IL-9 and T$_h$9 cells: progress and challenges

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Abstract

T$_h$9 cells are a new subset of helper T cells, and the signature cytokine for T$_h$9 cells is IL-9. Both T$_h$9 cells and T$_h$9 products are implicated in multiple disease settings. Thus, a clear understanding of how T$_h$9 cells are induced and controlled is an important and clinically relevant issue. There are different molecular pathways identified thus far in the induction of T$_h$9 cells, and activation of such diverse pathways requires integration of signals from TGF-β and IL-4 cytokine receptors as well as costimulatory molecules. These signals converge on the induction of multiple transcription factors that collectively drive the development of T$_h$9 cells.

Keywords: allergy, IL-9, inflammation, T$_h$9

Introduction

IL-9 was originally cloned in 1989 from murine helper T-cell clones ([1]), so it is by no means a newly discovered cytokine. IL-9 *per se* has been extensively studied; IL-9 belongs to a family of cytokines that use the common IL-2Rγc for signal transduction, and similar to other family members (i.e. IL-2, IL-4, IL-7, IL-15 and IL-21), IL-9 was believed to be a T-cell growth factor and its chief function was to drive T-cell proliferation. But later studies showed that IL-9 has a weak effect in proliferation of primary T cells ([2]), despite the fact that proliferation of certain T-cell clones can be strongly stimulated by IL-9. Instead, IL-9 exhibits other functions, most noticeably in proliferation of mast cells, goblet cells and airway mucin-producing cells. Thus, in many ways, IL-9 is different from other γc cytokines as a T-cell growth factor. IL-9 signals through the JAK/STAT system. Specifically, upon binding to its cell surface receptor, which consists of a private IL-9Rα chain and the common γc, IL-9 induces recruitment of JAK1 and JAK3 to the IL-9Rα chain and the common γc, respectively, followed by cross-phosphorylation and activation of JAK1 and JAK3. This leads to the activation of STAT1, STAT3 and STAT5. Consequently, STAT1 and STAT5 form homodimers, while STAT1 and STAT3 form heterodimers, and such dimeric complexes translocate to the nucleus to drive transcription of IL-9-inducible genes ([3]). These gene products are involved in cell survival, proliferation and secretion of inflammatory mediators.

IL-9 is often seen in the context of T$_h$2 cells *in vitro* or T$_h$2-associated inflammatory conditions *in vivo*, especially in allergic inflammation ([4], [5]). Thus, for a long time, IL-9 was considered just another T$_h$2 cytokine and thought to be redundant among other T$_h$2 cytokines (i.e. IL-4, IL-5

and IL-13) ([6], [7]). Furthermore, IL-9 is not confined to T$_h$2 cells, and other cell types including mast cells, NKT cells, T$_h$17 cells or even T$_{reg}$ cells can become IL-9 producers ([1], [8]–[15]). Moreover, a recent study demonstrated by using IL-9-Cre reporter mice that even innate lymphoid cells are significant producers of IL-9 ([16]). So, IL-9 seems to be one cytokine of many sources, and therefore, interest in IL-9 biology and in its significance is diluted, and study of IL-9 has lagged behind that of others. The recent discovery that IL-9-producing cells are in fact a unique subset of CD4+ helper T cells that is different from other subsets, with distinct features and transcriptional controls, generates renewed interest in the field.

In this review, we summarize the latest advances in the study of T$_h$9 cells, discuss the evolving conditions that promote their differentiation as well as the *in vivo* relevance of T$_h$9 cells and finally we highlight some outstanding issues that remain to be resolved.

Defining T$_h$9 cells

Naive CD4+ T cells can be further specialized into functionally different subsets upon activation (e.g. T$_h$1, T$_h$2, T$_h$17, T$_h$22 and T$_{reg}$ cells), which are often measured by the distinct cytokine profiles they express ([17]–[20]). Subset specialization is driven primarily by the texture of cytokines in the local environment where naive the T cells are activated, with the induction of lineage-specific transcription factors as a critical event in further development of specific subsets ([21]). T$_h$9 cells are a recently described new helper T-cell subset; the signature cytokine for T$_h$9 cells is IL-9 (without IL-4). T$_h$9 cells, together

with other helper T-cell subsets, form a complex array of effector mechanisms in the immune system.

