**Immune Regulation of Interleukin-27 in Malignant Pleural Effusion**

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**Abstract**

**Background:** Interleukin (IL)-27 has been reported to have anti-proliferative and anti-angiogenic activities on cancer cells. However, the involvement of IL-27 in malignant pleural effusion (MPE) remains unknown. Thus, in this research, we compared the immune functions of IL-27, interferon (IFN)-γ, and IL-17 on lung cancer cells and revealed the regulatory mechanism of IL-27 in MPE.

**Methods:** The distribution of IL-27 in both MPE and blood was evaluated by enzyme-linked immunosorbent assay and flow cytometry. The expressions of cytokine receptors and the levels of the phosphorylated signal transducer and activator of transcription (STAT) signalings were detected by flow cytometry. As well as the effects of proliferation, apoptosis, migration, and adherent activity of IL-27, IFN-γ, and IL-17 on lung cancer cells were also explored.

**Results:** The expression of IL-27 was increased in MPE when compared with blood (147.3 ± 25.1 pg/ml vs. 100.3 ± 13.9 pg/ml, \( P = 0.04 \)). IL-27 was noted to suppress both proliferation (18.33 ± 0.21 vs. 27.77 ± 0.88, \( P = 0.0005 \)) and migration (1.82 ± 0.44 vs. 3.13 ± 0.07, \( P = 0.04 \)) of A549 cells, but obviously promoted apoptosis of A549 cells (9.47 ± 1.14 vs. 4.96 ± 0.17, \( P = 0.02 \)) by activating STAT1 signaling. Interestingly, IL-27 played totally opposite effects on A549 cells by activating STAT3 pathway. Moreover, IL-27 exerted different intercellular adherent activities of A549 cells to pleural mesothelial cell monolayer by activating different STAT signalings.

**Conclusions:** IL-27 might exert an important immune regulation on lung cancer cells in human pleural malignant environment.

**Key words:** Interleukin-27; Lung Cancer; Malignant Pleural Effusion; Signal Transduction

**INTRODUCTION**

Malignant pleural effusion (MPE), an advanced malignancy disease, is diagnosed by the present of malignant cells on the pleura or in the pleural fluid. The current researches suggest that MPE is due to lymphatic obstruction caused by pleural metastatic tumor and inflammation of tumor and host.[1-3] Moreover, the major metastatic tumor is adenocarcinoma, especially from lung, and lung cancer patients with MPE have the shortest survival time.[4,5] Since current treatments are invasive and cause adverse effects,[7] further studying the etiology of MPE may help to develop a more accurate individual therapy for MPE.

Interleukin (IL)-27, as a new member of IL-12 family, has been increasing interest to investigate in recent years.[8] It is identified a heterodimeric cytokine composed of the Epstein–Barr virus-induced gene 3 and the p28 subunit.[9] Glycoprotein 130 and WSX-1 (T-cell cytokine receptor [TCCR]) constitute a heterodimeric receptor complex for IL-27.[10] The recent studies suggest that IL-27 could activate Janus kinase/signal transducer and activator of transcription (STAT) signaling molecules by combined with receptor subunits, and it might play a potent role of anti-tumor and anti-metastatic activity.[11,12] Thus, IL-27 could be a novel agent applicable to cancer therapy.

Though the anti-proliferative and anti-angiogenic effects of IL-27 have been reported in various tumor models,[12,15] the functions and mechanisms of IL-27 exerted in MPE are not understood well. In our previous studies, we proved that interferon (IFN)-γ and IL-17 in MPE were significantly increased when compared with blood, and while IFN-γ showed anti-tumor role, IL-17 probably facilitated the development of tumor.[16,17] In this research, we investigated the distribution of IL-27-producing CD4⁺ T-cells in MPE, as well as the functions and mechanisms of IL-27 on lung cancer cells.

