28, 37]. However, a more recent report identified a STAT1-IRF1 module as being required for the ability of Th9 cells to respond to IL-1β and amplify both IL-9 and IL-21 production from Th9 cells [38]. STAT1 activation by IL-1β relied on Myd88 and Fyn [38]. IRF1 bound directly to the *Il9* promoter and IRF1 was required for IL-1β-induced *Il9* expression [38] (Fig. 2).

The Smad/RBP-Jκ/Notch-ICD module

The Smad proteins Smad2, Smad3, and Smad4 function downstream of the TGFβ receptor and are necessary component of the development of Th9 cells. Published studies have utilized a number of models, and I have tried to stipulate the specifics for each with the results. IL-9 production is partially diminished in T cells that lack Smad2, Smad3 or Smad4 (using $Smad2^{fl/fl}$ CD4-Cre or Lck-Cre T cells, $Smad3^{-/-}$ T cells or $Smad4^{fl/fl}$ CD4-Cre T cells) [39, 40]. IL-9 production is more severely compromised in T cells that lack Smad2 and either one or two alleles of Smad3 (using $Smad2^{fl/fl}$ Lck-Cre, $Smad3^{+/-}$ or $Smad2^{fl/fl}$, $Smad3^{fl/fl}$ CD4-Cre T cells) [40, 41]. *Il9* mRNA was also decreased in vivo in the lungs of $Smad2^{fl/fl}$ Lck-Cre, $Smad3^{+/-}$ mice subjected to the OVA-alum model of airway inflammation [40].

The mechanisms of Smad function are still not entirely elucidated. Each of the Smads 2, 3, and 4 can bind to the *Il9* promoter and to CNS regions [39, 40](Fig. 2). One study identified diminished histone acetylation and H3K4 trimethylation in Th9 cells from $Smad2^{fl/fl}$ Lck-Cre, $Smad3^{+/-}$ mice [40]. Another study identified modest increases in H3K27me3 at the *Il9* locus coincident with increased EZH2 association in the absence of Smad2 or Smad4 [39]. This conflicted with the first study that showed no change in H3K27me3 [40]. Although both mechanisms might contribute to regulation of the *Il9* gene, the decreased histone acetylation observed in the Smad2/Smad3 compound mutant mice was associated with a greater loss of IL-9 production [40]. In addition to direct affects on the *Il9* locus, deficiency in Smad2/Smad3 resulted in increased expression of T-bet and Rorγ, both of which can repress IL-9 production [24, 40].

Smad3 was also observed binding to a region about -4kb from the *Il9* transcriptional start site [42](Fig. 2). This was observed in a system where Notch ligands were stimulating IL-9 production, and Smad3 association at this site was dependent on the cooperative binding of recombination-signal-binding protein for immunoglobulin-κ J region (RBP-Jκ) and the Notch intracellular domain (NICD)[42]. It is not yet clear if the RBP-Jκ/NICD factor are required for Smad binding to other sites in the *Il9* locus including the promoter, nor if this is the only effector of Notch signaling in the production of IL-9.

Downstream of the TGFβ activated kinase TAK1

The TGFβ activated kinase TAK1 mediates Smad-independent TGFβ signaling. TAK1 activates MAPK signaling pathways, although it is not clear whether MAPK pathways are important for IL-9 production. However, TAK1 can impact other pathways that regulate IL-9.

The E box transcription factor inhibitor Id3 represses IL-9 production [43]. Th9 cells that are deficient in Id3, or are treated with Id3 siRNA, have increased IL-9 [43]. Chemical or siRNA inhibitors of TAK1, or genetic deletion of TAK1, decrease IL-9 production and increase expression of Id3. Id3-deficiency increased binding of E2A and GATA3 at the *Il9* promoter, suggesting a potential mechanism of activity [43](Fig. 2). Twist1 also inhibits E2A activity, but Th9 differentiation appeared normal in Twist1-deficient T cell cultures [44], suggesting that there is specificity in the ability of Id3 to inhibit IL-9 production.

