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Generation and Differentiation of IL-17–Producing CD4+ T Cells in Malignant Pleural Effusion

Zhi-Jian Ye,*1 Qiong Zhou,*1 Yong-Yao Gu,*† Shou-Ming Qin,† Wan-Li Ma,* Jian-Bao Xin,* Xiao-Nan Tao,* and Huan-Zhong Shi*

IL-17–producing CD4+ T (Th17) cells have been found to be increased in some human cancers; however, the possible implication of Th17 cells in regulating antitumor responses in malignant pleural effusion (MPE) remains to be elucidated. In the current study, distribution and phenotypic features of Th17 cells in both MPE and peripheral blood from patients with lung cancer were determined by flow cytometry or double immunofluorescence staining. The impacts of cytokines on Th17 cell generation and differentiation were explored. The chemotactic activity of chemokines CCL20 and CCL22 for Th17 cells in vitro was also observed. It was found that the increased Th17 cells could be found in MPE compared with blood. The in vitro experiments showed that IL-1β, IL-6, IL-23, or their various combinations could promote Th17 cell generation and differentiation from naive CD4+ T cells. MPE was chemotactic for Th17 cells, and this activity was partly blocked by anti-CCL20 and/or CCL22 Abs. Our data also showed that the accumulation of Th17 cells in MPE predicted improved patient survival. It could be concluded that the overrepresentation of Th17 cells in MPE might be due to Th17 cell differentiation and expansion stimulated by pleural proinflammatory cytokines and to recruitment of Th17 cells from peripheral blood induced by pleural chemokines CCL20 and CCL22. Furthermore, the accumulation of Th17 cells in MPE predicted improved patient survival. These data provide the basis for developing immune-boosting strategies based on ridding the cancer patient of this cell population. The Journal of Immunology, 2010, 185: 000–000.

Malignant pleural effusion (MPE) is frequently observed in some cancers, especially lung cancer, and appearance of an MPE is an ominous prognostic sign for cancer patients, because the presence of MPE indicates that the tumor is incurable by surgery and life expectancy is short (1). Although there have been no epidemiologic studies, the annual incidence of MPE in the United States is estimated to be >150,000 cases (2). Although MPE is more and more common, very little information is available on the immune mechanisms that are involved in its development. An accumulation of lymphocytes, especially CD4+ helper T cells, frequently occurs in MPE secondary to direct pleural involvement and/or metastases from malignancies (3, 4).

IL-17 (also known as IL-17A) was originally cloned as CTLA8 from a rodent T cell hybridoma by Rouvier et al. (5) in 1993 and was identified by Yao et al. (6) 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 (7). The subset of CD4+ T cells that produce both IL-17A and IL-17F is now defined as a separate subset IL-17–producing CD4+ T (Th17) cells. Distinct from Th1 and Th2 cells, Th17 cells are reported to be generated from naive T cells by IL-6 and TGF-β (8, 9) and are expanded and stabilized further by IL-23 (10) and by virtue of expressing the orphan nuclear receptor RORγt as a critical transcription factor (11). Th17 cells have been found in some human cancers (12–15), however, the possible implication of Th17 cells in regulating antitumor responses in MPE remains to be demonstrated. The present study investigated the idea that Th17 cells could be involved in the control of the local immune response in MPE. In this study, we provided the insightful mechanism by which Th17 cells are generated and regulated by cytokines secreted from pleural cells and their immune infiltrates in MPE.

Materials and Methods

Subjects

The study protocol was approved by our institutional review board for human studies, and informed consent was obtained from all subjects. Pleural fluid samples were collected from 30 patients (age range, 32–81 y) with newly diagnosed lung cancer with MPE (Table I). Histologically, 19 cases were adenocarcinoma and 11 were squamous cell carcinoma. A diagnosis of MPE was established by demonstration of malignant cells in pleural fluid and/or on closed pleural biopsy specimen. The patients were excluded if they had received any invasive procedures directed into the pleural cavity or if they had suffered chest trauma within 3 mo prior to hospitalization. At the time of sample collection, none of the patients had received any anticancer therapy, corticosteroids, or other nonsteroid anti-inflammatory drugs.

