

REVIEW

Th9: A New Player in Asthma Pathogenesis?

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CD4⁺ T helper (Th) cells act as important regulators of the mammalian adaptive immune response. CD4⁺ Th cells were originally characterized as either Th1 or Th2 types, based on the cytokines they produce. Over the past two decades, however, we have attained a much more detailed understanding of CD4⁺ T-cell differentiation, functions, and gene expression profiles, which led to the identification of additional types, such as the Th17 and induced regulatory T cells. Recently, researchers have characterized yet another novel and distinct population that exists among these immune-modulatory cells. The 'Th9' cells were first identified as a Th2 subpopulation that produced exceptionally large quantities of the Th2-specific cytokine interleukin 9. However, experimental analysis revealed that Th9 cells had divergent regulatory capabilities and were critically involved in different immune processes. In particular, we now know that Th9 acts as a major contributor to the onset and progression of allergies, especially asthma. Here, we will review the heterogeneity and biology of CD4⁺ Th cells and summarize the characteristics known to date of interleukin 9 and Th9 cells to build a framework for better understanding of their roles in disease processes, especially in allergy-induced asthma.

Keywords Th9, IL-9, PU.1, allergic asthma, autoimmune diseases

INTRODUCTION

Asthma is one of the most common atopic diseases in the world and substantially impacts annual healthcare expenditures, especially in developed nations (1). As a complex inflammatory disease, asthma is characterized by intermittent and reversible airflow obstruction, bronchial hyperresponsiveness (BHR), and airway inflammation. Sufferers experience wheezing, breathlessness, chest tightening, and coughing. Evidence from human and animal studies have demonstrated that CD4⁺ T helper (Th) cells, specifically imbalances in the numbers and functions of Th1 and Th2 subpopulations, play a pivotal role in the pathogenesis of asthma (2). Th2 cells normally orchestrate asthmatic inflammation by secreting a series of cytokines, including interleukin (IL)-4, IL-5, and IL-13. These particular cytokines then modulate their various target cells, such as the B cells, eosinophils, mast cells, airway epithelial cells, and other CD4⁺ T cells, to elicit the pathological and physiological changes of asthma (3).

Among those cytokines known to be related with asthma, IL-9 was originally described as a growth factor that stimulated T cells and mast cells in mice (4, 5). Subsequent studies in mouse models and humans have extended our understanding of IL-9 functions in the pathogenesis of atopic asthma. Genetic and experimental analyses have correlated IL-9 expression and protein activity to the physiological features of asthma (6–8). The fact that IL-9 is a multifunctional and pleiotropic cytokine likely explains the possible relationship with allergic asthma, which has been linked to multiple cell sources including T lymphocytes, eosinophils, and mast cells (9–11).

Since Coffman's discovery of the heterogeneity of effector T cells nearly two decades ago (12), the CD4⁺ T-cell subset has remained classified as one of only two types: Th1 or Th2, according to their expressed cytokine profiles. In 2003, IL-17-expressing T cells (termed Th17) were recognized as a third lineage of Th cells; since then, other novel and distinct lineages have been characterized, including the Foxp3⁺ regulatory T (Treg) cells, T follicular helper (Tfh) cells, and Th9 cells (12–17). The Th9 cells were identified by their capacity to produce large amounts of IL-9. As IL-9 was widely considered to be a Th2-specific cytokine, Th9 cells were hypothesized to have arisen from the Th2 lineage. However, detailed studies into the function of Th9 cells have revealed that they express significantly lower levels of other common Th2-type cytokines and have distinct biological features.

Investigations into the heterogeneity and plasticity of CD4⁺ T cells have enhanced our understanding of many immunological characteristics of inflammatory diseases. Despite each of the CD4⁺ Th subsets having distinct profiles of expressed cytokines and biological functions, it is possible that many of those disease processes are associated with more than one Th type. Currently, the precise contribution of Th9 cells in allergic disease remains unknown. This review will describe our current understandings of the source, genesis, and functions for Th9 and its cytokine profile, and then draw on the recent literature to conjecture about the possible relationship between Th9 cells and allergic asthma.

INDUCTION AND DIFFERENTIATION OF TH9 CELLS

Heterogeneity of CD4⁺ Th Cells

In 1972, Parish *et al.* demonstrated the existence of at least two different populations of CD4⁺ effector T

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cells mediating adaptive immune responses in Wistar rats (18). Shortly thereafter, Mosmann *et al.* announced the coincident result that murine CD4⁺ T-cell clones could be divided into two major groups: Th1 and Th2 (12). Moreover, the Th1 and Th2 cells exhibited distinct but central activities in immune responses by means of their unique secreted cytokine profiles. This same phenomenon was observed in human CD4⁺ T cells by another group later on (19).

CD4⁺ Th lineages do not only differ by their cytokine profiles but have unique panels of surface markers and collections of expressed transcription factors. The Th1 type produces interferon (IFN)- γ ; this signature cytokine is responsible for activating macrophages, promoting opsonization, and stimulating production of complement-fixing antibodies. Thus, Th1 cells play a significant role in host defense against obligate intracellular infectious agents and in cell-mediated immune responses. Excessive or underexpression of Th1 products results in improper inflammatory responses and has been associated with many organ-specific autoimmune diseases (20). In terms of Th1-cell differentiation, IFN and IL-12 are powerful inducers (21, 22) and act by up-regulating the expression of the T-box family transcription factor T-bet, which regulates lineage commitment.

