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Th2 heterogeneity: Does function follow form?

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Abstract

Th2 immune responses are required for the two fundamental pathological processes characteristic of allergic disease: IgE mediated hypersensitivity and eosinophilic inflammation. The three established Th2 cytokines, IL-4, IL-5, and IL-13, each play a non-redundant role in allergic disease pathology. The recent explosion of T helper subpopulations combined with the wide availability of polychromatic cytokine staining has facilitated the discovery of Th2 lineage heterogeneity. In this Rostrum article, we review Th2 heterogeneity and ask at what point do these subpopulations graduate from in vitro curiosities to immunologically robust therapeutic targets? We propose criteria to establish a T cell subset as a biologically relevant entity, and address the evidence to support these Th2 subpopulations having a unique function, or specific contribution to allergic pathology or host defense.

The Th2 lineage- definitions and background

In the quarter century since the first description of T helper (Th)1 and Th2 cells, an enormous body of data has established the basic paradigm of CD4 T cell lineage heterogeneity, with each lineage having distinct molecular, cellular and functional properties.[1, 2] The original Th1/Th2 dichotomy has expanded to include a minimum of four different CD4 T cell lineages: Th1, Th2, Th17, and induced T regulatory. Each lineage expresses a unique cytokine profile, which along with other expressed genes, results in its functional characteristics. Each of the first 3 lineages generates a characteristic inflammatory response; Th1, Th2 and Th17 cells respectively causing macrophage-rich, eosinophil-rich, and neutrophil-rich inflammation. The concept of neatly pigeonholed stable irreversibly differentiated lineages requires some reassessment with recent findings of plasticity among various CD4 subsets.[3] However, the basic Th1/Th2 concept of functional modularity of CD4 immune responses has withstood the test of time.

Differentiation of naïve CD4 T cells into a given lineage is the product of multiple integrated signals, including T cell receptor signal strength, costimulatory and innate immune signals, and cytokine milieu.[2] During T cell differentiation, key genes, for example those encoding cytokines and lineage specific transcription factors, undergo epigenetic changes, including changes in histone and transcription factor binding and DNA methylation. Specific epigenetic changes can activate or repress a given gene, ultimately resulting in the transcription of specific gene products that confer the functional properties of that lineage. For example, in Th2 cells, there is increased binding of $H3K4me^3$ and decreased binding of $H3K27me^3$, respectively activating and repressing forms of histone 3, to Th2 gene promoters.[4] In Th1 cells reciprocal histone binding patterns are observed. In this manner, heterogeneity at the epigenetic level could ultimately result in Th2 heterogeneity. In contrast to cytokine gene promoters, the promoters for lineage specific

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transcription factors, such as the Th2 specific transcription factor GATA-3, demonstrate "bivalent" markings, with both activating and repressive histone binding.[4] These latter findings suggest that by maintaining their capacity to alter lineage specific transcription factor expression, differentiated T cells retain lineage plasticity.

Th2 cells were initially characterized as expressing IL-4, IL-5, and IL-13.[5] Although Th2 cells can express many other cytokines, including IL-2, IL-3, IL-9, IL-10, GM-CSF, and TNF, these cytokines are also expressed by other CD4 lineages. Thus, the original three cytokines, IL-4, IL-5, and IL-13, remain the established and generally agreed upon Th2 cytokines. The three Th2 cytokine genes are located in a syntenic region in human chromosome 5q31 and mouse chromosome 11. The *Il4* and *Il13* genes are adjacent to each other, whereas the *Il5* gene is 120 kilobases telomeric of these genes and in the opposite orientation.

