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REVIEW

Subpopulations of helper T lymphocytes in tuberculous pleurisy

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SUMMARY

Although it is curable, tuberculosis continues to be is a major global public health problem, especially in developing countries. Tuberculous pleural effusion (TPE) is one of the most common forms of extrapulmonary tuberculosis. It has been well documented that CD4+ T lymphocytes are dominant leukocytes present in TPE. Traditionally, CD4+ T cells have been classified into two functionally distinct subsets, helper T-cell type 1 (Th1) and Th2 cells, based on their cytokine production profiles. Recently, regulatory T cells, Th17 cells, Th9 cells, and Th22 cells have been added to the ‘portfolio’ of Th cells. In this review, we summarize recent findings regarding the phenotypic characteristics of the above Th cells, the mechanisms of differentiation and recruitment of Th cells into pleural space, and the immune regulation of Th cells in TPE environment. We also describe the interplay between different Th cells, as well as between Th cells and other type of cells, such as pleural mesothelial cells in TPE. Further studies should be directed at identifying the mediators and mechanisms involved in the immunoregulatory properties of pleural Th cells in tuberculosis infection.

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1. Introduction

Tuberculosis is the leading cause of death from a curable infectious disease. There were an estimated 8.8 million incident cases of tuberculosis globally in 2010, 1.1 million deaths among HIV-negative cases of tuberculosis and an additional 0.35 million deaths among people who were HIV-positive.1 In China, the prevalence of active pulmonary tuberculosis in 2000 was 367 per 100,000, the prevalence of smear positive pulmonary tuberculosis was 122 per 100,000, and the prevalence of bacteriological positive pulmonary tuberculosis was 173 per 100,000.2 Between 1990 and 2010, prevalence rates were halved, mortality rates were cut by almost 80% and incidence rates fell by 3.4% per year in China.1

Tuberculous pleural effusion (TPE) results from Mycobacterium tuberculosis (MTB) infection of the pleura and is characterized by an intense chronic accumulation of inflammatory cells in pleural space.3,4 Actually, tuberculosis is the major cause of pleural effusions in areas of high tuberculosis prevalence, and CD4+ T cells are a dominant population in TPE.3,5 More recently, it has been shown that after stimulation with MTB-specific antigens, CD4+CD69+ T cells expressed significantly higher levels of IFN-γ, IL-2 and TNF-α than CD4+CD69- T cells did, demonstrating that CD4+CD69+ T cells were MTB-specific helper T cells (Th1) cells.5

Since the identification of Th1/Th2 lineage more than two decades ago, regulatory T cells (Treg cells), Th17 cells, Th9 cells, and Th22 cells have been added to the ‘portfolio’ of Th cells. The involvement of these Th cells in MTB infection, including TPE, has been studied extensively. For example, Caramori and colleagues have determined the numbers of inflammatory cells, particularly Th cells (Th1/Th2/Th17/Treg cells) in biopsies of parietal pleura obtained from patients with TPE compared with a control group of subjects with nonspecific pleurisy, and have found that increased CD3+, CD4+, CCR4+ cells as well as Th17 cells and decreased mast cells as well as GATA-3+ cells (GATA-3 is a lineage-specific transcription factor for Th2 cells) in the parietal pleura distinguish patients with TPE from those with nonspecific pleurisy.6 Since Th1/Th2 cells had well been documented in the previous review,7 in this review, we concentrated on recent studies that provide insights into the presence of Treg, Th17, Th9 and Th22 cells, the mechanism of differentiation and recruitment of these Th cells, and the immune regulation of Th cells in TPE environment.

2. Treg cells

Studies ongoing for more than a decade have provided firm evidence for the existence of a unique Treg cells that actively and dominantly prevent both the activation and the effector function of autoreactive T cells that have escaped other mechanisms of tolerance.10,11 Murine and human natural Treg cells are CD4-positive and characterized by constitutive expression of high levels of the
IL-2Rx-chain (CD25) and the transcription factor Foxp3 and low expression of IL-7R (CD127). The involvement of such Treg cells in human tuberculosis has been documented recently. We and the others have demonstrated that the numbers of Treg cells in TPE were much higher than those in autologous blood and blood from normal controls, and that Treg cell numbers in tuberculous blood were significantly higher than those in normal control blood.