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The expression of markers on T-cells from MPE and blood was determined by flow cytometry as described previously. After surface or intracellular staining with anti-human-specific Abs conjugated with FITC, PE, or APC. These human Abs included anti-CD3, -CD8, -IL-27. Intracellular staining was performed on T-cells stimulated with PMA (50 ng/ml), ionomycin (1 μmol/L), GolgiStop, and GolgiPlug for 5 h, and then stained with anti-IL-27 with PE. Flow cytometry was performed on a FACS Canto II (BD Biosciences, Franklin Lakes, NJ) and analyzed using FCS Express 4 software (De Novo Software, Los Angeles, USA).

### Cell isolation
Isolation of pleural mesothelial cells (PMCs) was performed as described previously. PMCs were seeded and cultured in complete medium without epidermal growth factor, and then supernatants were harvested 72 h later.

### Signal transductions in lung cancer cells in vitro
The receptors of A549 lung adenocarcinoma cell line (A549 cells) (purchased from ATCC, Manassas, USA) were surface staining with Abs conjugated with PE, CF, or PerCP. A549 cells were incubated in the presence of medium alone, or with IL-27 (50 ng/ml), IFN-γ (50 ng/ml), IL-17 (50 ng/ml) for 40 min, then intracellularly stained with anti-pSTAT mAbs in order to determine the phosphorylated STAT signaling in A549 cells, samples were determined by flow cytometry.

### Effects on proliferation of lung cancer cells in vitro
A549 cells were incubated in the presence of medium alone, or with IL-27 (50 ng/ml), IFN-γ (50 ng/ml), IL-17 (50 ng/ml) in the absence or presence of STAT inhibitors (30 μmol/L each); in some experiments, A549 cells were incubated in the presence of MPE alone, or MPE mixed with anti-IL-27 (100 ng/ml), anti-IFN-γ (100 ng/ml), anti-IL-17 (100 ng/ml), or IgG irrelevant control for 48 h. After incubation, A549 cells were stained intracellularly for Ki-67 and tested by flow cytometry.

### Effects on apoptosis of lung cancer cells in vitro
A549 cells were cultured in the presence of medium alone, or with IL-27 (50 ng/ml), IFN-γ (50 ng/ml), IL-17 (50 ng/ml) in the absence or presence of STAT inhibitors (30 μmol/L each); in some experiments, A549 cells were incubated in the presence of MPE alone, or MPE mixed with anti-IL-27 (100 ng/ml), anti-IFN-γ (100 ng/ml), anti-IL-17 (100 ng/ml), or IgG irrelevant control for 48 h. After incubation, A549 cells were stained intracellularly for Ki-67 and tested by flow cytometry.

### Effects on lung cancer cell migration in vitro
A549 cells were incubated in the presence of IL-27 (50 ng/ml), IFN-γ (50 ng/ml), IL-17 (50 ng/ml) with or without STAT inhibitors (30 μmol/L each), or medium alone; in some experiments, A549 cells were incubated with APC-conjugated Annexin V for 15 min, then stained with propidium iodide. Finally, the apoptosis rate was determined by flow cytometry.

### Methods

#### Subjects
Pleural fluid and blood samples were collected from patients (age range: 40–86 years) with diagnosed lung cancer with MPE. A diagnosis of MPE was established by demonstration of malignant cells in pleural fluid or/and on local anesthetic thoracoscope pleural biopsy specimen. Before sample collection, none of the patients had received any anti-cancer therapy, corticosteroids, or other nonsteroid anti-inflammatory drugs. The study protocol was approved by the Institutional Review Board for human studies of Tongji Medical College, China, and informed written consent was obtained from all subjects.