A number of Th2 markers have emerged in the past decade. CRTH2, the type 2 prostaglandin D2 receptor, is preferentially expressed on Th2 cells and is the most well accepted Th2 surface marker. Ligation of this receptor results in augmentation of Th2 cytokine expression and chemotaxis.[6] Accordingly, CRTH2 antagonists are being examined in a number of diseases. CCR3, CCR4, and CCR8 are all preferentially expressed on Th2 cells and play a role in Th2 specific chemotaxis.[7] In a mouse model of allergic asthma, CCR4 but not CCR8, was required for Th2 trafficking.[8] Th2 heterogeneity in regard to chemoattractant receptor expression has not been studied. The IL-33R (IL-1 receptor like-1, IL1RL1, also known as ST2) and the IL-25R (IL-17RB) are highly expressed in activated, but not resting, Th2 cells.

Th2 cells have generally been approached as a homogeneous population; however, recent reports provide evidence for subpopulations within the Th2 lineage.[9–12] Given the various experimental systems and methods used to study Th2 cells it is not surprising that different approaches provide varying evidence for Th2 heterogeneity. However, at what point do these subpopulations graduate from in vitro curiosities to immunologically robust therapeutic targets?

We propose the following criteria to establish a Th subset as a biologically relevant entity:

1. **Generalizability.** The first level of proof requires that the Th candidate subpopulation be demonstrated in multiple disease states, model systems, and species, using several different experimental approaches.
2. **Disease association.** Although not formal proof of function, disease association is certainly evidence that the proposed subset may have clinically relevant pathological function.
3. **Function.** Lastly, demonstration of function either *in vitro* or *in vivo* is needed to truly demonstrate that a given Th subset may actually play a unique role in disease pathogenesis.

The following review examines the literature regarding Th2 heterogeneity using the above criteria, with an emphasis on the potential roles for Th2 subpopulations in disease pathogenesis.

Experimental approaches in the study of lineage heterogeneity

T cell biology is clonal in nature; consequently the determination of T cell cytokine expression is most informative when determined at the single cell level. To this end, the original characterization of Th1 and Th2 cells used T cell clones to demonstrate CD4 T cell

cytokine heterogeneity. Such clones are essentially a single T cell that has been proliferated to the point that conventional bulk cytokine assays, such as ELISA or PCR, can be used to examine that single cell's cytokine phenotype. T cell clones are relatively labor intensive to generate, examine only a limited number of T cells, and are subject to long-term *in vitro* culture artifacts. When polyclonal T cell responses are measured using bulk cytokine assays, the results are inherently less informative than those from single cell assays. For example, when both IL-4 and IFN-γare present in a culture, using a bulk assay it is not possible to determine if they are being made by a single IL-4+, IFN-γ+ dual producing population or by separate single producing IL-4+ and IFN-γ+ populations. Another confounder is that some cytokines such as IFN-γare expressed at very high per cell quantities relative to IL-4, such that bulk assays tend to indicate a sizable Th1 response even though few IFN-γand many IL-4 producing cells may be present.

The combination of intracellular cytokine staining (ICCS) and polychromatic (5+ color) flow cytometry has emerged as the technique of choice in the investigation of T cell lineage heterogeneity.[13] ICCS can detect the expression of multiple cytokines and associated phenotypic markers at the single cell level, allowing the discrimination of complex cytokine phenotypes. A variety of T cell stimuli can be used, including antigens (Ag) and mitogens.[11, 14] Such approaches have proven useful in the study of viral and vaccine immunology[13], but have been less widely applied to Th2 responses.

An alternative flow based single cell technique is the commercially available Miltenyi Cytokine Secretion Assay, in which cytokines are captured on the surface of cytokine producing cells.[15, 16] This technique has the advantage that analyte cells are alive and can be sorted for cell culture and additional analyses. Technical limitations restrict this method to the labeling of one, or at most two cytokines, whereas ICCS can examine 3–6 cytokines simultaneously. The ELISA spot (ELISPOT) assay, which allows the single cell determination of cytokine secretion by cytokine capture on an antibody coated plate, is highly sensitive, but is limited to 1–2 cytokines and requires instrumentation and technical expertise that is not widely available.

