Immunology

A Subset Apart? Th9 cell regulation and current limitations to study

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Th9 cells are a CD4$^+$ T helper subset that has garnered considerable attention recently, due to their ability to produce large amounts of the cytokine IL-9. Many functional and regulatory roles associated with them are currently not fully understood. In this paper, we attempt to study several of the distinguishing features of Th9 cells to lead to a firmer understanding of the subset and its role in immunity. We consider and discuss some of the current limitations to studying Th9s, as well other Th9 properties of interest, on the levels of Th9 stimulation/differentiation, transcription factor interplay/ internal protein regulation, and cytokine secretion. In particular, we highlight the role of some potential co-stimulatory factors, as well as the utility of confirming a lineage-defining transcription factor. *Journal of Nature and Science, 1(5):e101, 2015*

Th9 | IL-9 | T helper differentiation | lineage-defining transcription factor

Introduction

T helper cells play a critical role in mammalian adaptive immunity, with the capability of shaping the immune response in many ways. Through their recognition of antigens caught and processed by antigen-presenting dendritic cells and macrophages (APCs), T helper cells can stimulate the activation of B cells and subsequent antibody production. T helper cells may also recruit many other immune cells to specific sites of inflammation through the release of potent chemoattractants, and also release cytokines to regulate immune cell function. CD4+ T helper cells undergo differentiation in response to environmental signals, with 6 subtypes (Th1, Th2, Th17, Th9, Treg, Tfh) being currently recognized commonly, and some (most notably Th22, but also Tfr) posited.[1] Each of these subsets has important, non-redundant functions in immune responses.[2] One of these subsets is T helper 9 (Th9), a phenotype which is deregulated in several inflammatory diseases, but which also plays an important role in the clearance of certain pathogens. In this review, we discuss in detail this phenotype's differences from other T helper phenotypes on several different levels, as well as potential information that may permit us to more fully understand the phenotype under scrutiny.

Th9 Stimulation

One of the key aspects of study regarding T helper cells is the manner by which they are induced, and consequently the scenarios in which they may arise. Understanding the cytokines necessary for stimulating differentiation can also provide vital information for conducting useful *in vitro* experiments, and give hints as to the signal transduction networks that are critically involved in the cell type. Generally however, each cell type can be stimulated from a variety of different conditions, making such analysis difficult. For instance, Th17 cells are commonly induced by a combination of tumor growth factor-β (TGF-β) and interleukin-6 (IL-6).[3,4,5] Cells induced in such a manner are positive for the "lineage-defining" transcription factor retinoic acid-related orphan receptor gamma t (RORγt), and secrete substantial amounts of IL-17.[6,7,8] However, RORγt$^+$ IL-17 producing cells may also be induced without TGF-β, in the presence of IL-6, IL-23, and IL-1b.[9,10,11] Interestingly, cells induced in the latter fashion may also be more pathogenic than those stimulated in the classical manner.[12] Similarly, Th9 cells are also subject to distinct differentiation conditions that may have important ramifications for their effects. Typically differentiated using a combination of IL-4 and TGF-β, Th9 cells have also been shown to favorably arise in the presence of IL-21, IL-6, and IL-21.[13,14,15,16] No clear consensus differences have currently been found recognized between these alternate polarizations. As such, it seems clear that a confluence of different factors may drive differentiation, and that the differentiation can follow from the effect of distinct pathways. The involvement of TGF- β is also additionally interesting due to the known role of Th9 cells in combatting helminthes. One paper has suggested that helminthes may secrete a compound that can mimic the effect of TGF- β to suppress T effector function—the possibility that Th9 cells may also be able to use the signal as an evolutionary defense against helminthes is quite fascinating.[17]