#### Reagents
The following antibodies were used as follow: For flow cytometry determination, CD3, CD8, CD54 (intercellular adhesion molecule [ICAM]-1), CD106 (vascular adhesion molecule [VCAM]-1), CD11a (lymphocyte function-associated antigen-1 [LFA]-1), CD29 (integrin β1), IL-27, IFN-γ, IL-17, phosphorylated (p)-STAT1 (pY701), p-STAT1 (pS727), p-STAT2 (pY689), p-STAT3 (pS727), p-STAT3 (pY705), p-STAT4 (pY693), p-STAT5 (pY694), p-STAT6 (pY641), TCCR-WSX-1 (IL-27 receptor), IL-17 receptor (R), IFN-γR1, and Ki-67 mAbs were purchased from BD Biosciences (Franklin Lakes, USA), eBioscience (San Diego, USA), or R and D systems (Minneapolis, USA); for cytokine neutralization, IL-27, IFN-γ, IL-17, ICAM-1 and VCAM-1 mAbs were from eBioscience.

Recombinant human IL-27, IFN-γ, IL-17A were purchased from R and D systems or eBioscience. Fluorescent dye CFSE was from Enzo Life Sciences (Farmingdale, USA). Annexin V Apoptosis Detection Kit was from eBioscience. PMA and ionomycin were from Sigma-Aldrich (St. Louis, USA), and GolgiPlug and GolgiStop were from BD Bioscience. Foxp3 fixation/permeabilization concentrate and diluent for intracellular staining were from eBioscience. S14-95 and STAT3 inhibitor Galiellalactone were from R and D systems or eBioscience. STAT1 inhibitor S14-95 and STAT3 inhibitor Galiellalactone were from Enzo Life Sciences (Farmingdale, USA). Annexin V Apoptosis Detection Kit was from eBioscience.

### Sample collection and processing
Two hundred milliliters of MPE samples from each patient were collected through a standard thoracocentesis technique within 24 h after hospitalization. Twenty milliliters of blood were drawn simultaneously. MPE and blood specimens were centrifuged at 1,200 × g for 5 min. The cell pellets were resuspended in Hanks’ balanced salt solution, and mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden).

#### Cytokine measurements
Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentrations of IL-27 (eBioscience, San Diego, USA) in MPE and serum according to manufacturer’s instructions. The minimum detectable concentration of IL-27 was 9.5 pg/ml.
in 24-well Transwell chambers (Corning Costar, Corning, USA), A549 cells were placed into the top chamber \((1 \times 10^4)\) resuspended in RPMI 1640 medium with 0.5% FBS in the final volume of 100 μl, supernatants of cultured PMCs were placed in the bottom chamber in a volume of 600 μl, and the chambers were incubated at 37°C in 5% CO₂ atmosphere for 24 h. Finally, the nonmigratory cells were scraped off and washed gently in PBS, the migratory cells on the bottom surface of the transwell membrane were fixed in cold methanol for 30 min and underwent crystal violet staining, then photographed under a digital microscope (Olympus BX51; Olympus, Tokyo, Japan). The migratory index was calculated by dividing A549 cell numbers migrated in response to supernatants of cultured PMCs by A549 cell numbers migrated in response to RPMI-1640 medium control.

**Effects on lung cancer cell adhesion**

\(10^6\) A549 cells were incubated with IL-27 (50 ng/ml), IFN-γ (50 ng/ml), or IL-17 (50 ng/ml) in the absence or presence of STAT inhibitors (30 μmol/L each), or medium alone for 48 h prior to adhesion assay. Simultaneously, \(10^6\) PMCs were seeded in 12-well plates confluent and in the same conditions as used for A549 cells. A549 cells were labeled by CFSE and seeded into the washed PMC monolayer for 12 h. In some experiments, anti-ICAM-1 (2 μg/ml) or/and anti-VCAM-1 (2 μg/ml) were added. Finally, nonadherent A549 cells were removed by washing. Total adherent cells containing PMCs and A549 cells were analyzed by flow cytometry.

**Statistics**

Data are expressed as mean ± standard error of mean. Comparisons of the data between different groups were performed using Mann–Whitney U-test. For variables in MPE and in corresponding blood, paired data comparisons were made using a Wilcoxon signed-rank test. Analysis was completed with SPSS version 17.0 Statistical Software (Chicago, IL, USA), and \(P<0.05\) were considered to indicate statistical significance.