The characterization of Th2 subpopulations in human allergic disease vs. mouse models is currently the subject of active investigation. Human allergic diseases, such as asthma, are typically associated with long-term recurrent allergen exposure, whereas mouse models of allergic disease typically use short term Ag challenges. The chronicity and Ag dose typical of human allergen exposure may generate different Th2 subpopulations relative to mouse models of allergic disease.

Th0 cells

Soon after the original description of Th1 and Th2, T cell clones expressing both IFN-γand IL-4 were designated Th0.[17] The term "Th0" is often used incorrectly to designate naive or precursor T cells. Naïve T cells express very low levels of effector cytokines such as IFN-γand IL-4 and thus do not fulfill the Th0 definition. Whether Th0 cells are an intermediate stage in the differentiation of native T cells into Th1 or Th2, or whether they represent plasticity of Th1/Th2 cells that then acquire additional cytokine expression is the subject of active investigation. The plasticity of Th2 cells converting to Th0 has been demonstrated in vivo[18, 19]. In the latter study, virus specific Th2 cells were adoptively transferred to mice and upon subsequent viral infection were converted to Th0 cells; these Th0 cells were described as "Th2+1" to emphasize the acquisition of IFN-γexpression by plastic Th2 cells. This conversion was limited to viral Ag specific T cells and was mediated through both types I and II IFNs. The Th0 state required the expression of both GATA-3 and Tbet, and was stable for up to 60 days *in vivo*. In theory, such dual expressing cells could have potent

inflammatory effects, similar to the potentiation noted when Th1 cells are added to the existing Th2 response in a mouse asthma model.[20] An analogous human scenario could possibly exist in an allergic asthmatic patient with concurrent viral upper respiratory infection and aeroallergen exposure, in which allergen specific Th2 cells are activated by Ag in an interferon rich milieu, leading to an allergen specific Th0 response.

Most other studies citing substantial Th0 responses are subject to two major limitations. First, such investigations have typically used ELISA or other bulk assays, such that it is impossible to determine if a true Th0 response exists at the single cell level, as discussed above. Second, such studies often use extended in vitro culture, which may artifactually induce IFN-γexpression in Th2 cells. Allergen proliferated T cell lines cultured in vitro for greater than 3–4 days typically exhibit a greater frequency of Th0 cells due to *in vitro* differentiation and plasticity (Prussin, unpublished data). Whether Th0 responses play an important role in human disease has not been established.

Th2/Th17 coexpressing cells

Th17 cells, which express IL-17A, IL-17F and IL-22 as their signature cytokines are critical for host defense against fungal and extracellular bacterial pathogens, particularly at barrier interfaces such as skin and mucosa.[21, 22] Notably, autosomal dominant hyper-IgE syndrome is caused by hypomorphic mutations of STAT3, resulting in the absence of Th17 lineage differentiation and immunodeficiency.

IL-4 antagonizes Th17 cell differentiation, supporting the concept that the Th2 and Th17 responses are mutually exclusive.[21] However, two recent reports provide evidence for hybrid Th2/Th17 cells. Recently, Cosmi and colleagues generated human T cell clones from CD4+, CD161+, CCR6+ T cells known to be enriched in Th17 cells, and found a small fraction had a mixed Th2/Th17 phenotype.[12] The mixed phenotype was confirmed by the coexpression of the lineage specific transcription factors GATA-3 and RORγT. Th2/Th17 cells were present at very low frequencies in the blood of healthy controls (0.025%), but were significantly increased in asthmatic subjects (0.06%). Similar Th2/Th17 cells were found within the CRTH2+, CCR6+ subset in a variety of allergic mouse models.[23] Whether such Th2/Th17 cells represent a functional subset *in vivo* and if they play a role in asthma pathogenesis is the subject of active investigation.