In many aspects, T$_h$9 cells are a unique helper T-cell subset. For example, in most studies, the frequency of T$_h$9 cells is very low (~5%), even under optimal polarizing conditions *in vitro* (22). This often casts considerable concerns over whether T$_h$9 cells are truly a distinct helper T-cell subset. Also, T$_h$9 cells are closely associated with T$_h$2 cells, as T$_h$2 cells co-express both IL-4 and IL-9 in the early phase of T$_h$2 differentiation, and the T$_h$2 cytokine IL-4 provides one of the key signals for T$_h$9 induction (23).

Furthermore, some of the transcription factors in T$_h$2 development are also involved in T$_h$9 induction. A clear example is that STAT6 knockout CD4$^+$ T cells fail to develop to T$_h$2 cells; they also fail to become T$_h$9 cells (24). However, T$_h$9 cells are not T$_h$2 cells. As discussed below, the culture conditions and cytokine milieu that lead to T$_h$2 and T$_h$9 cells are very different. In some T$_h$2 cultures, CD4$^+$ T cells that express IL-4 (T$_h$2 cells) and IL-9 (T$_h$9 cells) are completely segregated in that only those that lose the ability to express IL-4 will become IL-9 producers (23). Interestingly, only a small fraction of T$_h$2 cells acquire the ability to continually express IL-9. Importantly, the transcriptional regulation mechanisms of T$_h$9 and T$_h$2 cells are strikingly different from each other, thus clearly setting T$_h$9 and T$_h$2 cells apart (23).

Are T$_h$9 cells progeny of T$_h$2 cells? In most reports showing low levels of T$_h$9 cells under TGF-β and IL-4 culture conditions, T$_h$9 cells often co-express IL-10, which is another T$_h$2 cytokine (25). It is likely that such T$_h$9 cells are derivatives of T$_h$2 cells as a consequence of induction of additional transcription factors such as PU.1 (purine-rich box 1) and IRF4 (interferon regulatory factor 4) (26–29), which shut off IL-4 and turn on IL-9 (see below). In this setting, T$_h$2 cells are likely intermediaries that may further differentiate to T$_h$9 cells. However, our own studies suggest another pathway of T$_h$9 induction in which naive CD4$^+$ T cells can be directly converted to T$_h$9 cells at high levels (up to 80% of the CD4$^+$ T cells) by TGF-β and IL-4 when OX40 costimulation is engaged (30). Furthermore, we demonstrated that the non-canonical NF-κB (RelB–p52) pathway (the canonical pathway involves RelA–p50) rather than PU.1 and IRF4 is essential to T$_h$9 induction (Fig. 1).

T$_h$9 induction

Cytokines

A complex cytokine milieu is required for T$_h$9 induction, and integration of multiple cytokine signals is critical to optimal T$_h$9 development. The best cytokine mixture for T$_h$9 induction is a combination of TGF-β and IL-4, which contrasts sharply to the role of individual cytokines. TGF-β alone without IL-4 promotes T$_{reg}$ cells by inducing Foxp3, whereas IL-4 alone without TGF-β supports T$_h$2 induction. This highlights the complexity of T$_h$9 induction and also places T$_h$9 cells as a unique subset that is different from T$_h$2 and T$_{reg}$ cells. IL-4 activates STAT6 and IRF4, whereas TGF-β activates PU.1 and represses T-bet (T-box expressed in T cells) and GATA3 (GATA-binding protein 3), and the integration of those events eventually drives IL-9 expression (18, 31–35).

In some models, IL-1 favors induction of T$_h$9 cells; so does IL-25 or IL-33 (16, 36–38), although the exact mechanism remains to be defined. On the other hand, IFN-γ and IL-23 are potent inhibitors of T$_h$9 induction. Also, cytokines that stimulate IFN-γ production such as IL-12 and IL-18 also inhibit the induction of T$_h$9 cells. These cytokines most likely act through the induction of T-bet, which promotes T$_h$1 cells and opposes other helper T-cell lineages including T$_h$9 cells. Thus, the texture of cytokines fine-tunes the production of different helper T-cell subsets.
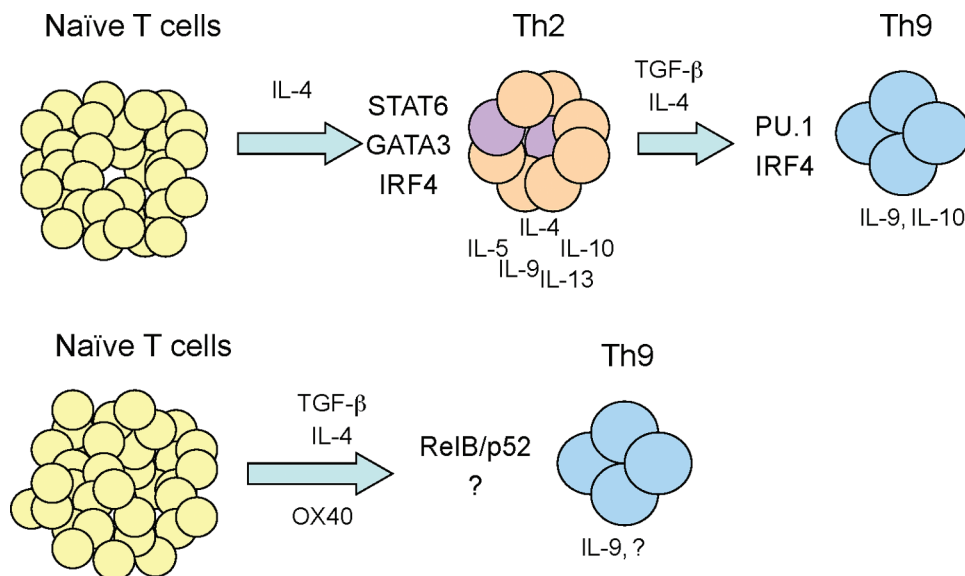