Sample collection and processing

The pleural fluid samples were collected in heparin-treated tubes from each subject, using a standard thoracocentesis technique within 24 h after hospitalization. Thirty milliliters of peripheral blood was drawn simul-
staining of pleural CD4+ T cells indicated that some CD4+ T cells are Th17 cells, some are Th1 cells, and protocol. Eventually, Th17 cells were obtained with a purity ranging from Biotec) was used to isolate Th17 cells, according to the manufacturer’s flow cytometry.

controls. Top panels mounting medium was used for cell nuclei staining. rhodamine-labeled goat anti-rabbit IgG (red), DAPI rabbit polyclonal Ab targeted against IL-17, and then mouse IgG (green). IL-17 expression was detected by IFN-γ

2 TH17 CELLS IN MPE

FIGURE 1. Presence of Th17 and Th1 cells in MPE. A, Expression of IFN-γ was detected by mouse anti–IFN-γ mAb and then fluorescein-labeled goat anti-mouse IgG (green). IL-17 expression was detected by rabbit polyclonal Ab targeted against human IL-17 and mouse anti-human IFN-γ mAb (both were purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate species-matched Abs were used as isotype controls. As secondary Abs, rhodamine-labeled affinity-purified goat anti-rabbit IgG was used for labeling the rabbit anti–IL-17 Ab, and fluorescein-labeled affinity-purified goat anti-mouse IgG was used for labeling the mouse anti–CD3, -CD68, and -EMA mAbs.

Flow cytometry

The expression markers on T cells from MPE and blood were determined by flow cytometry after surface staining or intracellular staining with anti-human-specific Abs conjugated with either PE or FITC. These human Abs included anti-CD3, anti-CD4, anti-CD45RA, anti-CD45RO, anti-CD62L, anti-CCR2, anti-CCR4, anti-CCR5, anti-CCR6, anti-CCR7, anti–IL-17, and anti–IFN-γ mAbs, which were purchased from BD Biosciences or eBioscience (San Diego, CA). Intracellular staining for IL-17– or IFN-γ–producing T cells was performed on T cells stimulated with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (1 μM; Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences) for 5 h, and the intracellular IL-17 or IFN-γ was then stained with anti–IL-17 or IFN-γ conjugated with

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PE (eBioscience). Flow cytometry was performed on a BD FACSCalibur flow cytometer using FCS ExpressV3 software.

**Measurement of cytokines and chemokine**

The concentrations of cytokines, including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-23, IFN-γ, and TGF-β1, as well as chemokine CCL20, in both pleural fluids, and sera were measured by ELISA kits according to the manufacturer’s protocols (all kits were purchased from R&D Systems, Minneapolis, MN). All samples were assayed in duplicate.

**Generation and differentiation in MPE of Th17 cells**

Purified naïve CD4+ T cells (5 × 10^5) were cultured in 1 ml complete medium containing human IL-2 (2 ng/ml) in 48-well plates and stimulated with plate-bound anti-CD3 (OKT3; 1 µg/ml) and soluble anti-CD28 mAbs (1 µg/ml) for 7 d. The exogenous cytokines used were TGF-β1 (5 ng/ml), IL-6 (100 ng/ml), IL-1β (10 ng/ml), and IL-23 (10 ng/ml). Recombinant human IL-1β, IL-2, IL-6, IL-23, and TGF-β1 were purchased from R&D Systems.

**Th17 cell chemotaxis assay**

Chemotaxis assays were performed using 8-µm pore polycarbonate filters in 24-well Transwell chambers (Corning Costar, Corning, NY). Briefly, Transwell membranes were coated with fibronectin (5 µg/ml; Chemicon International, Schwalbach, Germany) for 30 min at 37°C. Th17 cells isolated from peripheral blood were added to the top chamber resuspended in RPMI 1640 medium plus 0.5% BSA at 1 × 10^5 cells/ml in a final volume of 100 µl. MPE from five lung cancer patients were placed in the bottom chamber of the Transwell in a volume of 600 µl, and the chamber was incubated at 37°C in 5% CO₂ atmosphere for 3 h. At the end of incubation, the filter was washed by HBSS lightly, fixed, stained, and mounted on a glass microscope slide. The chemotaxis index was calculated by dividing the numbers of cells migrated in response to test MPE or recombinant human chemokines by the numbers of cells migrated in response to medium alone. To demonstrate that CCL20 or CCL22 was responsible for Th17 cell migration, blocking experiments were performed by mixing the MPE with 100 ng/ml anti-CCL20, anti-CCL22, both anti-CCL20 and anti-CCL22 mAbs, or mouse IgG irrelevant isotype control (R&D Systems).