Regulatory and inflammatory Th2 cells

IL-10 has potent anti-inflammatory effects *in vitro* and there is abundant evidence from mouse models that lack of IL-10 results in unchecked inflammation and autoimmune disease. This is underscored by the finding that a severe childhood onset form of inflammatory bowel disease is caused by a mutation in the IL-10 receptor.[24] IL-10 is expressed by multiple cell types, including monocytes, B cells, dendritic cells (DCs), and T cells. Soon after its initial characterization, IL-10 was found to be expressed in mouse Th2 cells and was initially classified as a Th2 cytokine.[25] However, in the intervening years extensive murine and human data demonstrate that IL-10 can be coexpressed by a variety of T cell subpopulations, including both Th1 and Th2 cells.[26] This induction of IL-10 in effector T cells may reflect an autoregulatory mechanism to negatively feedback on effector T cell function.

A number of regulatory-like Th2 populations have been identified. T regulatory 1 (Tr1) cells were initially described when naïve CD4 cells were differentiated in an IL-10 rich milieu, leading to their acquisition of regulatory activity and the expression of both IL-10 and IL-5.[27] Woodfolk and colleagues have identified a “modified Th2” response, characterized by Fel dI specific IgG4 and IL-10 expressing T cells, which are associated with the clinical

tolerance found in individuals exposed to high levels of cat allergen and in patients treated with cat immunotherapy.[28]

Using in vitro differentiated Th2 cells, Liu and colleagues have described dichotomous subsets of "regulatory" and "inflammatory" Th2 cells. Th2 cells differentiated in the presence of untreated DCs develop into regulatory Th2 cells that express the classic Th2 cytokines and IL-10, but not TNF.[9] In contrast, Th2 cells differentiated in the presence of thymic stromal lymphopoietin (TSLP) treated DCs (TSLP-DCs) develop into inflammatory Th2 cells that express TNF, but not IL-10. In addition to TNF, TSLP-DCs increase Th2 cell expression of Charcot-Leydon crystal protein, prostaglandin D synthase, and IL-17RB.[29] IL-25 secreted by eosinophils and basophils, signals through IL-17RB to augment Th2 cell proliferation and cytokine expression.[30] The increased IL-17RB expression found in TSLP-DC treated Th2 cells further amplifies this effect. The multiple inflammatory properties of TNF, including upregulation of adhesion molecules and eotaxin family members, suggests that TNF expression by Th2 cells could amplify their pro-inflammatory function. However, no functional or disease association studies have been performed on these "regulatory" and "inflammatory" Th2 subsets to further validate this concept.

IL-4neg Th2 cells

IL-4 is the signature Th2 cytokine. In addition to its expression by Th2 cells, IL-4 plays a fundamental role in Th2 differentiation. That being said, a number of studies have described Th2 cells lacking IL-4 expression ("IL-4neg Th2 cells"). The most well established example of IL-4neg Th2 cells is in the context of IL-33. IL-33 is an IL-1 family member and is expressed by epithelial and endothelial cells in response to innate immune signals. Activated Th2 cells express the IL-33 receptor, ST2. IL-33 activation of Th2 cells via ST2 results in the induction of IL-5 and IL-13, without substantial IL-4 expression.[31–33] In this manner the IL-33/ST2 pathway may be an important mechanism for innate immune signals to amplify an existing Th2 immune response.

IL-4 is expressed less abundantly than most other cytokines and is also consumed in culture. Thus, absent or low IL-4 expression in an otherwise Th2 dominated response may reflect the technical difficulties of IL-4 detection, rather than true IL-4neg Th2 cells. In such situations, ICCS, which is less affected by these issues than is ELISA, is generally more able to detect IL-4.

IL-5+ and IL-5neg Th2 cells

In our own work we have noted that using a variety of stimuli IL-4 and IL-13 are largely coexpressed, whereas IL-5 expression is limited to a subset of these IL-4+, IL-13+ Th2 cells. This pattern of coexpression led us to hypothesize that heterogeneity of IL-5+ expression within the Th2 pool may confer different function to IL-5+ Th2 cells. IL-5+ Th2 cells were found in greater amounts in eosinophilic gastrointestinal disease patients,[11] suggesting these cells may preferentially contribute to eosinophilic inflammation. Similar findings of dichotomous IL-5 expression have been found in a variety of clinical settings, suggesting this may be a generalizable phenomenon (Prussin unpublished results). Whereas IL-4 is expressed by both central and effector memory subsets, IL-5 expression is limited to the effector memory subset, suggesting that IL-5+ Th2 cells may be more differentiated[34]. Further work is needed to demonstrate that IL-5+ and IL-5neg Th2 cells have different functional properties, or whether other genes are differentially expressed in IL-5+ Th2 cells.