Similar to the ability of TAK1 to inhibit Id3, it also inhibits the expression of SIRT1 [45]. A recent report identified SIRT1, a histone deacetylase that is metabolic sensor in multiple tissues, as a modulator of Th9 differentiation by functioning as a repressor of mTOR and Hypoxia Inducible Factor 1a (HIF1α) [45]. IL-9 production was greater in SIRT1-deficient T cells that had greater expression of glycolytic enzymes, and blocking the glycolytic pathway in SIRT1-deficient Th9 cells reduced IL-9 production to amounts near those detected in wild type cells [45]. The regulation of IL-9 production by SIRT1 was linked to HIF1α, which bound to a site in the *Il9* promoter 1.2 kb upstream of the transcriptional start site, and activated the *Il9* promoter [45]. Double deficiency in SIRT1 and HIF1α resulted in IL-9 production that was similar to amounts in wild type cells [45]. Whether HIF2α, which also regulates IL-9 [46], is also in this pathway is not clear. How metabolism contributes to the development of Th9 cells, as it clearly plays a role in other Th subsets [47], still needs to be further defined.

The ETS family: PU.1 and ETV5

PU.1 is an ETS family member that was the first transcription factor found to play a role in IL-9 production in Th9 cells. T cells that lacked PU.1 expression had diminished IL-9 production, and ectopic expression of PU.1 increased IL-9 production as it decreased Th2 cytokine production, suggesting that it might be a switch factor between the subsets [12]. PU.1 bound directly to the *Il9* promoter and regulated chromatin remodeling at the *Il9* locus, being responsible for recruitment of histone acetyltransferases (HAT) and histone acetylation [12, 48, 49](Fig. 2). PU.1 directly interacted with the HAT Gcn5 [48]. In the absence of PU.1, Gcn5 was no longer associated with the *Il9* locus, and ectopic expression of full length PU.1 in PU.1-deficient cells, but not PU.1 lacking a transactivation domain, was able to rescue Gcn5 association [48]. The PU.1-dependent IL-9 production in T cells is linked to pathology in models of allergic airway inflammation and inflammatory bowel disease [12, 50, 51], each discussed in more detail in other articles in this issue.

However, PU.1 function is not strictly limited to Th9 cells. As mentioned above, PU.1 represses Th2 cytokines and affects heterogeneity of cytokine expression in Th2 cells by

interfering with GATA3 and IRF4 activity in subpopulations of cells [52–54]. As a consequence of greater GATA3 activity, even in naïve cells, PU.1-deficient T cells have increased TCR expression and increased IL-2 production compared to wild type cells when antigen receptor stimulation is limiting [53]. PU.1 also represses genes that are important for the function of T follicular helper (Tfh) cells including IL-21, PD-1 and CD40L [55]. Thus mice with PU.1-deficient T cells have increased numbers of Tfh and germinal center B cells, and increased serum immunoglobulin concentrations, compared to wild type mice. Thus, PU.1 might also indirectly promote Treg differentiation by limiting other differentiation programs.

More recently, another ETS family member, ETV5, was shown to have parallel effects on IL-9 as observed in PU.1-deficient T cells. IL-9 production was decreased in the absence of ETV5, and ectopic expression of ETV5 increased IL-9 production [56]. ETV5, which also promotes IL-17A/F production in Th17 cells [57], significantly bound to the *Il9* gene at regions distinct from the promoter that bound PU.1 [56](Fig. 2). Binding of the HAT p300 was dependent on ETV5, but Gcn5 binding was normal in the absence of ETV5 [56]. Th9 cells that were doubly deficient in PU.1 and ETV5 had less IL-9 production than Th9 cells from either single deficient strain. Interestingly, the effects on repression of IL-4 were not cumulative in double-deficient T cells [56]. In vivo, PU.1 and ETV5 seemed to have significant effects on some overlapping but also some distinct parameters of allergic inflammation. Notably, PU.1 seemed to have the dominant effect on IL-9 production from cells isolated ex vivo [56].

Despite parallel function, the expression of each factor appeared to be regulated by separate pathways. PU.1 expression in Th9 cells was induced by TGFβ but was independent of IL-4/STAT6 signaling [12, 24, 27]. However, PU.1 expression did not rely upon Smad2 or 3 [40]. ETV5 expression was dependent on IL-4/STAT6 signaling and IRF4 expression [56]. PU.1 and ETV5 had reciprocal inhibitory effects on each other's expression [56].

The finding that PU.1 and ETV5 had parallel functions in Th9 cells raised the possibility that ETS factors in general might have the ability to regulate IL-9. Based on microarray data [27], two additional ETS family factors, Elk3 and Etv6, were identified as being expressed in Th9 cells. However, transduction of either Elk3 or Etv6 into Th9 cells did not alter IL-9 production [56]. This suggested that even though PU.1 and ETV5 belong to distinct parts of the ETS family [58], there is specificity in the ETS family proteins that are involved in IL-9 regulation.