No data concerning the mechanisms of generation and differentiation of Treg cells in TPE environment are available so far. It has been shown that Treg cells expand from CD4+ T cells from healthy tuberculin reactors, but not from CD4+ T cells from tuberculin-negative donors, indicating that Treg cell expansion is MTB antigen-specific and that expanded Treg cells inhibit IFN-γ production by T cells. Recently, Periasamy and colleagues identified that MTB infection induces development of Treg cells fromCCR4+ cells through a process that depends on programmed death-1 and cytokine inducible SH2-containing protein. Furthermore, MTB-induced Treg expansion requires dendritic cells and toll like receptor-2- and dendritic cell-SIGN-dependent induction of programmed death ligand–1 expression on these immune cells.

It has been reported that Treg cells generated ex vivo are able to induce CD4+CD25+ T cells to become Treg cells. It is possible that inflammation-related factors induce transient Foxp3+ T cells from CD4+CD25+Foxp3- effector T cells; however, their suppressive function is thought to be temporary, not intrinsic and unstable. We speculated that an increased percentage of Treg cells in TPE might be due to active recruitment. We have provided direct evidence that IL-16 is capable of inducing CD4+ T-cell infiltration into the pleural space. Therefore, as a subpopulation of CD4+ T cells, Treg cells might also be recruited in TPE by local production of IL-16, since IL-16 level is significantly higher in TPE than in serum.

While chemokine receptors are important for T cell migration, it has been unclear how they are regulated in Treg cells. In consistent with the results reported by the other authors, our previous data showed that Treg cells strongly express CCR4, a chemokine receptor for CCL22 or CCL27, etc.) produced as a method for TPE diagnosis. Guyot-Revil and colleagues have reported that depletion of Treg cells from peripheral blood mononuclear cells resulted in increased numbers of MTB antigen-specific IFN-γ-producing T cells in tuberculous patients. In another study, Ribeiro-Rodrigues and colleagues have shown that a role for Treg cells in depressed IFN-γ production during tuberculosis was substantiated in depletion experiments, where CD25-depleted CD4 T cells produced increased amounts of IFN-γ upon MTB stimulation compared to unseparated T cells. Further studies indicated that Treg cells significantly inhibited IFN-γ production by γδ T cells. More recently, Singh et al. have demonstrated that blocking programmed death-1 and its ligand-1 pathway abrogated Treg cell-mediated suppression of IFN-γ production, suggesting that Treg cells depressed IFN-γ production via a programmed death-1-dependent pathways. It should be mentioned that most of these experiments where performed with peripheral blood cells where MTB-specific T cells are not as high in proportion as they are in TPE.

### 3. Th17 cells

IL-17 (also known as IL-17A) was identified in 1995 as a cytokine produced by activated human CD45RO+ memory T cells. IL-17F, a closely related member with 50% amino acid sequence homology to IL-17A, was later discovered and is also expressed in activated CD4+ T cells. The subset of CD4+ T cells that produce both IL-17A and IL-17F are now defined as a separate subset Th17 cells. Distinct from Th1 and Th2 cells, Th17 cells are reported to be generated from naïve T cells by IL-6 and TGF-β and are expanded and stabilized further by IL-23 and by virtue of expressing the orphan nuclear receptor RORγt as a critical transcription factor.

It has been reported that compared with healthy volunteers, patients with TPE had a higher numbers of Th17 cells in peripheral blood, and that Th17 cell numbers in TPE were much higher than those in autologous blood. Development of Th17 cells and Treg cells is closely linked, and human Treg cells can differentiate into Th17 cells. Actually, the development and differentiation of Th17 cells

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**Figure 1.** Trafficking of Th cells into pleural space from peripheral blood in patients with tuberculous pleural effusion. Chemokines (CCL20, CCL22, or CCL27, etc.) produced by local T cells, macrophages, and pleural mesothelial cells (PMCs) recruit Th cells via binding to their corresponding chemokine receptors (CCR4, CCR6, or CCR10, etc.) expressed on the cell surface.
IL-9 and IL-10 and develop from naïve CD4+ T cells via a TGF-β-dependent mechanism. However, the process of human Treg cells differentiating into Th17 cells was enhanced by exogenous cytokines such as IL-1β and IL-6, and inhibited by TGF-β. IL-17-producing Treg cells strongly inhibit the proliferation of CD4+ responder T cells, and maintain their suppressive function via a cell–cell contact mechanism. Treg cells can modulate Th17 responses even in patients with latent tuberculosis infection. Therefore, we investigated the distribution of Th17 cells in relation to Treg cells in a recent study and found that like Treg cells, Th17 cells were also increased in TPE when compared with their compartments in blood.