**Statistics**

Data are expressed as mean ± SEM or median (range). Comparisons of the data between different groups were performed using a Kruskal-Wallis ANOVA on ranks. For data in MPE and in the corresponding blood, paired data comparisons were made using a Wilcoxon signed-rank test. Correlations between variables were determined by Spearman’s rank correlation coefficients. Survival was estimated by the Kaplan-Meier method. Analysis was completed with SPSS version 16.0 Statistical Software (Chicago, IL), and p < 0.05 was considered to indicate statistical significance.

**Results**

Th17 and Th1 cells were significantly increased in MPE

To visualize the distribution of Th17 and Th1 cells in MPE, double immunofluorescence staining was performed on the pleural cell pellets with both anti-CD4 and anti–IL-17 or IFN-γ Abs. Because rabbit polyclonal Ab targeted against human IL-17 was used in the current study, and such a polyclonal Ab is notorious for lacking specificity, we performed positive controls using purified Th17 cells, which showed that the immunofluorescent technique used in our study was reliable (Fig. 1A, top panels). We noted that both Th17 and Th1 cells were found in MPE, and some of these pleural CD4+ T cells were Th17/Th1 cells (Fig. 1A, bottom panels). Consistent with the immunofluorescent findings, Th17 cells, Th1 cells, and Th17/Th1 cells in MPE could also be identified by flow cytometry (Fig. 1B). Percentages of Th17 cells represented the higher values in MPE (3.74 ± 0.34%), showing a significant increase in comparison with those in the corresponding blood (0.59 ± 0.06%) (n = 30; Wilcoxon signed-rank test; p < 0.001) (Fig. 1B–D). Similarly, a significant increase in Th1 cells was observed in MPE (29.81 ± 2.49%) compared with blood (2.92 ± 0.35%) (Wilcoxon signed-rank test; p < 0.001) (Fig. 1B–E). It was noted that pleural Th17 cell frequency was correlated positively with Th1 cell frequency (r = 0.865; p < 0.001) (Fig. 1F).

To identify the phenotypic characteristics of Th17 in MPE, we analyzed the expression profiles of CD45RO, CD62L, and CCR7 and observed that most pleural Th17 cells expressed high levels of CD45RO (89.6 ± 1.3%) but not of CD62L (3.7 ± 0.6%) or CCR7 (5.1 ± 0.7%), indicating that they were effector memory cells (Fig. 2).

**Generation and differentiation of Th17 cells in MPE**

We determined some cytokines that are thought to be related to the generation and differentiation of human Th17 cells in MPE. As shown in Fig. 3A, high levels of IL-1β, IL-6, IFN-γ, and TGF-β1 could be found in MPE, and they were significantly higher than their compartments in the corresponding serum (n = 14; all p < 0.01). The above results suggested that these proinflammatory cytokines might promote the generation and differentiation of Th17 cells in MPE.

To evaluate the contribution of cytokines to the generation and differentiation of pleural Th17 cells, we purified naïve T cells and cultured them in the presence of one or more of IL-1β, IL-6, IL-23, and TGF-β. With IL-2–containing medium provided a baseline for comparison, IL-1β, IL-6, or IL-23, but not TGF-β, could promote the differentiation of Th17 cells from naïve CD4+ T cells (Fig. 3B). The combination of IL-1β plus IL-6, IL-1β plus IL-23, IL-6 plus IL-23, or IL-1β plus IL-6 plus IL-23 significantly
increased the percentage of Th17 cells at higher extents compared with any single one of above cytokines. Surprisingly, although high concentration of IFN-γ was found in MPE, which was much higher than that in serum, IFN-γ did not affect Th17 cell numbers (Fig. 3B). Likewise, a significant high concentration of TGF-β was also found in MPE, but it did not promote the differentiation
of Th17 cells; in contrast, TGF-β could reduce the increased percentage of Th17 cells stimulated by the above cytokines (Fig. 3B).

We noted that abovementioned proinflammatory cytokines or their combinations could cause generation and differentiation of Th17 cells from blood naive CD4⁺ T cells in a similar manner (Fig. 3B). We also noted that IL-1β plus IL-6 could induce more Th17 cell generation and differentiation from MPE naive CD4⁺ T cells than from blood naive CD4⁺ T cells.