Unlike the Th1 cells, Th2 cells contribute to humoral immunity and protect against extracellular pathogens. These cells mainly produce cytokines IL-4, IL-5, and IL-13, but also express significant amounts of IL-10, tumor necrosis factor- α , and modest amounts of IL-2. Under certain conditions, Th2 cells can also express appreciable amounts of IL-9. Th2-cell function has been implicated in allergic reactions, mucosal immunity, immunoglobulin E (IgE) production, eosinophil recruitment, and defense against parasitic helminthes (23, 24). Aberrant expression of the Th2 cytokines is strongly associated with development and progression of chronic inflammatory disorders and allergic diseases (25). IL-4, in particular, has been evidenced as the most potent cytokine-mediating Th2 differentiation (26); it stimulates the activation of STAT6, which in turn promotes the expression of the transcription factor GATA-binding protein-3 (GATA3), the master regulator for Th2 differentiation (27–29).

The Th1/Th2 paradigm has dominated the typing of CD4⁺ T cells for more than 15 years, until it was discovered that transforming growth factor (TGF)- β and IL-6 were able to drive the expression of IL-17A in a subset of CD4⁺ T cells. This finding led to the identification of a third lineage of CD4⁺ effector Th cells (13, 16, 17), designated as Th type 17 (Th17). Th17 cells were further characterized by their capacity to produce IL-17A, IL-17F, IL-21, and IL-22 as signature cytokines, representing a significantly distinct profile from the classic Th1 and Th2 types (30–33). Studies identified the principal functions of Th17 cells as being the induction and propagation of autoimmunity; these processes were defined at the molecular level as being mediated principally by the secreted IL-17 cytokine, which itself has been associated

with multiple sclerosis, rheumatoid arthritis, psoriasis, and inflammatory bowel disease (34, 35). Furthermore, Th17 cells appear to play a role in host defense mechanisms in response to extracellular bacteria and fungi and can enhance the cytotoxic T-lymphocyte (CTL) responses against cancer. Th17 cells have also been implicated in the pathogenesis of various allergic diseases, such as allograft transplantation rejection (36, 37). Within the Th17 cells, IL-6 and TGF- β activation of STAT3 can induce expression of the master transcription factor retinoic acid-related orphan receptor (ROR) γ t; activated STAT3 and ROR γ t can then induce the expression of the Th17 cytokines IL-17A/F and IL-22 (38–41). Recently, a novel subset of Th2 cells was described that co-express GATA3 and ROR γ t and that produce cytokines associated with both Th17 and Th2 (42); notably, these IL-17-producing Th2 cells were also found to be increased in asthma patients and associated with robust induction of inflammatory cytokines (42).

The definition of particular cell surface markers and/or cytokine profiles represents a well-established method to distinguish the Th cell types. Another Th subtype, the Tregs, was identified in this manner. Since then, Tregs have been divided into two main categories: the naturally occurring Tregs (nTregs) and the induced Tregs (iTregs). The nTregs develop in the thymus following T-cell receptor stimulation, IL-2 and TGF- β signaling, together with co-stimulation of CD28 stimulation through its cognate ligand on antigen-presenting cells (43). The discovery of the Th17 cell type led the investigators to recognize an additional subset of T cells that had the characteristics of Tregs but which differentiated from naive T cells; thus, the so-called inducible Tregs (iTregs) were identified (44). The forkhead protein Foxp3 appears to act as the master transcription factor of the Tregs. Distinct from all the other Th cell types described above, the most important function of Tregs involves the maintenance of self-tolerance and immune homeostasis through their immunosuppressive properties. As such, Treg dysfunction has been associated with particularly high risks for developing autoimmune and inflammatory pathologies (43, 45).

Currently, it is widely accepted that Th lineages comprise four distinct subsets: Th1, Th2, Th17, and Tregs (Figure 1). Based on the vast body of experimental research that has been carried out to date, one can conclude that each of the Th cell lineages arises from particular physiological milieu, composed of unique combinations of master transcription factors, surface markers, and cytokine profiles that may be shared by the other types. Such complex and dynamic interplays complicate our ability to determine whether a population of Th cells actually represents a novel Th lineage. To this end, the following criteria may be useful: (i) naive CD4⁺ T cells differentiate independently into each lineage *in vitro* and *in vivo*; and (ii) each lineage harbors a collective gene expression signature that is distinct and heritable. Accordingly, the Th9 cells appear to represent a novel Th subset.