The classification of these pathways is also particularly intriguing in the context of understanding the effects the various T helper subtypes may have. Prior to the discovery of Th17 cells, T helpers were classified into a Th1/Th2 dichotomy, in which Th1 cells were to be mainly responsible for mediating inflammation, while Th2 cells were to protect against such inflammation.[18,19] While the discovery of additional subtypes has obviously complicated the picture, it is nonetheless still tempting to align the other subsets into that model. After all, some signaling pathways such as the IL-12 pathway that drives interferon gamma (IFNγ) production by Th1 cells seem to be clearly aligned to either be pro- or anti-inflammatory. [20,21] The standard pathway of Th9 cell differentiation challenges that clarity however, as both the IL-4 and TGF-β pathways are normally recognized as anti-inflammatory, yet drive the production of the pro-inflammatory IL-9. This result is particularly interesting given that IL-4 is a Th2 produced cytokine. [22,23] Further work may identify additional pathways that may lead to Th9 differentiation.

In addition, it must be noted that many other environmental factors beyond cytokines may also play important roles in controlling Th9 differentiation. In particular, metabolism-related factors could be a special interest to T helper cell stimulation.[24] After all, factors as diverse amino acid starvation, succinate, and high concentration of sodium have been shown to potently affect Th17 cells in opposite ways and in a manner distinct from other T helper subtypes.[25,26,27,28,29] Some degree of hypoxia has also been shown to help drive Th17 differentiation over differentiation into other phenotypes.[30,31] Tregs have similarly been shown to react differently than other T helper subtypes in the face of non-cytokine stimuli.[32] In addition, the Treg phenotype has been shown to have a lower metabolic profile than T effector phenotypes, and would thus likely respond differently than the other phenotypes to antimetabolic stimuli.[33,34] It is thus quite likely that Th9 cells will also exhibit some distinct responses to certain non-specific environmental stimuli than other T helper phenotypes. In fact, one such differential response has already been reported; Th9 cells have been found to have increased expression in the presence of nitric oxide (NO), while Th17 cells were previously found to be significantly suppressed by NO.[35,36] Of additional interest is the observation by some groups that thymic stromal lymphopoietin (TSLP) may actually encourage Th9 differentiation, despite TSLP being generally regarded as a factor suppressing T effector differentiation.[37,38] There may also be an additional time-related factor to Th9 differentiation that is distinct from other T helper cells. After all, while T helper phenotypes are generally polarized *in vitro* for three days in the presence of the polarizing cytokines prior to experimentation, several papers dealing with Th9 cells

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have used a double polarization process. During this process, Th9 cells are first cultured in the presence of IL-4 and TGF-β for three days, and then re-stimulated for an additional 2-3 days prior to experimentation.[35,39] It is not entirely clear why the double polarization process leads to clearer results, especially since other T helper subtypes generally begin to undergo apoptosis at that point due to a number of reasons.[40] It is possible that the additional polarization can add a step of selection. Further study may yield a more complete explanation.

Transcription Factor Interplay

After T helper cells are polarized, the next level at which they are typically studied is the protein level. The polarization process can be observe through investigation of the signal transduction networks inside the cell, as it undergoes chromatin remodeling and begins the production of its functional proteins. Transcription factors play a critical role in managing this process, as they regulate the expression of different proteins. As such, analysis of the transcription factors active in T helper cells has been an integral part of their study. Each T helper subtype is generally recognized to possess some transcription factor(s) that are unique to its lineage, as distinct from those shared among all subtypes. These transcription factors have been identified to be T-bet, GATA-3, RORγt, Foxp3, and Bcl6 for Th1, Th2, Th17, Treg, and Tfh, respectively.[41,42,43,44] In short, these transcription factors respond to the signaling from the stimulating cytokines to drive the cell to one fate, while suppressing the possibility of others. It should be noted, however, that such suppression is not by any means absolute.