We noted fewer Th17 cells than Treg cells in TPE, although the numbers of Th17 cells and Treg cells were both increased in TPE. Interestingly, we further noted that the numbers of Treg cells and Th17 cells are inversely correlated in TPE, but not in blood, suggesting that there could be a dynamic interaction between Th17 cells and Treg cells in TPE.

It was not surprising that immune response against MTB was more intensive in pathologic site than in peripheral blood. For example, Chatterjee and colleagues have demonstrated that MTB antigen and TGF-β protein induced production of IL-6 and TGF-β in dendritic cells in a toll-like receptor-2- and MyD88-dependent manners, which generates an environment that is conducive for Th17 cell differentiation in the infectious sites.

TGF-β is a key cytokine involving in regulating generation and differentiation of Th17 cells and Tregs. TGF-β is synthesized in cells as a pro-TGF-β precursor. Following homodimerization, pro-TGF-β is cleaved into two fragments: the C-terminal homodimer corresponds to mature TGF-β, while the N-terminal homodimer is latency-associated peptide (LAP). Mature TGF-β and LAP remain non-covalently bound to each other in a complex called latent TGF-β. Latent TGF-β is inactive because LAP prevents mature TGF-β from binding to its receptor, and hence from transducing a signal. We observed that pleural Treg cells could be induced to express LAP on their surface, and that in the in vitro coculture of naïve CD4+ T cells and Treg cells, a blocking mAb against LAP was able to revert the inhibitory effect exerted by Treg cells, suggesting that pleural Treg cells inhibit generation and differentiation of Th17 cells via a LAP-dependent mechanism.

Th17 cells contribute to the adaptive immune response to mycobacteria in exposed persons and in patients with tuberculosis. Regulation of Th17 and Th1 responses during tuberculosis is essential to promote anti-MTB immunity and prevent extensive immunopathological consequences. On the other hand, Th17 cells can also provide IFN-γ-independent protection against MTB, and this effect may contribute to the early control of MTB infection.

4. Th9 cells

The cytokine IL-9 was identified, and its basic features were described more than two decades ago. IL-9 has long been thought to be a Th2 cytokine, as it promotes allergic inflammation and is associated with various Th2 responses. More recent studies revealed the multifunction activities of this cytokine. Of significant importance are the recent discovery Th9 cells distinct from Th1, Th2, or Th17 cells. Th9 cells are characterized by production of IL-9 and IL-10 and develop from naive CD4+ precursors driven by the combined effects of TGF-β and IL-21. Although some studies have demonstrated that Th9 might elicit inflammation and contribute to the development of allergic diseases, the role of this new Th subset in infection immune, including tuberculosi, remains to be further elucidated.

More recently, we have demonstrated that Th9 cells were present in TPE, and the numbers of Th9 cells represented in TPE were much higher than those in the corresponding blood. The molecular mechanisms underlying the generation and differentiation of human Th9 cells are not elucidated completely. It has been reported that the transcription factor PU.1 and IFN-regulatory factor 4 were required for the development of murine Th9 cells. In human, memory CD4+ T cells could be induced to become Th9 cells. Similar to the findings in mouse studies, TGF-β and IL-4 induced the differentiation of human Th9 cells in vitro; and IL-1β, IL-6, IL-10, IFN-α, IFN-β or IL-21 could augment Th9 differentiation, while IFN-γ and IL-27 partially suppressed Th9 differentiation. Our study draw the similar conclusion, showing that TGF-β was essential for Th9 differentiation from naïve CD4+ T cells isolated from TPE or blood, addition of IL-4, IL-1β, or IL-6 augmented IL-9 production, and the production of IL-9 induced by TGF-β was in a dose- and time-dependent manner. Consistent with Wong et al., we showed that IFN-γ significantly suppressed IL-9 production induced by TGF-β. Surprisingly, although higher concentration of IFN-γ was present in TPE, the numbers of Th9 cells were higher in TPE than in blood. These data indicated that there was a complex cytokine network in the regulation of Th9 cell differentiation in TPE, and that there might be other mechanisms besides local differentiation leading to Th9 cell increase in TPE.

