Interplay of Th1 and Th17 Cells in Murine Models of Malignant Pleural Effusion

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Abstract

**Rationale:** IFN-γ–producing CD4⁺ T (Th1) cells and IL-17–producing CD4⁺ T (Th17) cells have been found to be involved in multiple malignancies; however, the reciprocal relationship between Th1 and Th17 cells in malignant pleural effusion (MPE) remains to be elucidated.

**Objectives:** To explore the differentiation and immune regulation of Th1 and Th17 cells in the development of MPE in murine models.

**Methods:** The distribution and differentiation of Th1 and Th17 cells in MPE were investigated in IFN-γ−/−, IL-17−/−, and wild-type mice. The effects of Th1 and Th17 cells on the development of MPE and the survival of mice bearing MPE were also investigated.

**Measurements and Main Results:** We have demonstrated that increased Th1 and Th17 cells could be found in MPE as compared with blood and spleen. Compared with wild-type mice, Th17 cells were markedly augmented in MPE from IFN-γ−/− mice, and improved survival could be seen in IFN-γ−/− mice, and increased survival could be seen in IL-17−/− mice. The in vitro experiments showed that IFN-γ deficiency promoted Th17-cell differentiation by suppressing the STAT3 pathway and that IL-17 deficiency promoted Th1-cell differentiation by suppressing the STAT1 pathway.

**Conclusions:** In mouse models of MPE, IFN-γ inhibited Th17-cell differentiation, whereas IL-17 inhibited Th1-cell differentiation. IL-17 inhibited the formation of MPE and improved the survival of mice bearing MPE; in contrast, IFN-γ promoted MPE formation and mouse death.

**Keywords:** lung cancer; malignant pleural effusion; Th1 cells; Th17 cells

Malignant pleural effusion (MPE) is frequently observed in some malignancies, and lung adenocarcinoma is the most frequent underlying malignancy (1). The appearance of MPE in patients implies systemic spread of cancer and reduction of life expectancy and quality (2, 3). Although MPE is becoming more common, very little information is available on the immune mechanisms involved in its pathogenesis (4). Improved understanding of the pathogenesis of MPE may be helpful for the development of safer and more effective therapeutic options for MPE. In MPE, CD4⁺ T lymphocytes are dominant, and the proportion of CD8⁺ T cells is much lower than that of CD4⁺ T cells (5). The subset of CD4⁺ T cells...
that produce IL-17A and IL-17F have been defined as a separate subset Th17 cells (6), and Th17 cells have been documented to be involved in the pathogenesis of cancers (7, 8). The development and differentiation of Th17 cells was described to be linked to that of Th1 cells in a reciprocal fashion because it has been demonstrated that the inhibition of the transcription factor T-bet expressed in Th1 cells suppresses differentiation of Th1 and Th17 cells (9) and that T-bet directly regulates transcription of the IL-23 receptor and influences the fate of Th17 cells, which depend on optimal IL-23 production for survival (10). In the previous studies, our data have shown that the numbers of Th17 and Th1 cells were increased in human MPE compared with the corresponding blood and that pleural Th1 cell numbers were correlated with pleural Th17 cell numbers. The underlying mechanisms by which Th1 and Th17 cells are involved in MPE formation are unclear.

**What This Study Adds to the Field:**
IL-17 inhibited Th1-cell differentiation by suppressing the STAT1 pathway, whereas IFN-γ inhibited Th17-cell differentiation by suppressing the STAT3 pathway in mouse models of MPE. IL-17 deficiency promoted angiogenesis and proliferation activity of pleural tumors as well as pleural vascular permeability and thus promoted MPE formation, whereas IFN-γ deficiency showed contrary effects. IL-17 deficiency accelerated the death of mice bearing MPE, whereas IFN-γ deficiency was associated with improved survival.

**At A Glance Commentary**

**Scientific Knowledge on the Subject:** It has been reported that the numbers of Th1 and Th17 cells were increased in human malignant pleural effusion (MPE) compared with the corresponding blood and that pleural Th1 cell numbers were correlated with pleural Th17 cell numbers. The underlying mechanisms by which Th1 and Th17 cells are involved in MPE formation are unclear.

**Methods**

Further details are provided in the online supplement.