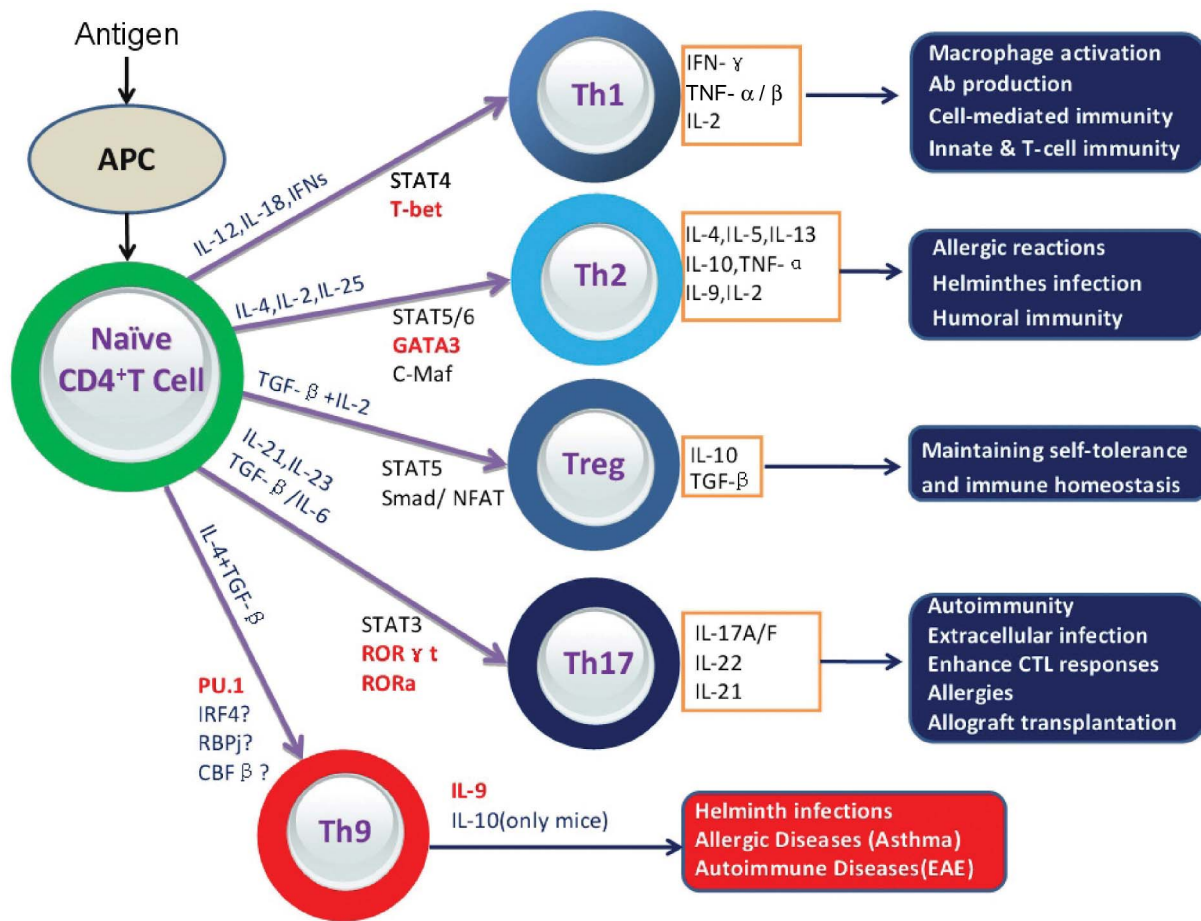


FIGURE 1.—The heterogeneity of CD4⁺ Th lineages. Following antigen-presenting cell-induced activation, naïve CD4⁺ T cells undergo clonal expansion and differentiate into various subsets of functional Th cells. Th1 cells are characterized by high production of IFN- γ and the specific transcription factor T-bet and are involved in macrophage activation, antibody production, cell-mediated immunity, and innate and T-cell immunity. For Th2 cells, IL-4 triggers differentiation; Th2 cells then produce IL-4, IL-5, and IL-13 and participate in humoral immunity, clearance of parasites, and allergic reactions. The combination of TGF- β and IL-6 triggers the differentiation of Th17 cells; Th17 cells then produce large amounts of IL-17A/F, IL-21, and IL-22 and play functional roles in autoimmunity, extracellular infection, enhancement of CTL responses, and allograft transplantation. In addition, Th17 cells act on macrophages and CTLs and are indispensable for cellular immunity and clearance of intracellular pathogens. TGF- β alone is the principal factor that induces the differentiation of iTregs, which specifically express Foxp3 as their signature transcription factor. The iTregs are involved in maintaining self-tolerance and immune homeostasis. Recently, the combination of IL-4 and TGF- β was found to induce a novel IL-9-producing subset, called the Th9 cells. PU.1 was demonstrated as being responsible for this lineage commitment. IRF4, RBPJ, and CBF β represent candidate signature transcription factors of these cells. Th9 cells may play critical roles in helminth infection and inflammatory diseases, such as allergic asthma and EAE.

Th9: A Novel CD4⁺ Th Lineage

As cited above, IL-9 was originally categorized as a Th2-associated cytokine, based on its being largely produced by Th2 cells and involved in Th2-like inflammation and pathology (46). When IL-9-deficient mice were created, however, researchers were surprised to find no shortage in T-cell development; moreover, there was a marked absence of antibody responses to and clearance of the helminth *Nippostrongylus brasiliensis*. It was concluded that the functions of IL-9 were, at least partially, redundant in Th2 immune defense (47). Many non-Th2 cells, including Th1, Th17, Tregs, and some memory T cells, had been shown to preferentially produce IL-9 under different conditions. Thus, the generation of IL-9 was hypothesized to be highly regulated by the local cytokine milieu. The coordination of TGF- β and IL-4 was found to significantly enhance IL-9 production from activated naïve CD4⁺ T cells, whereas IFN- γ was found to inhibit

this signaling pathway (48). In addition, similar molecular events were observed when TGF- β and anti-IFN- γ coexisted, but the extent of the IL-9 production was not as robust. Furthermore, IL-9 production was found to be influenced by Th1 response and Th1 IFN- γ production in IL-10-deficient mice (48). Another group reported that a culturing technique using ovalbumin, IL-2, IL-18, and antigen-presenting cells could lead to increased IL-9 and IFN- γ production by memory Th1 cells (49).

More recently, IL-9 was identified as one of the major cytokines produced by and able to functionally influence the Th17 cells (50, 51). It was also found to be capable of facilitating the differentiation and development of Th17 cells in response to TGF- β through JAKs/STAT3 signaling pathways, independently of IL-6 signaling; IL-23 appeared to negatively regulate this effect by reducing the amount of IL-9 secreted from Th17 cells (51). Meanwhile, suppressive effects of IL-9 on Th17, through

the STAT5 pathway, were observed *in vitro* and *in vivo* (52). Although the precise mechanism by which IL-9 acts on Th17 cells is still not clear, it is evident that IL-9 from Th17 cells plays critical roles in Th17 generation and responses. IL-9 has also been shown to be produced by nTregs in peripheral tissues and by iTregs that were generated in the presence of TGF- β (53). High levels of IL-9 production appear to act as a survival mechanism for nTregs; exogenous addition of IL-9 has been shown to partially rescue Treg cells from apoptotic cell death and to significantly increase the number of viable cells in culture (51). The reason for such discrepant findings could be that peripheral CD4⁺CD25⁺ T cells actually represent a mixture of committed Foxp3⁺ Tregs and recently activated Th cells that already produce IL-9. Similarly, activation of CD4⁺CD25⁺ T cells, which are a mixture of naive and effector/memory T cells, in the presence of TGF- β gives rise to both Foxp3⁺ iTregs and Foxp3⁻ effector T cells that subsequently produce IL-9 upon activation (54).