On the other hand, an increase in numbers of Th9 cells in TPE might also be due to Th9 cell recruitment from peripheral blood. We have evaluated whether chemokine/CCR axis was responsible for the influx of Th9 cells, and found that all pleural mesothelial cells (PMCs) from TPE expressed CCL20, and that CCL20 concentration in TPE was much higher than those in serum, and that Th9 cells in both TPE and blood expressed high level of CCR6 on their surface. These data suggested that CCL20/CCR6 axis might be related to the accumulation of Th9 cells in TPE. Indeed, an in vitro migration assay further confirmed that both TPE and supernatants of cultured PMCs could induce the migration of Th9 cells, and that anti-CCL20 mAb significantly inhibited the ability of TPE or supernatants to stimulate Th9 cell chemotaxis. Therefore, PMC-produced CCL20 might be able to chemoattract Th9 cell recruitment into pleural space during MTB infection.

Our data also showed that the numbers of Th9 cells in TPE positively correlated with that of Th17 cells, but not of Th1, Th2, or Tregs. We supposed the finding that IL-9 together with TGF-β promoted Th17 differentiation from naïve CD4+ T cells might account for this correlation. In addition, Zhou et al. has reported that IL-9 promoted Th17 cell migration into the central nervous system via CCL20 produced by astrocytes, and we speculated that IL-9 may also contribute to Th17 migration into TPE, which need to be further investigated.

The pathophysiological functions of Th9 cells in MTB infection have not been investigated. Exogenous IL-9 reduced IFN-γ expression in peripheral blood mononuclear cells from patients with latent tuberculosis infection and neutralization of IL-9 restored the IFN-γ expression. Since PMCs are an important component of the pleural environment, they may collaborate with the other kinds of cells, including Th9 cells, in the generation of local cell-mediated immunity to various pathogens, including MTB. The mesothelium is a slowly renewing tissue that can be stimulated by a variety of agents and direct physical damage to increase its turnover rate. An understanding of the balance between proliferation and apoptosis of PMCs exposed to environmental inflammation is critical to further understanding of the mechanisms and patterns of pleural injury and fibrogenesis, which occur frequently during MTB infection. Our data showed IFN-γ not only impaired PMC wound healing but also induced PMC apoptosis; in contrast, neither IL-9 nor IL-4 affected apoptosis of PMCs, both cytokines further inhibited IFN-γ-induced PMC apoptosis.
5. Th22 cells

IL-22 is a member of IL-10 cytokine family and primarily produced by Th17 cells. Expression of IL-22 has recently been reported in a number of human diseases, including mucosal-associated infections and inflammatory disorders of the intestine, skin, and joints. The relationship between IL-22 and IL-17, as well as between IL-17 and IFN-γ, is of particular interest, and their expressions are often linked to proinflammatory processes. Volpe and colleagues have demonstrated that production of human IL-17 and IL-22 is differentially regulated during cytokine induced Th cell differentiation, and that IL-22 is not a Th17-specific cytokine, and may be more broadly implicated in Th1 cell mediated immune responses. Actually, IL-22 can also be produced by non-Th17 cell types independently of IL-17 production. It has been confirmed that aryl hydrocarbon receptor (AHR) was required for the development of Th22 cells.

IL-22 was readily detected in TPE and exceeded the matching blood levels, suggesting that IL-22 may be involved in the pathogenesis of TPE. Moreover, Th22 cells have also been found to be present in TPE. More recently, we have confirmed that numbers of Th22, Th17, and Th1 cells represented in TPE were much higher than their counterparts in blood, and that most Th22 cells from TPE displayed the phenotype of effector memory cells, indicative of expressing high levels of CD45RO, as well as low levels of CD45RA and CD62L.