The BATF/IRF4 module

Both BATF and IRF4 are potent activators of IL-9 production and Th9 cells that are deficient in either factor are defective in their ability to produce IL-9 and to promote the development of allergic inflammation [27, 59, 60]. Both factors bind to the *Il9* promoter and activate IL9 reporter plasmids and are downstream of the IL-4/STAT6 signal during differentiation [24, 27](Fig. 2). IRF4 is also a target of STAT5 in Th9 cells when cells are stimulated with IL-2 or TSLP [29, 30, 34], but it is not yet determined if BATF is similarly induced.

BATF and IRF4 expression is not restricted to Th9 cells, though both seem to be expressed in higher amounts in Th9 cells than in other T helper subsets [27, 59]. The expression of both factors in additional Th subsets contributes to differentiation programs distinct from Th9 differentiation, and mice with T cells deficient in either factor have defects in Th2 cell, Th17 cell, and Tfh cell development [61–66]. This overlap in function is linked to the cooperative binding of BATF and IRF4, which has been demonstrated extensively in Th17 cells [67–69]. Cooperative binding is also seen in Th9 cells where deficiency in either factor results in decreased binding of the reciprocal factor to the *Il9* promoter, and ectopic expression of BATF also increased binding or IRF4 to the *Il9* promoter [27]. Consistent with the requirement for cooperation between these factors, transduction of BATF or IRF4 into cells deficient for the reciprocal factor have less of an effect than transduction into wild type cells [27].

IRF4 also provides a link between IL-4 and TGFβ signaling in the generation of IL-9-secreting T cells. TGFβ enhances IRF4 binding to the *Il9* gene [40]. Smad2/Smad3 and IRF4 bind to the *Il9* regulatory elements cooperatively, providing at least one link between the two differentiating cytokine signals [40]. Smad3 and IRF4 also physically interact and can be co-precipitated. Importantly, IRF4 cannot induce IL-9 in Th9 lacking expression of Smad2/Smad3, and Smad3 cannot induce IL-9 production in IRF4-deficient T cells [40]. Thus, there is a considerable degree of cross-talk among IL-9-inducing transcription factors.

NF-κB in T cell receptor and TNF superfamily signaling

The outcome of the Th differentiation process is that loci encoding lineage-specific cytokines are programmed for rapid induction following antigen receptor signaling. Antigen receptor signaling pathways function cooperatively and include induction of NFAT proteins and the NF-κB pathway. At the *Il9* locus, NFAT1 is required for chromatin accessibility and the ability of NF-κB p65 to bind the *Il9* promoter [70]. IL-9 was also decreased in mice that lacked NFATc1/NFAT2 in T cells following sensitization and challenge in an OVA/alum model [71]. Each of these factors contributes to rapid induction of IL-9 when Th9 cells are activated after differentiation.

The NF-κB pathway is also induced by TNFR superfamily members and this pathway is remarkably potent in the induction of IL-9. However, the mechanisms and transcription factors that are activated by TNFRSF members are quite different. The first TNFRSF member identified with this function was OX40. Antigen presenting cells expressing OX40L, or antibodies to OX40 are potent inducers of IL-9 production in Th9 cultures that are differentiated from naïve T cells [72]. OX40 functions by activating a TRAF6/NF-κB RelB-p52 pathway [72] where a RelB-p52 heterodimer binds directly to the *Il9* promoter. OX40 activated IL-9 production was also dependent on STAT6 but independent of PU.1 [72].

GITR induces IL-9 production from Treg cells by repressing Foxp3 expression as it induces IL-9 through a TRAF6/NF-κB-dependent pathway [73, 74]. Repression of Foxp3 requires NF-κB p50 through recruitment of histone deacetylases [74]. GITR induces phospho-STAT6, BATF, PU.1 and IRF4. However, repression of Foxp3 did not require any of these

factors. Induction of IL-9 required STAT6 and was partially dependent on IRF4, but independent of BATF and PU.1 [74]. Because GITR induced IL-9 even in cells lacking IL-4R, it is possible that GITR is activating STAT6 through a pathway that is independent of the usual cytokines [73]. The STAT6-dependent activation of the *Il9* locus was linked to recruitment of the HAT p300 to the gene [74]. Interestingly, the ability of GITR to induce STAT6 activation was dependent on NF-κB p50 [74]. Since Foxp3 inhibits IL-9 production [24, 74] it is important to note that the repression of Foxp3 and the induction of IL-9 were separable events because STAT6-deficient T cells were able to repress Foxp3 following GITR stimulation, but not induce IL-9 [74].