The identification of these unique transcription factors is extremely useful to conducting subtype research, for they essentially serve as unique subtype markers. For instance, Th17 cells can be determined to exist through evidence on many different levels, including, but not limited to, qPCR for RORγt and IL-17 mRNA, flow cytometry using IL-17 and RORγt gates, ELISA for IL-17 protein, and western blotting for RORγt protein. Based on gene expression profile studies, Th9 cells are somewhat similar to Th2s and more distantly related to Treg, but also display many novel elements.[39] Unfortunately, no clear lineage-defining transcription factor has been found for Th9 cells. As such, current options for studying Th9 cells are more limited, and largely rely on detecting IL-9. Although 2 proteins (interferon regulatory factor 4 (IRF4) and Pu.1) have been posited as potential candidates, neither appears to be fully consistent with characteristics of the other transcription factors.[15,45] After all, IRF4 is broadly expressed by all T helper subtypes through the course of differentiation, and IRF4-/- mice are generally immunodeficient.[46,47,48] Pu.1 is more promising, as it is not so highly expressed in other T helper phenotypes, but is still highly expressed by myeloid and B cells during the course of their development.[49,50,51] As such it seems that IRF4 and Pu.1 are less specific, but still vital proteins for Th9 cells, leaving open the possibility of the existence of other transcription factors that are lineage defining. This analysis holds when the other common characteristic shared by lineage-defining transcription factors is considered, namely that they can bind to the promoter regions of the signature cytokines that the cells secrete.[52,53,54,55] Both IRF4 and Pu.1 fulfill that characteristic, being capable of binding to the IL-9 promoter.[16,56] However, eukaryotic promoter regions are long regions that can attract the binding of many different proteins. As such, there may well exist some other protein that binds to the IL-9 promoter and is also specific to the Th9 lineage. However, given that the current understandings have shifted from declaring proteins "master regulators" to merely identifying them as "lineage-defining", it may be only necessary that a protein be a marker of some sort.

Beyond the search for a lineage-defining transcription factor, the interplay of various transcription factors in Th9 cells may yield interesting mechanistic insights. For instance, the potent suppressing protein Bcl6 has been shown to transiently downregulate during the process of Th9 differentiation, raising questions as to the means by which that downregualtion is induced.[57] The curious expression pattern of Bcl6 also raises the possibility that its re-emergence after several days is in fact marking the onset of apoptosis/exhaustion for the subtype. In addition, similar to how other STAT family members have been shown to aid in T helper activity, STAT6 has been demonstrated to enhance IL-9 transcription and influence Th9 activity in several ways.[58,59,60,61] The Notch and Smad pathways have also been identified to have a role in Th9 differentiation.[62,63] IRF1 has also been shown to play an important role in Th9, especially in causing it to also produce IL-21.[64] The effects of these proteins and others on Th9 cells have been mostly reviewed in-depth elsewhere.[65,66,67,68,69] Post-translational modifications (PTMs) on these proteins, and their subsequent impact on interactions between other proteins that strongly interact with modified residues may also yield curious results. After all, it is currently understood that IRF4 and Pu.1 binding along the IL-9 promoter induce changes in chromatin modeling, via proteins such as the histone acetyltransferase Gcn5.[70,71] As such, there may be some PTMs (and the corresponding PTM-inducing proteins) that are unique to Th9 cells and which are useful for their study.

Cytokine Secretion

From the complex interplay of the various transcription factors and other proteins in regulating Th9 cells arises the secretion of large amounts of interleukin 9 (IL-9), the signature of the Th9 phenotype. In fact, as another group has suggested, the existence of $CD4^+$ $IL\text{-}9^+$ is perhaps the most convincing evidence that the subtype does indeed exist.[58,72,73] After all, although Th2 and Th17 have some capability of secreting IL-9, they cannot produce it at such a high level as Th9.[74] Besides IL-9, Th9 cells have also been shown to be capable of producing some amounts of IL-21 and IL-10.[75] Study of the impact of these other cytokines in affecting Th9 cell function is more difficult, however, since Th9 cells have fewer known markers than other T helper subtypes. This leads to a complication in fully comprehending the Th9 phenotype because of its wide range and irregularity of function in comparison to other T helper subsets. As such, most of the work done on the effects of Th9 cytokine secretion thus far has been focused on IL-9.