Overall, a complex plasticity and heterogeneity exist among IL-9-producing cells. In all probability, a distinct subset of cells that produces large amounts of IL-9 is present in the living system. In support of this idea, IL-4 has been shown to be capable of inducing IL-9 production from CD4⁺ T cells in the presence of TGF- β (48). Over a decade later, Veldhoen *et al.* (15) demonstrated that the combination of TGF- β and IL-4 was able to induce the differentiation of naive CD4⁺ T cells into a distinct subtype of IL-9-producing subset Th cells, designated as the 'Th9 cells.' This observation was later confirmed in studies by Dardalhon *et al.* (14).

In vitro studies using quantitative PCR analysis to evaluate murine highly polarized effector T cells revealed that these IL-9-producing T cells emerged in response to TGF- β 1 together with IL-4, distinct from the IL-6 response of Th17 cells. Furthermore, naive CD4⁺ T cells cultured in conditions suitable for Th1, Th2, iTreg, or Th17 cells did not lead to IL-9 expression. Reverse transcription-PCR- and ELISA-based studies revealed that IL-9 mRNA and protein expression were concordant. Furthermore, the presence of recombinant IL-9 was demonstrated to promote further expression of IL-9, indicating an autocrine positive-feedback mechanism at work. Meanwhile, neither nTregs cultured in IL-2 with/without TGF- β nor iTregs from mice treated with TGF- β presented detectable IL-9 signals. In contrast, naive CD4⁺ T cells differentiated toward lineages of IL-9-producing cells in culture when exposed to a combination of TGF- β and IL-4; however, a small proportion of these cells did co-express Foxp3, IL-4, or IL-17. Reverse transcription-PCR by another group indicated that these IL-9-producing cells did not express any of the traditional CD4⁺ T-cell transcription factors: T-bet, GATA3, ROR γ t, or Foxp3.

The combination of IL-4 and TGF- β treatment appeared to induce murine Th2 cells to shut off the production of IL-4, IL-5, and IL-13 and led to a gain in production of IL-9 and IL-10. These reprogrammed IL-9⁺IL-10⁺ T cells presented no regulatory properties, but produced abundant amounts of IL-10, the only cytokine related (but not specific) to Th2 cells; interestingly,

STAT6 and GATA3 were still required for their development. It is known that both Th1 and Th17 cells can produce IL-10 under certain conditions (55). Further analysis of the differentiation properties indicated a dependence of IL-9-secreting T cells on the presence of TGF- β and IL-4, because naive CD4⁺ T cells isolated from mice expressing a dominant negative TGF- β receptor II (CD4dnTGFbRII) or from STAT6^{-/-} mice were unable to develop into IL-9-producing cells even when cultured in the presence of IL-4 and TGF- β . Collectively, the data described above support the theory that another CD4⁺ Th lineage exists that is distinct from the traditional subsets (Th1, Th2, Th17, Tregs). This new cell type, named as Th9, appears to differentiate from a particular cytokine milieu, which includes TGF- β and IL-4, and is referred to as 'Th9 conditions' (15) (Figure 1).

As is often the case with scientific discoveries, unrelated groups observe new physiological phenomena almost simultaneously. A second group investigating the suppressive effects of IL-4 on TGF- β -induced Foxp3 expression noted that IL-4, in conjunction with TGF- β , induced the generation of IL-9⁺IL-10⁺Foxp3⁻ T cells (14). Although these cells produced considerable amounts of IL-10, *in vitro* studies indicated their apparent differences from the Foxp3⁺ Tregs. The new cells lacked anergic and suppressive functions associated with their own acute proliferation and were unable to facilitate the proliferation of other effector T cells. Likewise, *in vivo* studies revealed that IL-9⁺IL-10⁺ T cells significantly contributed to events of tissue inflammation, such as those observed in colitis and peripheral neuritis. Overall, these observations further confirmed that Th9 represented a novel lineage of CD4⁺ Th cells.

The requirements for the generation of human Th9 cells were only defined in this past year. Wong and colleagues confirmed that the *in vitro* Th9 conditions (TGF- β and IL-4) that induce the differentiation of Th9 cells from naive CD4⁺ T cells in mice are evolutionarily conserved in humans. Human Th9 cells share a similar, but slightly extended, cytokine profile with murine Th9 cells; specifically, human Th9 cells do not express IL-10 (56–58).

Other Regulatory Factors for Th9 Cell Development

Since the discovery of the Th9 cell type, significant efforts have focused on elucidating its regulatory mechanisms. Several factors have been identified that contribute to its lineage commitment. IL-25, a member of the IL-17 cytokine family, was previously characterized as an important factor in promoting the differentiation and function of Th2 cells through IL-4/STAT6 pathways (59). More recently, Angkasekwina *et al.* (60) identified IL-25 as a potential regulator of Th9 cells *in vitro*. Unlike IL-4, IL-25 was found not to be involved in the initiation of Th9 polarization *in vitro* because no IL-9 expression was detected in IL-25 stimulated CD4⁺ T cells, with or without TGF- β . Robust production of IL-9 by IL-25 requires the combination of TGF- β and IL-4 to induce the expression of IL-17RB, a key factor for IL-25 signaling, highlighting the significance of 'Th9 conditions' for

Th9 responses. Although the regulation of IL-25 in IL-9 production *in vivo* is confirmed in this article, we cannot determine where the elevated IL-9 comes from Th2, Th9, or other sources.