Together, these reports indicate that multiple components of the NF-κB pathway are involved in the induction of IL-9. This pathway is functioning on multiple levels that include direct binding and activation of the *Il9* gene, repression of repressors such as Foxp3, and activation of STAT6, though mechanisms that are still not entirely clear [70, 72–74]. It is interesting that mechanisms are so distinct, and it is noteworthy that as detailed above, another TNFSF/TNFRSF pair, TL1A/DR3, requires neither NF-κB or STAT6, but instead functions through a IL-2/STAT5 amplification pathway [31]. This suggests that responses to each of these ligands might be specialized to specific conditions in vivo.

Stability and transitioning to an IL-9-secreting phenotype

The stability and plasticity of Th subsets has been the focus of considerable work [75, 76]. Plasticity requires that T cells maintain cytokine receptor expression to facilitate responses to an altered cytokine environment and genes required for the acquisition of separate phenotypes having a poised chromatin configuration. Th9 cells appear to be less stable than other subsets and tend to lose IL-9 expression when maintained in culture [10], although Th9 cultures can acquire other cytokine-secreting phenotypes when they are switched to polarizing conditions after three days in vitro [10]. This lack of stability in vitro has made it challenging to define the transcription factors that might be important for maintaining the phenotype. It is possible that some of the negative regulatory factors (STAT3, BCL6, Foxp3) might promote instability and that is an active area of investigation. However, it is important to consider that in vitro derived Th9 cells can be adoptively transferred and retain IL-9-dependent functions in vivo [34, 59], suggesting that the IL-9-secreting effector phenotype can be maintained in vivo. It is still possible that there is an evolutionary rationale for IL-9 production being more transient to avoid the potential harmful effects of chronic IL-9 production.

Several other Th subsets can become IL-9-secreting T cells, though whether these are bona fide Th9 cells or rather represent Th cells that are transiently producing IL-9 is not clear. Th2 cells can become Th9 cells when they are exposed to TGFβ [25]. Both Smad proteins and PU.1 are important in repressing the Th2 cytokine program, as they activate *Il9* and other Th9 genes [12, 27, 39, 40]. STAT3 might be another switch factor in the Th2/Th9 balance, promoting Th2 cytokine production as it inhibits IL-9 production [36, 77]. GITR signals T regulatory cells to become IL-9 secreting T cells [74]. This transition requires both p50 and STAT6 [74]. Finally, Th17 cells can acquire an IL-9-secreting or mixed phenotype on culture with TGFβ, IL-4, and IL-2, a process that is enhanced by the action of OX40 [72,

78, 79]. This relies on STAT6 and likely the IL-2/STAT5 pathway that both represses Th17 and induces Th9 differentiation [80]. These additional pathways to the Th9 phenotype are indicated in Fig. 1.

Transcriptional regulation of genes in Th9 cells other than IL-9

As with most analysis of Th subset gene expression, much of the transcription factor network in Th9 cells has been defined by an ability to regulate the hallmark cytokine IL-9. However, differentiated Th subsets have enriched expression of a broad range of genes that contribute to the ability of a Th subset to survive, migrate to a site of inflammation, respond to particular cytokine milieus, and control the function of other cells during immune responses. In Th9 cells this subset of genes has been defined using a microarray approach and includes transcription factors, chemokines and chemokine receptors, cytokines and cytokine receptors, and genes with still undefined functions [27]. The requirement for transcription factors to regulate Th9 genes is distinct from the factors that are strictly required for *Il9* gene expression.

In gene expression analysis of in vitro differentiated Th subsets, there was a hierarchy of transcription factor dependence for expression in Th9 cells. STAT6 and BATF were required for a large portion of the genes examined, with IRF4 only impacting a subset of the STAT6 and BATF-dependent genes [27]. In contrast, PU.1 regulated only a small subset of the genes examined [27]. These initial analyses were performed on fewer than 20 genes, and larger scale analyses that could more thoroughly define the effects of each transcription factor still remain. Moreover, the effects of other critical factors including Smad2/3/4 and STAT5 have not been examined beyond effects on *Il9*.