**Animals**
Wild-type (WT) C57BL/6 mice were purchased from the Animal Center of Wuhan University, Wuhan, China. IFN-γ−/− mice in a C57BL/6 background were purchased from The Jackson Laboratory. IL-17a−/− mice in a C57BL/6 background were generated as previously

![Figure 1](image-url)

**Figure 1.** Formation of malignant pleural effusion (MPE) in wild-type (WT), IFN-γ−/−, and IL-17−/− mice. (A) Multiple tumor foci could be observed on the parietal and visceral pleura 14 days after intrapleural injection of Lewis lung cancer cells (top panels). The overall view of MPE in the three groups (bottom panels). (B) Positron emission tomography and computed tomography imaging of developing pleural tumors and MPE in WT (top panels), IFN-γ−/− (middle panels), and IL-17−/− mice (bottom panels). (C) The maximum standardized uptake values (SUV_{max}) on positron emission tomography were calculated (each n = 10). (D) Pleural permeability assay was calculated by MPE/serum Evan’s blue ratio (each n = 6). In C and D, data are presented as means ± SEM. *P < 0.05 compared with one another among three groups. The comparisons were determined by Kruskal-Wallis one-way ANOVA on rank.
Figure 2. Effects of deficiency of IFN-\(\gamma\) and IL-17 on expression of CD34 and Ki-67 in pleural tumors and survival of mice bearing malignant pleural effusion. Tumor tissues were harvested from wild-type (WT), IFN-\(\gamma\)^{-/-}, and IL-17^{-/-} mice 14 days after intrapleural injection of Lewis lung cancer cells. The paraffin sections were stained with Abs specific to CD34 (A, green) and Ki-67 (B, red), and 4',6-diamidino-2-phenylindole mounting medium was used for cell nuclei staining. Original magnification: \(\times 200\). Results are representative of five independent experiments. (C and D) Comparisons of CD34^{+} cells (C) and Ki-67^{+} cells (D) in the three groups (each \(n = 5\)). Data are presented as means \(\pm\) SEM. *\(P < 0.05\) compared with one another among three groups. The comparisons were determined by Kruskal-Wallis one-way ANOVA on rank. (E) Overall survival of WT, IFN-\(\gamma\)^{-/-}, and IL-17^{-/-} mice (each \(n = 15\)) was estimated by the Kaplan-Meier method and compared using the pairwise log-rank tests.
intravenous injection and were killed 1 hour later. Evans’ blue concentrations in pleural fluid and serum were determined by measuring absorbance at a wavelength of 630 nm in comparison to the standard Evans’ blue concentration.

**Immunofluorescence Staining**
To investigate angiogenesis and proliferation activity of pleural tumors, double immunofluorescence staining of CD34 and Ki-67 was performed on paraffin sections of tumor tissues removed from the pleural cavity. Further details are provided in the online supplement.

**Flow Cytometry**
Intracellular detection of cytokines was determined by flow cytometry. Further details are provided in the online supplement.

**Quantitative Real-Time PCR**
mRNA expression of IFN-γ, IL-17, T-bet, and RORγt in MPE cells was detected by quantitative real-time PCR. Further details are provided in the online supplement.

**In Vitro Differentiation of Th1 and Th17 Cells**
Differentiation of Th1 and Th17 cells from naive CD4+ T cells isolated from mouse spleens were determined in vitro. Further details are provided in the online supplement.

**Signal Transductions of IL-17 and IFN-γ**
Intracellular expression of phospho-signal transducer and activator of transcription (p-STAT)1 or p-STAT3 during differentiation of Th1 or Th17 cells was determined by flow cytometry. Further details are provided in the online supplement.

**Statistics**
Data are expressed as mean ± SEM. Statistical methods are discussed in the online supplement.

**Results**
Fourteen days after injection of LLC cells, pleural tumors were found to reside equally on the visceral and parietal pleura in WT, IFN-γ−/−, and IL-17−/− mice (Figure 1A, upper panels). Bloody pleural fluid could be directly visible through the diaphragm, surrounding tumor foci in all three groups (Figure 1A, bottom panels). The numbers of pleural tumor foci and the volume of MPE were increased in the IL-17−/− group and were decreased in the IFN-γ−/− group.