Signaling via the IL-9 receptor, IL-9 can transduce effects through a JAK/STAT pathway to alter the expression of many genes in a variety of immune-related cell types.[76,77] In particular, the role of IL-9 in recruiting mast cells and enhancing the resulting release of histamines has been noted.[78] IL-9 has been shown to play an important role in several diseases, acting as an antagonist in asthma/bronchial hyperresponsiveness, atopic dermatitis (AD), and ulcerative colitis (UC), among others.[79,80,81,82] Beyond the obvious link between AD and asthma that has been previously noted, it is quite interesting that each of these autoimmune disorders also share the similarity of being Th2-related. As such, the common models used for studying Th9 cells *in vivo* are often based on oxazolone or ovalbumin, instead of other models such as DSS that more likely lead to Th1/Th17 responses.[83,84,85,86] This result is not surprising, given that Th9 cells require the presence of large amounts of IL-4 to differentiate, and IL-4 is commonly produced by Th2 cells.[87] However, it does complicate the study of Th9 cells significantly, especially in the context of using compounds to suppress their function. As such, Th9 cells might be better understood as a subtype that may arise slightly later, after Th2 cells have already formed at the site. Time-lapse experiments may be able to verify the authenticity of this hypothesis.

Regardless of the precise time that Th9 cells arise, it is clearly non-trivial that beyond the obvious need to clarify that a compound's effect in a certain animal model is suppressing IL-9 from being produced by Th9 cells instead of Th2 or Th17 cells. Th2 cell production of IL-4 would also have to be monitored in tandem. This additional factor also introduces the possibility of divergence between *in vivo* and *in vitro* experiments, as *in vitro* experiments would hold the level of IL-4 constant even if its secretion fluctuated *in vivo*. However, it is also possible that there may exist some sort of double positive population of Th2/Th9 cells,

due to plasticity between different T helper subtypes—a recently discovered population of Th2/Th17 double positive cells has been shown to be important in some diseases.[88] Beyond that concern, it is also unclear exactly how long Th9 cells are retained in tissue *in vivo*, with a recent report suggesting that they may have shorter effects than other T helper subtypes.[89] Such an observation also conflicts slightly with aforementioned techniques in *in vitro* experiments that culture the cells for longer than usual. Greater rigor is thus necessary in studying Th9 cells than there may appear at first analysis.

In addition, it is important to also recognize that IL-9 is also beneficial in responding to certain pathogens when properly regulated. For instance, IL-9 has been shown to have a strong protective capability in countering parasites such as helminthes, and such infections are endemic in many areas of the world.[90,91,92,93] In addition, IL-9 is also capable of attacking tumors, with that function being first detected in a study of melanoma.[94,95,96] Upregulation of IL-9 and Th9 may thus be beneficial in certain other conditions as well.[97,98] Th9 cell production of IL-3 may also be an important beneficial effect, as IL-3 has been shown to prolong DC survival.[99] This latter result may also be a feedback mechanism that Th9 cells can potentially use to survive for longer periods of time in tissue. That persistence is likely a key contributing factor to asthma.

Conclusion

Th9 cells are an important T helper subset with unique characteristics that defy the standard classification scheme for T helpers. In this paper, we discuss several of those distinguishing features in regards to Th9 stimulation, transcription factors involved, and Th9 cytokine secretion. We note some of questions that have yet to be answered regarding Th9 stimulation, including in terms of non-cytokine factors such as time. We also analyze the issue of Th9 cells currently lacking an accepted lineage-defining transcription factor, and the consequent difficulties that result. Further studies on Th9s may lead to new information that will eliminate the current limitations, and yield valuable data on potential therapies involving Th9s.

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