The human Th9 transcription factor network

Most of the studies summarized in this review have focused on murine cells for their ease of manipulation, access to gene-deficient cells, and because cell numbers are not limiting. However, the important research questions remain focused on human health, and as such it is important to test whether transcription factor function based on observations in mouse cells are conserved in human cells. Treatment of primary human T cells with siRNA for PU.1, BATF and IRF4 each decrease the amount of IL-9 produced in Th9 cultures [12, 27, 49, 59]. Inhibitors of Itk also diminish IL-9 production in Th9 cultures, similar to the effects of inhibitors and Itk-deficiency in murine cells [29]. A SNP of ITK is also associated with asthma [81]. SIRT1 inhibition by siRNA also increased IL-9 in human T cells in parallel to effects on glycolytic enzymes and similar to the effects of rapamycin and glycolytic inhibitors [45]. TAK1 inhibition decreases and Id3 siRNA increases IL-9, similar to the effects of these approaches in the murine system [43]. T cells from patients with mutations in STAT1 (Chronic Mucocutaneous Candidiasis) and STAT3 (Hyper-IgE Syndrome) have diminished IL-9 production, compared to healthy controls, suggesting somewhat distinct effects of these pathways between murine and patient systems [82].

There are also changes in expression of Th9 transcription factors that are linked to disease. NFATc1 and IRF4 expression are increased in asthmatic children [71]. Expression of PU.1

and BATF are increased in Th9 cultures derived from atopic infants, compared to non-atopic infants [27, 49, 83]. Thus, much of the framework of the Th9 transcriptional network that has been established in murine cells has been verified in human cells as well. This suggests that the knowledge gained in the murine studies will be helpful in guiding the development of potential Th9-targeted therapies.

Together, these studies suggest that there is broad conservation of IL9 regulation between mice and humans. Moreover, there is evidence of conservation of function of Th9 cells in human and mice. Th9 cells are observed in asthma, ulcerative colitis, and in tumors, parallel to function that has been demonstrated in mouse models of these diseases [1]. However, many of the pathways established in the murine system, including Smads, the Notch signaling pathway, and TNFSF-TNFRSF signaling have not been as thoroughly examined in human cells. These will be critical to define in the future.

Concluding remarks and future directions

IL-9-secreting T cells were first described over 20 years ago [28] and were rediscovered in 2008 as a subset separable from other established Th subsets [23, 25]. In almost ten years since that finding, we have discovered a multitude of signaling pathways and transcription factors that promote expression of *Il9* and a Th9 cell genetic program. Still, there are many questions left unanswered. The first is whether there is a lineage-defining factor associated with IL-9 expression in the same way that T-bet is linked to Th1 cells, GATA3 to Th2 cells, and RORγt to Th17 cells. Most of the transcription factors discussed, including BATF, IRF4, Smad2/3/4, and STATs, have functions in multiple T helper subsets. PU.1 and ETV5 are expressed in other subsets, and respectively have effects on T cell activation and Tfh cells, and in Th17 cells. Thus a truly specific factor remains elusive. The question of Th9 stability is also not entirely settled. Although Th9 cells appear unstable in vitro, there is evidence that there are Th9 cell memory responses in vivo, and in patients, suggesting that stability can occur under some circumstances. Defining the signals and transcription factors that control the maintenance of the Th9 phenotype will be an important milestone in defining the role Th9 cells in immune responses.