Fig. 1. Pathways of T$_h$9 induction. Naive CD4$^+$ T cells can be converted to IL-9-producing T$_h$9 cells via different molecular pathways. Depending on the presence or absence of OX40 costimulation, T$_h$9 cells can develop from a subset of T$_h$2 cells (shown in purple) or directly from CD4$^+$ T-cell precursors under TGF-β/IL-4-polarizing conditions.

Costimulatory signals

T-cell costimulatory signals control not only the status of T-cell activation but also the character of the T-cell response. We recently showed that OX40, a costimulatory molecule in the TNFR superfamily (TNFRSF), is surprisingly potent in promoting T$_h$9 cells (30), thus emphasizing the importance of costimulation, in addition to cytokines, in T$_h$9 induction. OX40 is expressed by activated, but not resting, T cells, especially activated CD4$^+$ T cells, and plays an important role in cell survival and proliferation (39). Specifically, we found that under T$_h$9-polarizing conditions, OX40 ligation on naive CD4$^+$ T cells resulted in a remarkable increase in T$_h$9 induction. Such T$_h$9 cells did not express detectable levels of IL-4 or IL-10 (30). Furthermore, OX40 ligation under T$_{reg}$- and T$_h$17-polarizing conditions potently inhibited the induction of Foxp3$^+$ cells and IL-17-producing cells (30). Thus, the effect of OX40 on T$_h$9 induction seems to be specific.

The role of OX40 in the induction of T$_h$9 cells was also observed *in vivo* where OX40L transgenic mice or injection of agonist anti-OX40 antibody induced allergic airway inflammation, as demonstrated by goblet-cell metaplasia and eosinophil infiltration (30). Mechanistically, OX40 signaling activates and sustains induction of the non-canonical NF-κB pathway (RelB–p52), which is critical to IL-9 transcription. This is further confirmed using both gain-of-function assays and loss-of-function assays. Under T$_h$9-polarizing conditions, overexpression of RelB–p52 in CD4$^+$ T cells led to much greater T$_h$9 induction by TGF-β and IL-4, and knockout of p52 drastically reduced T$_h$9 induction (30), which places RelB–p52 as a center piece in the induction of T$_h$9 cells. While interesting, these data also raise several questions on the role of other TNFRSF members in the induction of T$_h$9 cells, especially those that activate the non-canonical NF-κB pathway. Studies in this area deserve more attention.

Other costimulatory molecules that are known to affect T$_h$9 induction include the CD28 and Notch pathways. It has been shown that conditional deletion of Notch1 and Notch2 markedly decreased IL-9 production in T$_h$9 cultures (40). There are multiple ligands for the Notch receptors, and Jagged2 but not Delta-like 1 was shown to induce IL-9 production under TGF-β-based polarizing conditions. In an experimental autoimmune encephalitis (EAE) model, Jagged2-mediated IL-9 production was involved the EAE pathology, and conditional deletion of Notch1 and Notch2 in T cells attenuated the disease (40).