In addition, IL-12 or IL-21 can also promote IL-9 production in IL-4⁺TGF- β ⁺pCD3/sCD28-activated memory CD4⁺CD25⁺CD45RO⁺ T cells, but to a significantly lesser extent than IL-1 β (57). Both IFN- γ and IL-27 have been implicated in the inhibition of IL-9 production in human naive CD4⁺ T cells cultured with TGF- β and IL-4 (56). Although the precise mechanisms used by these cytokines remain unknown, the findings to date have provided useful insights into the differentiation and regulation of Th9 cells.

Th9-Specific Transcription Factors

It is known that the different lineages of CD4⁺ T cells are controlled by unique interplays of shared transcription factors and that the differentiation events culminate in the expression of distinct panels of cytokines and effector genes. Although the exact Th9-specific (signature) transcription factor has yet to be identified, it has been determined that it is not T-bet (Th1), ROR γ t (Th17), or FOXP3 (Tregs) (15). Recently, Chang *et al.* provided evidence that identified PU.1 as a potential Th9-specific transcription factor, effectively strengthening the proposed uniqueness of Th9 cells (61). PU.1 is an E-twenty six (ETS)-family transcription factor that is expressed specifically in the subpopulations of Th2 cells with relatively low IL-4 expression (62). Ectopic PU.1 expression was demonstrated to decrease the production of most Th2 cytokines and phenotype conversion from Th2 to Th9; however, PU.1 can modestly induce IL-9 production in iTreg, Th1, or Th17 cell cultures. In contrast, PU.1 deficiency induced by both short hairpin RNA targeting and gene knockout were shown to specifically accelerate total cytokine production by cells secreting two or more cytokines. Taken together, these observations indicate that PU.1 is capable of inhibiting Th2-related cytokine production in T cells and uniquely contributes to the Th9 phenotype. PU.1 function in Th9 cells may partly rely on limited interactions with transcription factors that support the Th2 phenotype, such as GATA3 and IFN-regulatory factor 4 (IRF4), and by targeting loci that encode Th9-related cytokines (62–64).

When cultured under Th9 conditions, PU.1-deficient murine T cells were found to produce significantly lower levels of IL-9, whereas ectopic expression of PU.1 by a bicistronic retrovirus resulted in higher levels of IL-9 being produced. These results supported the idea that PU.1 is important for Th9 cell development. Quantitative PCR and ELISA assays have revealed significantly high levels of PU.1 mRNA in Th9 cells, compared with that in Th1, Th2, or Th17 cells. Interestingly, PU.1 transcript levels detected in Th9 cells were lower than in iTregs. Further analysis determined that PU.1 deficiency in Tregs did not affect Foxp3 expression, indicating a specific role of PU.1 for Th9 phenotypes. Moreover, chromatin immunoprecipitation and DNA-affinity-precipitation assays followed by immunoblotting

demonstrated that PU.1 specifically bound to conserved noncoding sequences in the promoter of the IL-9 gene. Thus, it appears that PU.1 is required for chromatin modifications of the IL-9 locus. As importantly, these analyses also confirmed the Th9 conditions for human CD4⁺ Th cells and demonstrated that diminishing PU.1 expression in human Th9 cultures was associated with reduced IL-9 production *in vitro*. Finally, PU.1 was characterized as being indispensable for IL-9 expression in human Th9 cells, indicating the evolutionary conservation of development-related functions in human and murine Th9 cells (61).

Previous studies have proven that IL-9 can promote allergic inflammation in transgenic animal models (7, 65), and blocking of IL-9 effectively inhibits the development of inflammation (66). T-cell-specific PU.1-deficient mice were also found to present with attenuated pulmonary inflammation, compared with wild-type mice suffering from allergic airway inflammation. Specifically, fewer inflammatory infiltrates and decreased airway hyperresponsiveness (AHR) were identified in the PU.1-deficient mice. Interestingly, both Th2 and Th17 cells developed normally in these animals, and the specific absence of Th9 cells was functionally implicated in the observed response. In the ovalbumin-induced model, it was confirmed that blockade of IL-9 with antibodies led to decreases in allergic inflammation, suggesting that IL-9 was important for regulating the inflammatory response in the lung, although other PU.1-dependent cytokine expressions could not be ruled out as potential contributors to the inflammation events (61).

The above-described investigations into PU.1 involvement in Th9-mediated immunity indicated that this transcription factor acts as an important modulator of the Th9 phenotype and is critical for the development of allergic inflammation. However, PU.1 does not seem to fulfill the signature role as the sole and unique factor capable of promoting the differentiation and development of Th9 cells. Studies have shown that the transcription factor IRF4 is essential for the establishment and maintenance of Th9 cell development and function (58). CD4⁺ T cells from IRF4-deficient mice failed to differentiate into Th9 cells under 'Th9 conditions.' Likewise, down-regulation of IRF4 by IRF4-specific siRNA significantly inhibited IL-9 production by Th9 cells. Chromatin immunoprecipitation assays also revealed that IRF4 induced by TGF- β could directly induce Th9 differentiation and regulate the IL-9 expression by binding to the IL-9 gene promoter.