Chemokines CCL20 and CCL22 might be responsible for recruitment of Th17 cells into MPE

Besides local generation and differentiation, recruitment from peripheral blood might also contribute to the increase in pleural Th17 cells. Because lymphocyte migration is tightly regulated by chemokine–chemokine receptor interaction (16), we therefore sought to elucidate the effects of chemokine–chemokine receptor interaction on recruitment of Th17 cells into MPE. In the current study, our results showed that the expressions of CCR2, CCR4, CCR5, and CCR6 could be observed in pleural Th17 cells; among these chemokine receptors, CCR4 and CCR6 exhibited significant high levels of expressions (Fig. 4). The percentages of CCR2⁺ Th17, CCR4⁺Th17, CCR5⁺Th17, and CCR6⁺Th17 cells were 20.6 ± 2.7, 65.9 ± 24, 26.8 ± 2.2, and 48.3 ± 4.8%, respectively. We also noted that the expressions of these CCRs in Th17 cells was similar between MPE and blood (n = 14; all p < 0.05).

In a previous study, we have reported that all cancer cells, most of macrophages and T cells in MPE, expressed high level of CCL22 and that concentration of soluble CCL22 in MPE was much higher than that in serum (17). In the current study, we extended these observations and further demonstrated that significant expression of another chemokine CCL20 was found in all cancer cells identified by anti-EMA mAb; some macrophages identified by anti-CD68 mAb, and some T cells identified anti-CD3 mAb (Fig. 5A). These data suggested that malignant cells, macrophages, and T cells might be the cell sources of pleural CCL20. In addition, our data also showed that CCL20 concentration in MPE (79.6 ± 19.6 pg/ml) was also much higher than that in serum (26.2 ± 6.1 pg/ml; n = 14; p < 0.001) (Fig. 5B).

On the basis of our observation that Th17 cells expressed high levels of CCR4 and CCR6, and high levels of CCL20 and CCL22 were found in MPE, we hypothesized that Th17 cells could migrate into the pleural space in response to CCL20 and/or CCL22. Our results showed that MPE exerted a potent chemotactant activity for circulating Th17 cells and that either anti-CCL20 or CCL22 mAb significantly suppressed Th17 cell chemotaxis; the combination of both mAbs exhibited an even more strong suppressive effect (Fig. 5C). Taken together, these data suggested that chemokines CCL20 and CCL22 might be responsible for recruitment of Th17 cells into MPE.

Increase in pleural Th17 cells predicted improved survival of patients with MPE

To test the prediction that pleural Th17 cells would affect survival of patients with MPE, we analyzed survival time with the number of Th17 cells in 30 patient with MPE. The individuals were divided into two equal groups on the basis of Th17 cell numbers. The low Th17 cell group included all those with Th17 cell counts of ≤4% (n = 15) and the high Th17 cell group included those with Th17 cell counts > 4% (n = 15). As shown in Fig. 6, there was a significant correlation between pleural Th17 cell numbers and patient survival (p = 0.001). Patients with higher Th17 cells had significant longer overall survival (median, 5 mo) than patients with lower Th17 cells (median, 3 mo). Individuals in the low Th17 cell group experienced a 4.5-fold higher death hazard as compared with those in the high Th17 cell group (95% confidence interval, 1.8–11.3). The multivariate Cox proportional hazards analysis was performed, and variables that were associated with survival by univariate analysis were adopted as covariates (Table I). The multivariate analysis revealed that the number of pleural Th17 cells was an independent prognostic factor for survival (hazard ratio = 0.347; p = 0.002) (Table II). However, Th1 number, glucose concentration, and pH value were not independent prognostic factors for survival (all p > 0.05). Therefore, an increased in pleural Th17 cells was a significant predictor of decreased risk for death and for improved survival in patients with MPE.