The exact mechanism of how Notch promotes the generation of T$_h$9 cells remains unclear. Notch signaling is known to favor T$_h$2 cells (41, 42), which may indirectly promote T$_h$9 cells. This notion is supported by the finding that exogenous IL-4 could overcome the effects of Notch1 and Notch 2 deficiency. Other studies indicate that Notch may modulate TGF-β signaling by acting on Smad (small/mothers against decapentaplegic) proteins. For example, in humans, Smad3 binds Notch 1, whereas Jagged2 and Delta-like 1 bind Notch 2 (43). It should be noted that T$_h$9 cells induced upon Notch stimulation are also at low levels, but PU.1 and IRF4 appear not critical to Notch-mediated induction of T$_h$9 cells.

Transcription machinery

Cytokine signals and costimulatory signals converge to activate the transcriptional apparatus that eventually drives the differentiation of T$_h$9 cells. Unlike other helper T-cell subsets, there is a hierarchy of transcription factors involved in both induction and differentiation of T$_h$9 cells. But a single 'master' transcription factor, as shown in other subsets, has not been identified thus far for T$_h$9 cells.

The IL-9 promoter region plus two additional regions of conserved non-coding sequences upstream of the promoter region form the *cis*- and *trans*-regulatory elements collectively regulating IL-9 gene expression (26, 35). Sequence analysis identified binding sites for a plethora of transcription factors, which include PU.1, IRF4, STATs, NFAT (nuclear factor of activated T cells), GATA1, GATA3, Smads and Notch as well as NF-κB and AP-1 (activator protein 1) (35), highlighting the complexity of IL-9 gene regulation. In a broad sense, the transcriptional control of T$_h$9 cells induced by polarizing cytokines (TGF-β and IL-4) and the polarizing cytokines plus costimulatory signals appears to be very different.

Under TGF-β- and IL-4-polarizing conditions, PU.1 and IRF4 have been identified as key transcription factors in the induction of T$_h$9 cells (26, 27). Overexpression of PU.1 in CD4$^+$ T cells facilitates T$_h$9 induction by TGF-β and IL-4, and deficiency of PU.1 inhibits the induction of T$_h$9 cells. Furthermore, PU.1 knockout mice exhibit reduced allergic lung inflammation in which T$_h$9 cells are known to be involved (26). Using a similar experimental strategy, IRF4 was shown to display the same effect on T$_h$9 development as PU.1 (27). Mechanistically, both transcription factors have been shown to bind to the promoter region of IL-9 and are capable of promoting IL-9 gene expression.

It should be noted that T$_h$9 development requires signals from both TGF-β and IL-4 cytokine receptors. In the absence of IL-4, TGF-β promotes T$_{reg}$ cells, and in absence of TGF-β, IL-4 leads to the development of T$_h$2 cells. As T$_h$9 cells are closely related to T$_h$2 cells, IL-4-mediated induction of STAT6 and the STAT6 target gene GATA3 are both required for T$_h$9 development (24, 25). However, it is not clear how STAT6 and GATA3 function to promote T$_h$9 cells at the cost of T$_h$2 cells, nor it is clear how STAT6 and GATA3 collaborate with PU.1 and/or IRF4 in T$_h$9 induction. In addition, the target molecules downstream of TGF-β signaling pathways that are critical to T$_h$9 development are incompletely defined. An intriguing point is that TGF-β and IL-4 only convert a small fraction of T$_h$2 cells to T$_h$9 cells (26); what renders some T$_h$2 cells responsive to the switch while other cells are resistant under the same T$_h$9-polarizing conditions warrants further clarification. Interestingly, a recent study from Chen Dong's group uncovered the importance of cytokine-induced SH2 protein (CIS) in the control of T$_h$2 and T$_h$9 differentiation (44). CIS is induced by IL-4 and suppresses the activation of STAT3, STAT5 and STAT6 in T cells. They found that STAT5 and STAT6 promote IL-9 expression by directly binding to the IL-9 promoter, and therefore, CIS-deficient T cells exhibit enhanced differentiation into T$_h$9 cells. Consequently, CIS-deficient mice spontaneously develop airway inflammation in which T$_h$9 cells are required (44).

In our own studies, we identified a new molecular pathway by which T$_h$9 cells develop, and this pathway is triggered by

OX40-mediated costimulation (30). One striking feature is that when OX40 costimulation is delivered to CD4$^+$ T cells, up to 80% of the CD4$^+$ T cells can be converted to IL-9-producing T$_h$9 cells by TGF-β and IL-4. Unlike previously reported T$_h$9 cells, such T$_h$9 cells have no detectable levels of IL-10 and are highly pathogenic in a mouse model of allergic lung inflammation (30). Thus, we believe that T$_h$9 cells developed under OX40 costimulation are *bona fide* T$_h$9 cells. However, OX40 must act in concert with polarizing cytokines in T$_h$9 development. We showed that OX40 signaling blocks the induction of inducible T$_{reg}$ cells and T$_h$17 cells and selectively diverts the cells to a T$_h$9 phenotype. However, without the polarizing cytokines, OX40 signaling instead supports T$_h$1 and T$_h$2 development (30).