More recently, van den Ham *et al.* developed a novel analysis method to identify polarizing master genes of CD4⁺ Th cells (67). This method comprised a scoring system based on the specific behavior of master genes in the Th cells (such as up-regulation and polarization of expression level) to quantify the deviation from the diagonal, designated as a 'polar score.' This approach was demonstrated to be superior to the available generic approaches for identifying polarizing genes. The calculated polar scores supported the existence of Th9 cells and some other molecules related to Th9 differentiation, such as interleukin 1 receptor antagonist

(IL1RN), tumor necrosis factor ligand superfamily member 11 (TNFSF11), receptor activator of nuclear factor kappa-B ligand (RANKL), chemokine (C-C motif) ligand 17 (CCL17), and potential arachidonate 5-lipoxygenase-activating protein (ALOX5AP). Moreover, several candidate master transcription factors believed to direct the Th9 phenotype, including recombination signal binding protein for immunoglobulin kappa J. region (RBPJ) and CBF β , were also identified by this method. It was particularly striking that RBPJ, which is known to bind to the T-box protein 21 (TBX21) promoter and participate in Notch signaling, was identified as a potential master transcription factor of Th9 differentiation (23, 68).

Collectively, these conclusions have broadened our understanding of the mechanisms involved in Th9 fate decisions. Still, further experiments are needed to clarify the detailed relationship and pathways associated with such candidate master transcription factors. The immune functions of Th9 cells, however, are relatively well established as being involved in the protection against infections with helminth parasites and the pathogenesis of allergic inflammation (14, 15, 69). The next section, then, will describe the data obtained from investigations in human and animal models to determine the possible contribution of Th9 to the development and persistence of allergic asthma.

TH9 CELLS IN ALLERGIC ASTHMA

Asthma is a complex disease characterized by three major features: (i) intermittent and reversible airflow obstruction with recurrent episodes of wheezing, breathlessness, chest tightness, and cough; (ii) bronchial hyper-responsiveness; and (iii) airway inflammation (3). Many types of inflammatory cells, such as mast cells, B cells, eosinophils, and lymphocytes, and their secreted cytokines, are involved in the pathogenesis of asthma (70). Allergic asthma has been described as a chronic pulmonary inflammation caused by inhaled allergens; accumulation and activation of CD4⁺ T cells, eosinophils, and mast cells; and elevated IgE levels in the serum are used as clinical diagnostic indicators (37, 71). Th2 cells are responsible for orchestrating the asthmatic inflammation through release and action of their particular panel of cytokines, namely, IL-4, IL-5, and IL-13 (3). Recently, IL-9 was proposed to act as a significant modulator of the pathogenesis of allergic asthma, which has since been confirmed by a series of subsequent studies.

Genetic predisposition and environmental exposures are critical risk factors associated with the development of allergic asthma. Genetic studies along with experiments *in vitro* and *in vivo* have yielded some clues to the particular role that Th9 cells play in allergic asthma. As described above, human Th9 cells, unlike murine Th9 cells, produce IL-9 rather than IL-10 as the major effector molecule. IL-9 is a 14 kDa glycoprotein made up of 144 amino acids. The protein sequence includes a signal sequence of 18 amino acids, which contains a high proportion of cationic amino acid residues and 10 cysteines along with four N-linked glycosylation sites (72). IL-9 belongs to the

four-helix bundle cytokine family, and the human IL-9 gene lies within the Th2 cytokine cluster on chromosome region 5q31–35 (73). This region is also known to contain the genes encoding IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-13, IL-15, CD4, and granulocyte-macrophage colony-stimulating factor (72, 74, 75). Polymorphisms and linkage disequilibrium in this region have revealed a close association between IL-9 and asthmatic phenotypes, such as BHR and elevated total IgE levels, implicating IL-9 as a candidate gene for allergic asthma (76, 77).

Murine IL-9 genes, on the contrary, are located on chromosome 13 and share 69% homology with humans, having five exons and four introns that extend over approximately 4 kb. Although each of the five exons is identical in size among the species, respectively, they range in homology at the sequence level (72). Additionally, the diverse sequences in murine IL-9 genes in different strains of mice have been proposed to mediate the different asthmatic phenotypes observed among them (72, 73).

The human IL-9 receptor gene is located on the long arm of the XY pseudoautosomal chromosome region, which was subsequently shown to be closely associated with asthma and BHR (78). Human IL-9R gene transcripts present an intriguing heterogeneity. Alternative splicing mechanisms leading to the insertion of one or two alternative exons, 28 nucleotides downstream from the putative initiation codon, might be responsible for the genetic susceptibility to asthma (79). In contrast, the murine IL-9R gene is composed of nine exons and eight introns and shares many features with other cytokine receptor genes. The above-described genetic analyses strongly support a pivotal function of IL-9/IL-9R pathways in the complex pathogenesis of asthma.

With the exception of genetic evidence, data from different experiments *in vitro* and *in vivo* have provided more insights into the significance of IL-9 and Th9 in allergic asthma. For humans, IL-9 was found to be a critical regulator for inducing mucus hypersecretion events in asthmatic subjects (80). Bronchial biopsies revealed that when stimulated by allergens, cells in the bronchial mucosa of atopic asthmatics expressed significantly more IL-9 mRNA than normal controls, or individuals with chronic bronchitis and sarcoidosis; this event was also positively correlated with the number of eosinophils and mast cells present at the site of inflammation (8, 81). Moreover, IL-9 production from CD4⁺ Th cells in lung tissues of atopic subjects with allergic asthma was higher than in nonasthmatics (9, 81). It is important to note that the amount of Th2-type cytokines that are produced can elicit a similar response (9, 82). It has also been reported that segmental allergen challenge was able to induce increased IL-9 production, mainly from lymphocytes, in the bronchoalveolar lavage fluid of atopic asthmatics (9). In addition, the level of IL-9 secreted by peripheral blood mononuclear cells was correlated with the titer of allergen-specific IgE in adult atopic asthmatics (83). These presentations, however, do not diminish the importance of Th2 cytokines in the development of allergic asthma, but indicated that these two subsets can work in coordination to initiate

allergic inflammation (61). In short, findings from the human system have suggested that IL-9 can indeed play an enhancing role in the development and progression of atopic asthma, whereas a better understanding of whether and to what extent IL-9 is involved will rely on invasive examination in mouse models.