**Discussion**

In MPE, CD4⁺ T cells are dominant, and the proportion of CD8⁺ T cells is significantly lower than that of CD4⁺ T cells (18). In contrast, the proportion of CD4⁺ T cells in the pleural cavity of lung cancer patients without MPE is significantly lower than that of CD8⁺ T cells (19). Furthermore, the proportion of pleural CD4⁺
T cells may help to select patients who are likely to have a poorer prognosis after surgery and therefore may be suitable for consideration of adjuvant treatments (20). Classically, naive CD4+ T cells have been thought to differentiate into two main lineages, Th1 and Th2 cells (21, 22). Regulatory T cells represent only a small subset of CD4+ T cells in the peripheral circulation and are responsible for the balance of immune responses, which is essential for health (23). The identification of Th1/Th2 cell balance in MPE was first reported in a study by Higashi et al. (5). In that work, they showed that the ratio of Th1 to Th2 cells in MPE was higher than in peripheral blood. The mechanisms of accumulation of Th17 cells in the tumor microenvironment remain largely unknown. We speculated that an increased percentage of Th17 cells in MPE might be due to local differentiation and/or active recruitment. In the previous study, we provided direct evidence that IL-16 is capable of inducing CD4+ T cell infiltration into the pleural space (33). Therefore, as a subpopulation of CD4+ T cells, Th17 cells might also be recruited into MPE by local production of IL-16, because IL-16 level is significantly higher in MPE than in serum (33). T cell infiltration into the pleural space (33). Therefore, as a subpopulation of CD4+ T cells, Th17 cells might also be recruited into MPE by local production of IL-16, because IL-16 level is significantly higher in MPE than in serum (33).

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Although chemokine receptors are important for T cell migration, it has been unclear how they are regulated in Th17 cells. Recently, Su et al. (34) have demonstrated that tumor microenvironmental RANTES and MCP-1 secreted by tumor cells and tumor-derived fibroblasts mediate the recruitment of Th17 cells into the tumor sites. In the current study, we were prompted to evaluate whether pleural CCL20 and CCL22 might be responsible for the influx of Th17 cells into the pleural space. In the previous study (17) and the current study, our results showed that concentrations of both CCL20 and CCL22 in MPE were significantly higher than those in the corresponding serum and that malignant cells, macrophages, and T cells might be the cell sources of pleural CCL20 and CCL22. Moreover, Th17 cells in peripheral blood and MPE strongly express CCR4 and CCR6, two chemokine receptors for CCL22 and CCL20, respectively, on their surface. The above data suggested that CCL20 and CCL22 in MPE might be related to the accumulation of Th17 cells in MPE. Indeed, an in vitro migration assay further confirmed that MPE could induce the migration of Th17 cells and that either anti-CCL20 or CCL22 mAb significantly inhibited the ability of the MPE to stimulate Th17 cell chemotaxis, and the combination of both mAbs exhibited an even more strong suppressive effect. Therefore, CCL20 and CCL22 might be able to chemottract Th17 cell recruitment into pleural space.

It should be mentioned that besides Th17 cells, we also noted Th1 and Th1/Th1 cells were increased in MPE compared with

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**Table I. Characteristics of the study population**

<table>
<thead>
<tr>
<th>Characteristics, n (mean ± SEM)</th>
<th>Median (Range)</th>
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<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
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<tr>
<td>Histological type</td>
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<tr>
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<tr>
<td>Age, y</td>
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<tr>
<td>pH</td>
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<tr>
<td>Protein, g/ml</td>
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<td>Glucose, mg/dl</td>
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<td>LDH, U/l</td>
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<td>Total cell counts, × 10⁹/l</td>
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<td>Th1 cells, %</td>
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<tr>
<td>Th17 cells, %</td>
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LDH, lactate dehydrogenase.

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**Table II. Univariate and multivariate analyses of variables associated with survival**

<table>
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<th>Hazard Ratio</th>
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<th>Multivariate p</th>
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<td>Type</td>
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<td>Age</td>
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<td>Protein</td>
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<tr>
<td>LDH</td>
<td>0.001</td>
<td>0.347</td>
<td>0.177–0.682</td>
<td>0.002</td>
</tr>
<tr>
<td>Th17 cells</td>
<td>&lt;0.001</td>
<td>0.347</td>
<td>0.177–0.682</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Cl, confidence interval; LDH, lactate dehydrogenase; NA, not adopted.

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**FIGURE 6.** Accumulation of Th17 cells predicts improved survival in patients with malignant pleural effusion. Individuals with higher Th17 cell frequency group (n = 15) had a significant improved survival than those with lower Th17 cell frequency group (n = 15). Overall survival was estimated by the Kaplan-Meier method and compared by the log-rank test.

---

that facilitates the differentiation of Th17 cells should also be present in MPE microenvironments. Indeed, our data showed that the concentrations of some cytokines, including IL-1β, IL-6, and TGF-β1, in MPE were significantly higher than those in the corresponding serum. Furthermore, our data demonstrated that IL-1β, IL-6, and IL-23 each expanded the Th17 cells in naive CD4+ T cells. The combination of IL-1β plus IL-6, IL-1β plus IL-23, IL-6 plus IL-23, or IL-1β plus IL-6 plus IL-23 significantly increased the percentage of Th17 cells at even higher extents. Some studies demonstrated that TGF-β is required for human Th17 cell differentiation (29–31); however, our data are consistent with the findings reported in the other studies in which TGF-β suppresses the differentiation of Th17 cells (14, 32).