To evaluate the presence of pleural tumors and MPE in the living mice, we performed deoxyglucose-PET (FDG-PET) and CT scans. Increased radiotracer uptake on FDG-PET scanning was found in the areas of tumors and MPE (Figure 1B). Compared with WT mice, the PET imaging confirmed lower 18F-FDG retention in IFN-γ−/− mice and higher retention in IL-17−/− mice. The maximum standardized uptake values of WT, IFN-γ−/−, and IL-17−/− mice were 9.43 ± 0.72, 7.46 ± 0.52, and 12.95 ± 0.90, respectively, with statistical significance between any two groups of them (all \( P < 0.05 \) (Figure 1C).
To explore whether MPE accumulation was dependent on increased vascular permeability, we measured leakage of Evans’ blue dye into the pleural space. One hour after intravenous injection of 10 mg Evans’ blue into mice bearing MPE (n = 6 each group), serum Evans’ blue levels in WT, IFN-γ−/−, and IL-17−/− mice were 23.1 ± 1.3, 32.4 ± 1.1, and 21.5 ± 1.5 μg/ml, respectively. MPE Evans’ blue levels in WT, IFN-γ−/−, and IL-17−/− mice were 11.4 ± 0.8 μg/ml (50 ± 6% of serum levels), 4.8 ± 0.4 μg/ml (15 ± 1% of serum levels), and 13.9 ± 1.2 μg/ml (64 ± 5% of serum levels), respectively (Figure 1D). These results indicated that the absence of IFN-γ decreased pleural vascular permeability, whereas the absence of IL-17 increased pleural vascular permeability.

We noted that CD34 (Figure 2A) and Ki-67 (Figure 2B) were abundant in tumor tissues. CD34 expression was mainly detected on the cell membrane of newly born blood vessel, whereas Ki-67 was expressed in cell nucleus of proliferative tumor cells. Compared with WT mice, expression of CD34 (Figures 2A and 2C) and Ki-67 (Figures 2B and 2D) was increased in IL-17−/− mice and was decreased in IFN-γ−/− mice.

Similar results were seen in the MC38 model of MPE 11 days after intrapleural injection of MC38 cells (data not shown).

Effects of Th1 and Th17 Cells on Survival of MPE Mice
The median survival times of WT, IFN-γ−/−, and IL-17−/− mice bearing MPE induced by LLC cell instillation were 26, 31, and 19 days, respectively (Figure 2E). The overall log rank test for survival among the WT, IFN-γ−/−, and IL-17−/− mice was significant (P < 0.001), and pairwise log rank tests showed that each group was different from the others (WT vs. IFN-γ−/− mice log-rank, P = 0.036; WT vs. IL-17−/− mice log-rank, P < 0.001; IFN-γ−/− mice vs. and IL-17−/− mice log-rank, P < 0.001). The absence of IFN-γ was a significant predictor for improved survival of MPE mice, whereas the absence of IL-17 was associated with short survival.

Cytological Characteristics in MPE
In the LLC and MC38 models, MPE volume in WT mice was more than that in IFN-γ−/− mice and less than that in IL-17−/− mice (Table 1) (all P < 0.05). The cytological characteristics in MPE are illustrated in Table 1. The numbers of nucleated cells were significantly increased in IFN-γ−/− mice and decreased in IL-17−/− mice compared with WT mice (all P < 0.001). In addition, nucleated cell counts in IFN-γ+/+ mice were much higher than those in IL-17−/− mice (all P < 0.001). The absolute numbers of total nucleated cells and each cell types, including lymphocytes, neutrophils, monocytes, and eosinophils, in the whole MPE were not different in the three groups (all P > 0.05).
Th1 and Th17 Cells Were Significantly Increased in MPE

Th1 cells and Th17 cells in MC38 and LLC models were identified by flow cytometry (Figure 3A). In WT and IL-17−/− mice, a significant increase in Th1 cells was observed in the MPE of MC38 models (Figure 3B) and in the LLC model (Figure 3C) compared with those in the corresponding blood and spleen (all P < 0.05). Similarly, in WT and IFN-γ−/− mice, the percentages of Th1 cells represented an increase in MPE in the MC38 model (Figure 3D) and the LLC model (Figure 3E) compared with those in the corresponding blood and spleen (all P < 0.05). The percentages of Th1 and Th17 cells in blood and spleen showed no significant differences.

In the MC38 and LLC models, Th1 cells in MPE, but not in blood and spleen, from IL-17−/− mice were significant increased as compared with WT mice, whereas Th17 cells in MPE, but not in blood and spleen, from IFN-γ−/− mice were higher than those from WT mice (Figure 3).

Expression of IFN-γ, IL-17, T-bet, and RORγt mRNA in MPE

There was much higher mRNA expression of IFN-γ and T-bet in MPE from IL-17−/− mice than those from WT mice with the LLC model of MPE (Figure 4). Similarly, there was much higher mRNA expression of IL-17 and RORγt in MPE from IFN-γ−/− mice than from WT mice.