To date, several mouse models have been established to investigate the precise functions of IL-9 in the pathogenesis of allergic asthma (Table 1). Administration of intratracheal recombinant murine IL-9 in naive C57BL/6 mice led to lung eosinophilia, expression of IL-5Ra, and enhanced serum IgE levels (84). Furthermore, lung-specific overexpression of IL-9 led to more severe symptoms of asthma, even in the absence of allergen (7). IL-9 was found to promote the proliferation of bone marrow-derived mast cells in conjunction with IL-3 by inducing the expression of transcripts encoding mast cell-specific proteases and granzyme B proteases. IL-9 was also found to be able to enhance the mRNA expression of the α -chain of the high-affinity IgE receptor and induce IL-6 production by mast cells (5, 85, 86). IL-9 alone, however, did not affect IgE production by murine B cells *in vitro*, but when expressed together with IL-4, IgE expression from lipopolysaccharide-activated murine B cells was apparent (6). The mechanism(s) regulating these events remain elusive.

Studies on human subjects have yielded similar results. Exposure to anti-IL-9 antibodies led to reduced IgE production by IL-4- and IL-7-activated human peripheral blood mononuclear cells (92). These observations suggested that IL-9 might facilitate the later phase of IL-4-mediated IgE production. As with the accumulation of eosinophils, previous findings indicated that IL-9-induced

eosinophilia *in vivo* depends on the presence of IL-5. Moreover, IL-9 was shown to up-regulate the expression of eotaxin and other chemokines in lung epithelial cells, indicating possible direct and indirect influences on the proliferation and migration of eosinophils. Further studies, however, are required to confirm this supposition (93–95).

Allergic pulmonary inflammation and airway hyperactivity have been shown to mask the difference between IL-9^{-/-} mice and wild-type controls, suggesting that other Th2 cytokines can functionally substitute for IL-9 in the development of allergic asthma (88). However, blockade of IL-9 with specific antibodies significantly reduced allergic inflammation, as evidenced by decreased numbers of eosinophils and lymphocytes and production of IL-4, IL-5, IL-13, and macrophage-derived cytokines in bronchoalveolar lavage fluid (66). The discordant results may be due to the different models used in the two studies. The IL-9 knockout was generated during the embryonic stage of a mouse; considering the wide interactions between IL-9 and other targets, disruption of IL-9 at this early stage might have unexpectedly influenced the normal development of the murine immune system. In addition, reduction in IL-9-producing T cells, mostly Th9 cells, was associated with decreased allergic inflammation in the presence of a normally developed Th2 cell population (61).

Recently, through the establishment of the *Rag2*^{-/-} mouse models that were adoptively transferred with Th9 or Th2 cells, Th9 cells were characterized to have the capacity of mediating severe asthma symptoms comparable to that mediated by Th2 cells (58). Treatment of Th2 cell recipient *Rag2*^{-/-} mice with a neutralizing IL-9 antibody only slightly ameliorated asthma symptoms.

TABLE 1.—Experimental models of IL-9 in asthma pathogenesis.

Models	Major conclusions	References
IL-9 transgenic mice	IL-9-induced subepithelial fibrosis relies on eosinophils and profibrotic mediators in a concentration-dependent manner. Lung-specific overexpression of IL-9 led to more severe symptoms of asthma, even in the absence of allergen. IL-9, together with IL-4, increased the IgE expression from LPS-activated murine B cells	(6–7, 87)
IL-9 knockout mice	IL-9 was not obligatory for the development of eosinophilia and airway hyperresponsiveness. It is likely that other Th2 cytokines can act in a compensatory fashion	88
PU.1-deficient mice	Reduction in IL-9-producing T cells caused weakening of the allergic inflammation associated with a normally developed Th2 cell population	61
Rag2 knockout mice	Th9 and Th2 cells showed identical capacity for mediating allergic asthma <i>in vivo</i> . Stable Th9 cells exist <i>in vivo</i> and Th9-derived IL-9 plays crucial functions in the pathogenesis of allergic asthma <i>in vivo</i>	58
IL-13-deficient mice	In the absence of IL-13, IL-9 mediated mucus production and completely abolished the expression of normally up-regulated genes	89
Mast cell-deficient mice	Mast cells were involved in epithelial goblet cell hyperplasia, up-regulated expression of the mucin genes <i>muc5ac</i> and <i>muc5b</i> , and increased levels of lung collagen. IL-9 may indirectly influence mucus metaplasia and subepithelial fibrosis	90
C57BL/6(B6) mice	Increased level of IL-9 in the airway induced lung eosinophilia and serum total IgE levels. Murine trachea tissue exhibited <i>muc5ac</i> up-regulation and histopathological changes in response to administration of recombinant IL-9, in the absence of inflammatory cells	84, 91
Ovalbumin-sensitized BALB/c mice	Blockade of IL-9 with specific antibodies significantly reduced allergic inflammation	66
Allergen-challenged dogs	IL-9 directly induced the expression of <i>muc5ac</i> gene <i>in vitro</i> , a chief mucin in bronchial epithelia	66
A murine T-cell clone	IL-9 enhanced the mRNA expression of the α -chain of the high-affinity IgE receptor	86

In sharp contrast, application of the neutralizing IL-9 antibody to Th9 cell recipient *Rag2*^{-/-} mice resulted in a profound amelioration of Th9 cell-mediated asthma. These data clearly indicate the stability of IL-9-producing Th9 cells *in vivo* and that the pathophysiological role of Th9 cells in allergic asthma is predominantly mediated by IL-9 (58). Presumably, IL-9 plays only a marginal role in the asthma-promoting activity of Th2 cells. These results collectively indicate that Th9 cell-derived IL-9 is a significant contributor to the pathogenesis of asthma.