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blood, and Th17 cell numbers were correlated positively with Th1 cell numbers. In addition, IFN-γ released into MPE was also significantly increased. Recently, Yang et al. (35) have demonstrated that inhibition of the transcription factor T-bet expressed in Th1 cells suppresses the differentiation of both Th1 and Th17 cells. Furthermore, T-bet directly regulates transcription of the IL-23R and influences the fate of Th17 cells, which depend on optimal IL-23 production for survival (36). The insights into the interaction between Th1 and Th17 cells in human were largely unknown so far, and the investigation of impacts and regulation of Th1 cells on Th17 cell generation and functions in MPE is on the way in our laboratory.

Although Th17 cells have been found in tumor microenvironment, their physiological functions in cancer immunity have been poorly defined. It has been suggested that Th17 cells have an indirect role in antitumor immunity by promoting dendritic cell, cytotoxic T cell, and NK cell trafficking to, and retention within, the tumor microenvironment (37). Recently, Martin-Orozco et al. (38) have provided direct evidence for a protective role of Th17 cells in antitumor responses by using IL-17–deficient mice in a model of lung melanoma. Moreover, the adoptive transfer of tumor-specific Th17 cells protect against various tumors and, in the lung melanoma model, promote tumor-specific cytotoxic T cell responses (38). We noted in the current study that the accumulation of Th17 cells in MPE predicted improved patient survival, implying a new role of proinflammatory response in regulating tumor progression in human cancer. Taken together, these data provide the basis for developing novel immune-boosting strategies based on ridding the cancer patient of this cell population.

In conclusion, our data showed that Th17 cell numbers in MPE were significantly increased when compared with the corresponding peripheral blood. Although it cannot be excluded that over-representation of Th17 cells in MPE may be due to increased local Ag stimulation, our findings have demonstrated that pleural proinflammatory cytokines could promote the differentiation of Th17 cells and that chemokines CCL20 and CCL22 might be capable of inducing migration of Th17 cells to MPE.

Acknowledgments

We thank Dr. Jiao Lan, Medical Research Center, People’s Hospital of Guangxi Zhuang Autonomous Region, for her excellent flow cytometric assistance.

Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The images published in Fig. 5A were mistakenly selected from micrographs showing expression of CCL22 in macrophages, T cells, and malignant cells, respectively, from other experiments in which similar procedures were performed. The correct figure showing expression of CCL20 is published below; the entire figure is shown, but the only change is to panel A. The legend is correct as published and is also shown below for reference.

**FIGURE 5.** Chemokines CCL20 and CCL22 in MPE were chemotactic for Th17 cells. *A*, Expression of CCL20 in macrophages (*top panels*), T cells (*middle panels*), and malignant cells (*bottom panels*). Macrophages, T cells and malignant cells were incubated with mouse anti-CD68, anti-CD3, and anti-EMA mAbs, respectively, and then were stained with fluorescein-labeled goat anti-mouse IgG (green). CCL20 expression was detected by rabbit polyclonal Ab targeted against CCL20 and then rhodamine-labeled goat anti-rabbit IgG (red). Immunofluorescence doublestaining indicate that some macrophages (white arrows in the *top right panel*), some T cells (red arrows in the *middle right panel*), and all malignant cells express CCL20. Original magnification \( \times 1000 \).

*B*, CCL20 was present in MPE and serum from a patient with lung cancer (*n* = 14). CCL20 concentrations were measured with an ELISA. Horizontal bars indicate medians. Comparison of CCL20 levels between malignant pleural effusion and serum was made using a Wilcoxon signed-rank test.

*C*, Chemokines CCL20 and CCL22 were chemotactic for Th17 cells in vitro. MPE from patients with lung cancer (*n* = 5) were used to stimulate chemotaxis of peripheral blood Th17 cells isolated from healthy adults in the presence of anti-CCL20 or/and anti-CCL22 mAbs or an irrelevant isotype control. The comparisons were determined by Kruskal-Wallis one-way ANOVA on ranks. *p* < 0.01 compared with the irrelevant isotype control.

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