Mechanistically, OX40 ligation activates, and most strikingly sustains, activities of the non-canonical NF-κB pathway (RelB–p52), which potently mediates T$_h$9 induction by TGF-β and IL-4. In fact, the promoter region of IL-9 has multiple NF-κB binding sites and RelB–p52 is directly involved in IL-9 transcription (30). These studies uncover additional complexities in IL-9 transcription and further suggest that, for T$_h$9 cells, additional signals besides those downstream of cytokine receptors are critically important. Our studies also raise other questions. For example, are other costimulatory molecules that also activate the non-canonical NF-κB pathway involved in T$_h$9 development? RelB–p52 is not acting alone, and PU.1 and IRF4 are not involved in OX40-mediated induction of T$_h$9 cells (30). Thus, what are the molecular partners downstream of TGF-β and IL-4 receptors that conspire with RelB–p52 in driving development of T$_h$9 cells? Are there any roles for the classical T$_h$2 transcription factors STAT6 and GATA3 in this process? Clearly, more studies are required to further clarify these questions.

Clinical relevance

What are T$_h$9 cells made for? There are several clinical settings in which T$_h$9 cells are implicated in the disease process. This suggests that intervention of T$_h$9 development may be therapeutically important. Studies from many laboratories including our own highlight the importance of T$_h$9 cells in allergic lung inflammation (30, 45, 46). However, the inflammatory response in the lung also involves other cell types besides T$_h$9 cells, and most prominently T$_h$2 cells. The interactions among different cell types in development and progression of the disease remain unclear, but T$_h$9 cells appear to play a particularly important role in airway epithelial alterations, which include goblet-cell hyperplasia, mucus production and infiltration of the airspace by mast cells and eosinophils. Indeed, blocking T$_h$9 cells markedly reduced the airway pathology while that in the lung parenchyma was not significantly affected (30), suggesting that T$_h$9 cells are a part, but may not the entirety, of allergic lung inflammation.

The role of T$_h$9 cells in other inflammatory conditions, especially chronic inflammation, remains to be determined. There are reports supporting a role for T$_h$9 cells in certain autoimmune diseases including EAE (40), suggesting that targeting T$_h$9 cells may provide an additional approach in treatment of such autoimmune conditions. There are other conditions where promotion of T$_h$9 cells might be therapeutically beneficial. It has been shown that IL-9 from mast cells promotes transplant tolerance to skin allografts by recruiting Foxp3$^+$ T$_{reg}$ cells to the grafts (15). Thus, neutralizing IL-9 resulted in failure of tolerance induction (15). By the same token, T$_h$9 cells might also be tolerogenic in transplant settings. However, considering the inflammatory nature of mast cells, T$_h$9 cells and cells recruited by IL-9, the exact role of T$_h$9 cells and T$_h$9 cell products in immunity and immune tolerance deserves careful clarification.

Another area that T$_h$9 cells recently attracted considerable attention is cancer therapy. Two laboratories independently reported that T$_h$9 cells exhibit remarkable therapeutic efficacy in cancer models (47, 48). In a highly aggressive B16 melanoma model, it has been shown that induction of T$_h$9 cells is associated with potent anti-cancer effects and favorable outcomes of cancer-bearing mice (47, 48). This is a significant area, considering the growing incidence of cancers and the limited choices in treatment of cancer patients.

Conclusions

T$_h$9 cells are a new and evolving subset of helper T cells. There are different molecular pathways identified thus far supporting the development of T$_h$9 cells, and integration of multiple signaling pathways downstream of cytokine receptors and costimulatory molecules is essential for specification and induction of T$_h$9 cells. The greatest effect on T$_h$9 induction is achieved by engaging OX40 under TGF-β- and IL-4-polarizing conditions. T$_h$9 cells and T$_h$9 products are highly pathogenic in allergic lung inflammation as well as in some autoimmune conditions, but they may be therapeutically desirable in other conditions such as cancer therapies. However, compared with other T helper subsets, T$_h$9 cells are less well studied. Many questions regarding T$_h$9 induction, the molecular machinery involved, their relationships with other helper T-cell subsets, especially T$_h$2 cells, and the exact role of T$_h$9 cells in immunity and immune pathology deserve further attention in future studies.

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