The molecular mechanisms underlying the generation and regulation of Th22 cells in TPE remain unknown. Our data were not consistent with those reported by Volpe et al. which showed that none of IL-1β, IL-6, or TNF-α induced IL-22 production in vitro from naïve CD4+ T cells. We found that IL-1β, IL-6 or TNF-α could significantly promote the differentiation of Th22 cells from naïve CD4+ T cells, and that the combination of IL-1β and IL-6, IL-1β and TNF-α, IL-6 and TNF-α, or IL-1β and IL-6 and TNF-α promoted Th22 cell differentiation at even higher extents. In addition, IFN-γ did not affect Th22 cell numbers; in contrast, it could reduce the increased percentage of Th22 cells stimulated by IL-6 or/and TNF-α. There were two potential explanations for the discrepancies in our study as compared with those in Volpe et al.’s study. First, the subjects were normal healthy donors in their study whereas our subjects were patients with TPE. It is therefore possible that Th22 cell differentiation might be affected by MTB infection in our study. Second, other factors, such as different concentrations of cytokines used in the in vitro culture, could also account for discrepancies. In interpreting the results of our study, one should consider that Th22 cell differentiation promoted by proinflammatory cytokines in our study were similar to those reported for the patients with skin disorders. In addition, recruitment of Th22 cells into TPE could be induced by PMCs via CCL20—CCR6, CCL22—CCR4, and/or CCL27—CCR10 axes.

We were also interested in knowing whether PMCs could promote differentiation of pleural Th22 cells in MTB infection. If PMCs are to function in presenting antigen to T cells, they would need to express HLA-DR and co-stimulatory molecules. Our data of PMCs recovered from TPE confirmed that these cells exhibited the above mentioned requisite features. In the coculture with purified naïve CD4+ T cells and PMCs, promoted significantly proliferation of CD4+ T cells and differentiation of Th22 cells could be observed even in the absence of exogenous MTB-specific protein antigen; the addition of MTB antigen 85B yielded more intensive proliferation of CD4+ T cells and differentiation of Th22, Th17 and Th1 cells in CD80- and CD86-dependent means. In addition, antigen presentation of PMCs could also promote differentiation of Th9 cells. Although Th22 cells have been found to be increased in TPE, their pathophysiological functions have been poorly defined. It has been reported that Th22 cells can evolve to retain IL-22 on membrane and to exert efficient cell—cell interaction for anti-MTB effector function. Pleural mesothelium has tight intercellular junctions which promote cell—cell adhesion and contribute to the control of pleural permeability. Pleural mesothelial integrity is necessary in barrier maintenance against various pathogens including MTB. It has been reported that in TPE, mycobacteria caused release of vascular endothelial growth factor from PMCs and resulted in protein exudation by altering mesothelial adherent junction proteins. When PMCs were stimulated with bacille Calmette-Guérin in vitro, the expression of intercellular adhesion molecule-1 on PMCs could facilitate monocyte transmigration across the injured pleural mesothelium. Our previous findings showed that IL-22, IL-17, and IL-9 significantly improved wound healing and long term restoring of PMCs; in contrast, IFN-γ even severely impaired this wound healing and restoring. These data suggest that the interaction among different Th1, Th17, Th9 and Th22 cells play important immunoregulation in the pathogenesis of TPE.

6. Concluding remarks

Despite the recent identification of Treg, Th17, Th9, and Th22 cells, over the past few years we have made rapid and large advances in our understanding of the development, regulation and function of these Th cells. This has been particularly true in the context of autoimmune diseases, inflammation and cancer, where the pathogenic roles of Th cells have been well documented. Th cells have emerged as important effector cells in infection diseases including TPE. Our results, along with those reported by the others, provide insight on expansion and immune regulation of Th cells in TPE environment. Since Th cells, such as Th17 cells, can recruit granulocytes to the site of MTB infection to produce inflammation, these Th cells might be able play an important role in the early development of protective immunity in tuberculosis, including TPE.

However, despite this exciting new knowledge about Th cells in TPE, we think there are still many unsolved mysteries. Answering these questions will not only benefit our understanding of Th cell regulation, but also our knowledge of the pathogenesis of TPE. Now, there is agreement that some molecules involved in the induction of Th cells and their effector functions (i.e., TGF-β, IL-4, IL-6, IL-17, IL-21, IL-22, and IL-23, etc.) have been identified, and this knowledge will allow the rational development of strategies for modulating Th cells in TPE, and further would be helpful for developing new procedures for differential diagnosis and treatment of patients with TPE.

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283