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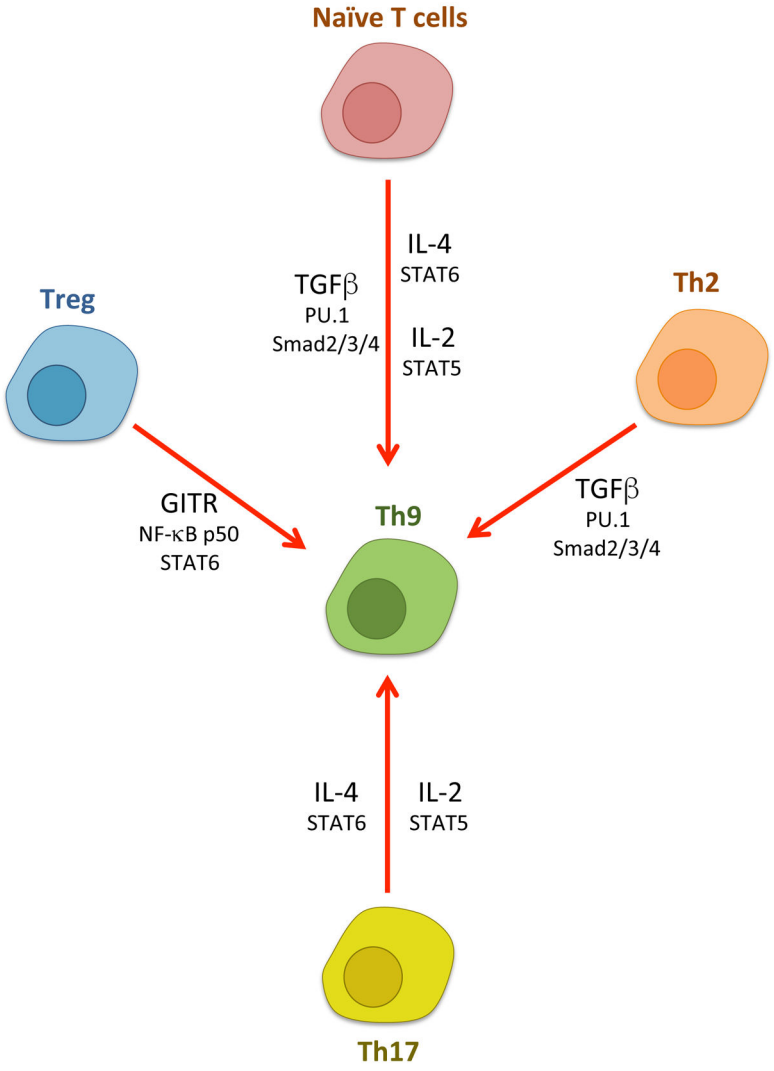

Figure 1.

Pathways to Th9. The diagram indicates the T cell types that are known to acquire an IL-9-secreting T cell phenotype. The cytokines and transcription factors that are downstream of those cytokines and critical for differentiation are indicated for each pathway.

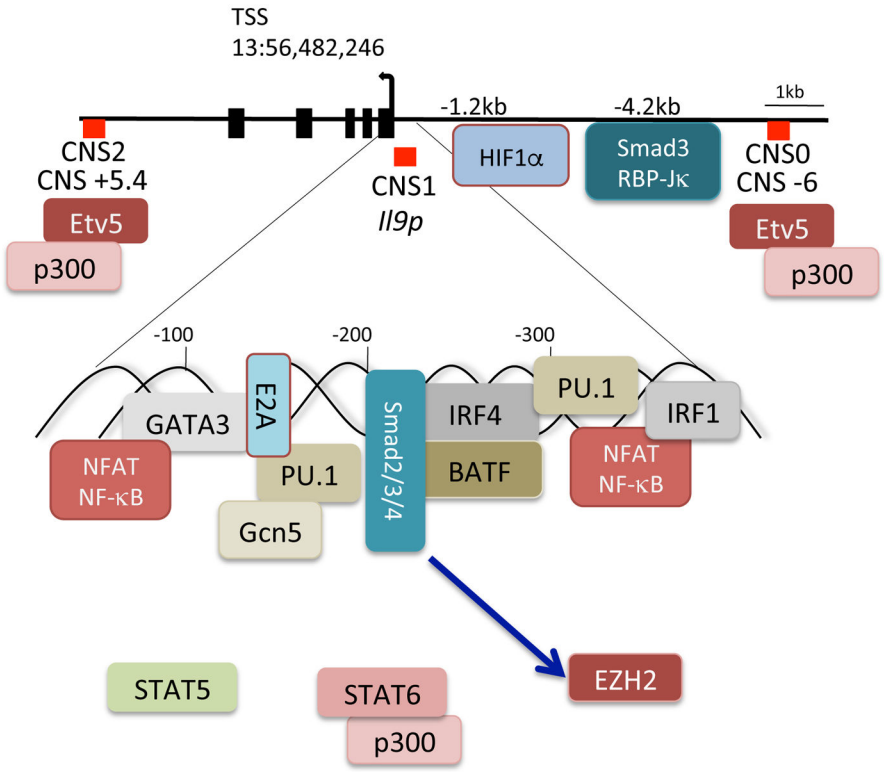

Figure 2.

Regulation of the *Il9* gene. Schematic of the Il9 locus indicating the three conserved non-coding sequences (CNS) that have been characterized thus far. The Il9 promoter/CNS1 is shown at a higher magnifications to allow visualization of factors known to bind the region. The approximate binding sites (not to scale) for transcription factors are based on published studies described in this review. The binding sites for some factors has not been strictly defined. For example, STAT5 and STAT6 clearly bind the promoter, and there is at least one consensus STAT site in the promoter, but binding to that site has not clearly been demonstrated.