Th2 follicular helper T cells

Follicular helper T cells (Tfh) are a distinct population of T cells that are found in germinal centers and supply cognate T cell help for B cell class switching, differentiation, and immunoglobulin production.[35] Tfh express the transcription factor bcl-6, the germinal center homing chemokine receptor CXCR5, as well as ICOS, PD-1, and IL-21. It is not presently understood if Tfh represent a separate lineage of CD4 T cells or if they originate from typical Th1/Th2 cells that then further differentiate and acquire Tfh features.

Human Th2-like Tfh have been described as CRTH2+, CXCR5+ T cells found in tonsils, but not in peripheral blood.[36] Th2-like Tfh have been described in mouse models of parasitic disease in which a subpopulation of Th2 cells express the Tfh signature genes CXCR5, ICOS, PD-1 and bcl-6.[37, 38] Tfh function was confirmed by the finding that B cells in T/B conjugates with Th2-like Tfh switch to IgE, whereas B cells in conjugates with Th1-like Tfh switch to IgG2a, a Th1 associated isotype.[39]

Th9 cells

IL-9 was originally described as a Th2 associated cytokine, with multifunctional activity on mast cells, T cells and epithelial cells. IL-9 is encoded on chromosome 5q31 in proximity to the established Th2 cytokine genes. When expressed under a lung specific promoter, IL-9 results in severe pulmonary inflammation with increased numbers of mast cells and eosinophils, airway hyperreactivity, mucus metaplasia, and subepithelial deposition of collagen.[40] Recently, the association of IL-9 and Th2 responses was clarified by the characterization of a new subpopulation of T cells, termed Th9 cells, which express IL-9 but not the established Th2 cytokines.[41, 42] Naïve T cells differentiate into Th9 cells through the combined actions of IL-4 and TGF-beta. Intriguingly, these investigators also showed that TGF-β“reprograms” existing Th2 cells into Th9 cells.[41] Despite this Th2 association, IL-9 can also be coexpressed with IL-17 and in this role appears to potentiate Th17 driven inflammation.[43, 44]

Clearly, IL-9 has multiple pro-allergic activities.[45] A more precise understanding of the relationship between the Th2, Th9 and Th17 cells will further clarify the role of IL-9 in disease pathogenesis. Anticipated clinical trials of anti-IL-9 therapeutics will address the criticality and non-redundancy of IL-9 as a mediator of allergic inflammation.[46]

Conclusions

As detailed in this review there is ample evidence for Th2 heterogeneity, although most of these Th2 subpopulations have yet to be described in more than a single human disease or mouse model system. As posed by this review’s title: *is there sufficient evidence to establish that any of these Th2 subpopulations have a unique function, or differentially contribute to allergic pathology or host defense?* Using the aforementioned criteria of generalizability, disease association; the present answer is still “no”. Despite these limitations, the field appears ripe for advancement, as in recent years the cellular and molecular tools to address such questions have made huge advances. If such Th2 subpopulations do exist, why are they even important? Presumably, Th2 subpopulations, such as “regulatory” IL-10+ Th2 or “pro-eosinophilic” IL-5+ Th2 cells, represent immunologically specific functional modules. A better understanding of the cellular and molecular biology of these Th2 subpopulations should provide more specific therapeutic approaches to block their contribution to disease.

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Abbreviations

Ag	Antigen
DC	Dendritic cell
ICCS	Intracellular cytokine staining
neg	Negative
Tfh	Follicular helper T cells
Th	T helper

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