Figure 4. mRNA of IFN-γ and IL-17 and their corresponding transcription factors are expressed in malignant pleural effusions (MPEs) in wild-type (WT), IFN-γ−/−, and IL-17−/− mice. The mononuclear cells were isolated from MPE, RNA was isolated, and quantitative PCR was performed for IL-17 mRNA (A), IFN-γ mRNA (B), T-bet mRNA (C), and RORγt mRNA (D). Electrophoresis photographs are representative of four independent experiments, and data are presented as mean ± SEM of four experiments. *P < 0.001 compared with the other groups. The comparisons were determined by Mann-Whitney test or Kruskal-Wallis one-way ANOVA on rank.

Discussion

It is generally believed that pleural fluid accumulates in pleural space when
production outweighs absorption, the latter mainly occurring via pleuropulmonary lymphatics (4). Removal of pleural fluid is reduced when tumors invade the drainage system (15). Recently, it has been suggested that inflammatory cells, mesothelial cells, and endothelial cells in the pleural microenvironment interact with tumor cells and contribute to MPE formation (4).

Despite recent advances in our understanding of the differentiation and function of Th cells, very little is known about their immune regulation in MPE. Given the fact that inflammation is linked to cancer development and progression and that Th17 and Th1 cells are increased in MPE (11), it is reasonable to predict that there might be interaction between proinflammatory Th17 and Th1 cells in the development of MPE. In the present study, our data showed that, consistent with the distribution of pleural tumors on the visceral and parietal pleura after intrapleural instillation of cancer cells, the volume of MPE was significantly increased in IL-17\(^{-/-}\) mice and was decreased in IFN-\(\gamma\)^{−/−} mice as compared with WT mice, indicating that MPE development could be promoted by IL-17 deficiency and inhibited by IFN-\(\gamma\) deficiency.

IL-17A and Th17 cells have been found to be present in multiple human malignancies (11, 16–20); however, their functions in tumor immunity remain controversial. Some studies have demonstrated that Th17 cells have antitumor activity (21–24), and other studies have supported the finding that Th17 cells can promote tumor growth (25, 26). On the other hand, Th1 cells were shown to have some antitumor activity in early experiments (27). In a murine B-cell cancer model, Th1 cells recently were reported to induce macrophages to secrete angiostatic chemokines CXCL9/MIG and CXCL10/IP-10, as well as proinflammatory cytokines IL-1\(\beta\) and IL-6, and to render macrophages directly cytotoxic to cancer cells, suggesting that inflammation driven by tumor-specific Th1 cells might prevent rather than promote cancer (28). The underlying mechanisms by which IL-17 and IFN-\(\gamma\) are involved in MPE formation are unclear. By determining the expression of CD34 and Ki-67, we noted in the present study that IL-17 deficiency enhanced tumor angiogenesis and proliferation, whereas IFN-\(\gamma\) deficiency played an inhibitory effect on these events. We also noted that the absence of IL-17 increased pleural vascular permeability, whereas the absence of IFN-\(\gamma\) decreased this event. Therefore, the opposing functions of IL-17 and IFN-\(\gamma\) extended our understanding that IL-17 inhibits MPE formation and that IFN-\(\gamma\) promotes MPE formation by affecting angiogenesis and proliferation of pleural tumor as well as pleural vascular permeability.

Although there are no data directly indicating the lineage association between the development of Th1 and Th17 cells, there is evidence demonstrating that Th1 and Th17 cells might be phenotypically, developmentally, and functionally linked in the tumor microenvironment (27).
IFN-γ has been found to be expressed by Th17-polarized mouse cells (21) and in primary Th17 cells in human tumors (29). Previously we have demonstrated that IFN-γ−/−IL-17−/−CD4+ T cells were also found in human MPE (11). In the present study, we observed that Th1 and Th17 cells were markedly increased in MPE compared with the corresponding blood and spleen. More importantly, compared with WT mice, a significant increase in Th1 cells was observed in MPE from IL-17−/−/− mice, and a significant increase in Th17 cells was observed in MPE from IFN-γ−/− mice. Our in vitro experiments further confirmed that exogenous recombinant mouse IL-17 inhibited IFN-γ production by CD4+ T cells from IL-17−/−/− mice, whereas exogenous IFN-γ inhibited IL-17 production by CD4+ T cells from IFN-γ−/−/− mice. These data indicated that IL-17 was able to inhibit the differentiation of Th1 cells, whereas IFN-γ was able to inhibit the differentiation of Th17 cells in the tumor environment in murine MPE models.