The above-described study, however, only delineated the asthma-related roles of IL-9 from Th2 and Th9 cells. Considering that there are so many potential cellular sources of IL-9 involved with asthma, we cannot exclude the possibility that IL-9 from other cell types is functionally involved. For instance, once reactivated by antigen and IL-18, Th1 memory cells were able to induce airway hyperresponsiveness and airway inflammation by up-regulating the expression of many asthma-associated factors, including IL-9 (49). Nevertheless, this experiment did not use Th9 cells as a control, thus it is difficult to compare the differential contributions to asthma of the memory Th1 cells and Th9 cells. Another study has demonstrated that invariant natural killer T cells (NKT) cells have the capacity to produce IL-9, whereas the precise correlation with asthma remains undefined (96). Finally, when eosinophils and mast cells are taken into consideration, the cellular sources of IL-9 relating to asthma pathogenesis seem to be complex and dynamic in nature, and further studies are needed to elucidate this issue.

Based on the findings available to date, we can draw the conclusion that IL-9 and Th9 play critical roles in the regulation and promotion of allergic asthma and inflammation. However, whether asthma-associated IL-9 originates from other potential sources besides Th9, and how Th9 cells affect allergic asthma, needs to be investigated further. Overall, targeting IL-9 or Th9 cells may be a viable strategy for developing novel therapies for treating patients with asthma and other allergic inflammation disorders (97).

IL-9 AND AIRWAY REMODELING

The epithelium of asthmatic airways is known to exhibit different levels of structural changes, a hallmark of allergic asthma referred to as 'airway remodeling.' These changes include epithelial cell mucus metaplasia, smooth muscle hypertrophy/hyperplasia, subepithelial fibrosis, and increased angiogenesis (98), processes that are mediated by cytokines, chemokines, and growth factors derived from inflammatory and structural cells in the airway (99). Among them, IL-9 has been proven to act as a key factor mediating increased mucus production from airway epithelia in asthma. IL-9 was evidenced *in vitro* to directly induce the expression of the *muc5ac* gene, a chief mucin in bronchial epithelia; IL-5 and IL-13 do not have this feature, indicating a distinct function of IL-9

in airway structural changes during Th2-induced asthma pathogenesis (100). Reader *et al.* (91) further demonstrated that administration of recombinant IL-9 led to *muc5ac* up-regulation and histopathological changes in murine trachea, in the absence of inflammatory cells. However, studies in IL-13-deficient mice suggested that an indirect mechanism of IL-9-induced mucus metaplasia was at work (89); in the absence of IL-13, IL-9-mediated mucus production and the up-regulated gene expression were completely abolished, indicating the existence of an IL-13-dependent pathway (89). In addition, evaluation of mast cell-deficient mice revealed that mast cells were involved in epithelial goblet cell hyperplasia, up-regulated expression of the mucin genes *muc5ac* and *muc5b*, and increased levels of lung collagen, suggesting another indirect influence of IL-9 on mucus metaplasia and subepithelial fibrosis (90). In addition, the IL-9 transgenic mouse model revealed that subepithelial fibrosis induced by IL-9 relied on the concentration of eosinophils and profibrotic mediators (87). Undoubtedly, a close functional relationship exists between IL-9 and airway remodeling in asthma; however, the precise mechanisms involved remain largely unknown (80, 100).

OTHER POSSIBLE TH9-ASSOCIATED DISEASES

The functions of IL-9-producing cells related to allergic disease and defense against intestinal nematode infection are relatively well characterized, as opposed to its roles in autoimmune diseases. Investigations involving the experimental autoimmune encephalomyelitis (EAE) mouse model revealed that Th9 cells, independently of Th1 and Th17 cells, could induce distinct central nervous system lesions. These lesions were characterized by less infiltration of small lymphocytes in the meninges but with massive and more evenly distributed parenchymal infiltrates associated with marked demyelination (101). Moreover, IL-9 was found to promote the development of peripheral nervous system (PNS) lesions, which may be related to Th17 cell differentiation events and Treg function (51). Together with TGF- β 1, IL-9 can induce Th17 cell differentiation from naive CD4⁺ T cells, which effectively enhances EAE. Moreover, the suppressive activity of Tregs is known to be up-regulated by IL-9 *in vitro* and weakened by neutralization of IL-9 *in vivo*. Blockade of IL-9 attenuates EAE and this event has been associated with decreases of Th17 cells and IL-6-producing macrophages in the central nervous system and of mast cells in the PNS (50, 51). These findings indicate the existence of a novel role for IL-9 as a regulator of autoimmune responses. Considering that the biology of IL-9 is complex and dynamic, Th9 may also play distinctive roles in all or many of the diseases associated with Th17 cells and Tregs.

SUMMARY

Investigations into the distinct CD4⁺ Th lineages and their particular cytokine profiles have afforded us a better

understanding of many related disease processes. The heterogeneity and plasticity of Th cells facilitates an intriguingly complex and dynamic milieu of mammalian physiology. As a novel member of the Th cell types, Th9 cells contribute to defense against parasitic infection, onset, and progression of autoimmune disorders and allergic inflammation, particularly asthma. The intricate biology of IL-9 empowers Th9 cells with these various functions. Recently, a series of master transcription factors have been proposed, and time will tell whether PU.1 represents the critical signature of the Th9 cell type. The precise functions of Th9 cells in relevant diseases have yet to be fully elucidated, and targeting the unique characteristics of Th9 cells might benefit patients with allergic asthma and other inflammation-related diseases.

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DECLARATION OF INTEREST

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