T-bet is a transcription factor that was first associated with the differentiation of Th1 cells and IFN-γ production by CD4+ T cells (30), and the differentiation of Th17 cell lineage requires the up-regulation of the orphan unclear receptor RORγt (31). Therefore, we examined mRNA expression of IFN-γ and Th17 simultaneously with mRNA expression of T-bet and RORγt and noted that there was much higher mRNA expression of IFN-γ and T-bet in MPE from IL-17−/−/− mice than those from WT mice. Similarly, there was much higher mRNA expression of IL-17 and RORγt in MPE from IFN-γ−/− mice than those from WT mice. Our in vitro experimental data further showed that, similar to the proliferation of Th1 and Th17 cells, the numbers of T-bet+CD4+ T cells in IL-17−/−/− mice and of RORγt+CD4+ T cells in IFN-γ−/−/− mice were significantly increased as compared with those in WT mice. Collectively, our data suggested that IL-17 inhibited Th1-cell differentiation by down-regulating T-bet expression and that IFN-γ inhibited Th17-cell differentiation by down-regulating RORγt expression.

It is well known that the activation of specific STAT proteins in CD4+ T cells is associated with the differentiation of T cells into distinct Th-cell lineages (32, 33). In view of this, IFN-γ is the major cytokine responsible for STAT1 activation. Such activation was almost completely suppressed in IFN-γ−/−/− mice (34), and IL-6–inducedSTAT3 signaling was critical in promoting Th17-cell differentiation (35, 36). We next investigated the molecular mechanisms of signal transduction in differentiation of Th1 and Th17 cells in MPE. We noted that STAT1 was phosphorylated in the presence of optimal Th1 conditions and that STAT3 was phosphorylated in the presence of optimal Th17 conditions. p-STAT1 expression was reduced when IL-17 was added into Th1 conditions, and p-STAT3 expression was suppressed when IFN-γ was added into Th17 conditions. These data suggested that IL-17 inhibited Th1-cell differentiation by suppressing the STAT1 pathway, whereas IFN-γ inhibited Th17-cell differentiation by suppressing the STAT3 pathway.
inhibited Th17-cell differentiation by suppressing the STAT3 pathway.

Th17 cells have been shown to possess antitumorigenic and protumorigenic functions, and Th17 cells’ functions in cancers are highly dependent on context (37, 38). Satisfactory explanations for these contradictory observations are not available. It is possible that the effects of Th17 cells vary with different tumors, which may be associated with distinct patterns of stromal, angiogenic, and inflammatory reactions. In addition to cells of the immune system, the cellular targets of IL-17 or IFN-γ in the tumor microenvironment can be tumor cells, stromal cells, and vascular endothelial cells (7). Furthermore, although IL-17 and IFN-γ are the signature cytokine of Th17 and Th1 cells, respectively, the biological activities of IL-17 or IFN-γ might not be equated with the biological activities of Th17 and Th1 cells in vivo.

The timing and numbers of Th17 cells in tumor environment, and the balance with Th1 immunity and other aspects of host defense, could also be critical (37). Our previous data have shown that the accumulation of Th17 cells in MPE predicted improved patient survival, implying a new role of the proinflammatory response in regulating tumor progression in humans (11). Consistent with this finding, we noted in the current study that IL-17 deficiency was a significant predictor for decreased survival of mice bearing MPE, whereas IFN-γ deficiency was associated with improved survival.

We have demonstrated that Th1 and Th17 cells were increased in MPE in mouse models and that IL-17 inhibited MPE formation and IFN-γ promoted MPE formation by affecting angiogenesis and proliferation of pleural tumor as well as pleural vascular permeability. Our data also showed that IFN-γ inhibited Th17 cell differentiation by suppressing the STAT3 pathway, whereas IL-17 inhibited Th1 cell differentiation by suppressing the STAT1 pathway. Given the poor prognosis of patients with MPE, our observations may provide a rationale that is probably worthy to be investigated in future clinical trials.

Figure 7. Signals of signal transducer and activator of transcription (STAT) in differentiation of Th1 and Th17 cells. (A) Purified naive CD4+ T cells isolated from spleens of wild-type (WT) and IL-17−/− mice were cultured in Th1 conditions (as described in MATERIALS AND METHODS) in the presence of anti-CD3 and anti-CD28 mAbs. (B) Purified naive CD4+ T cells isolated from spleens of WT and IFN-γ−/− mice were cultured in Th17 conditions in the presence of anti-CD3 and anti-CD28 mAbs. Forty-eight hours later, cells were stimulated for 30 minutes with designated cytokines alone or in various combinations. The results are reported as mean ± SEM from five independent experiments. The comparisons were determined by Kruskal-Wallis one-way ANOVA on rank. *P < 0.05 compared with medium control. †P < 0.05 compared with IL-2 plus IL-12. ‡P < 0.05 compared with IL-1β plus IL-6.

Author disclosures are available with the text of this article at www.atsjournals.org.

References