**RESULTS**

**Increased proportions of interleukin-27 in malignant pleural effusion**

We determined concentration of IL-27 in MPE and serum using ELISA, and found that concentration of IL-27 in MPE \((147.3 ± 25.1 \text{ pg/ml})\) was higher than that in serum \((100.3 ± 13.9 \text{ pg/ml}; n = 20; P = 0.04)\) [Figure 1a]. Next, we performed flow cytometry on mononuclear cells from MPE and blood with gating on CD3+ and CD8− T-cells to detected IL-27+ CD4+ T-cells. It showed that the percentage of IL-27+ CD4+ T-cells represented a higher value in MPE \((76.6\% ± 2.8\%\) in comparison with that in the corresponding blood \((70.5\% ± 3.2\%, n = 17, P = 0.04)\) [Figure 1b and c].

**Signal transductions in A549 cells**

As shown in Figure 2a and b, the medium level of TCCR‑WSX‑1, and high levels of IFN-γR1 and IL-17R were expressed on A549 cells. Simultaneously, we noted that IL-27 significantly phosphorylated STAT1 in the sites of tyrosine 701 (Tyr701) \((11.37 ± 1.48 \text{ vs. } 1.73 ± 0.40, P = 0.003)\) and serine 727 (Ser727) \((8.52 ± 0.47 \text{ vs. } 2.99 ± 0.57, P = 0.002)\), as well as STAT3 in the sites of tyrosine 705 (Tyr705) \((20.49 ± 1.37 \text{ vs. } 3.10 ± 0.71, P = 0.0004)\) and Ser727 \((98.07 ± 0.66 \text{ vs.}...

![Figure 1: Interleukin (IL)-27 increased in malignant pleural effusion (MPE). (a) Comparison of the concentration of IL-27 between MPE and blood by enzyme-linked immunosorbent assay \((n = 20)\) is shown. (b) The percentage of IL-27+ CD4+ T-cells in MPE and blood were determined by flow cytometry \((n = 17)\). Horizontal bars indicate means. (c) The representative flow cytometric dot-plots of IL-27+ CD4+ T-cells in MPE and blood are shown. CD4+ T-cells were identified based on their expression of CD3 and not of CD8.](image-url)
46.41 ± 0.64, \(P = 0.0003\), while IFN-\(\gamma\) also phosphorylated STAT1 in the locus of Tyr701 (13.63 ± 2.13 vs. 1.73 ± 0.40, \(P = 0.005\)), and IL-17 prefer to phosphorylating STAT3 in the locus of Ser727 in A549 cells (98.15 ± 0.46 vs. 46.41 ± 0.64, \(P = 0.0002\)). Other signals, including STAT2 (Tyr689), STAT4 (Tyr693), STAT5 (Tyr694), and STAT6 (Tyr641) were all negative during stimulation by IL-27, IFN-\(\gamma\), or IL-17 [Figure 2c and d].

**Effects on proliferation of A549 cells**

As indicated by Ki-67 expression levels, IL-27 (18.33 ± 0.21 vs. 27.77 ± 0.88, \(P = 0.0005\)) and IFN-\(\gamma\) (12.91 ± 0.41 vs. 27.77 ± 0.88, \(P = 0.0001\)) substantially suppressed proliferation of A549 cells, whereas IL-17 showed a promotion of A549 cells proliferation (53.30 ± 3.88 vs. 27.77 ± 0.88, \(P = 0.003\)). When STAT1 inhibitor was added into the culture of A549 cells in the presence of IL-27 (33.24 ± 3.22 vs. 18.33 ± 0.21, \(P = 0.0099\)) or IFN-\(\gamma\) (48.02 ± 9.91 vs. 12.91 ± 0.41, \(P = 0.02\)), the suppressive proliferation was reversed; when STAT3 inhibitor was added into the culture with IL-27 or IL-17, the promotive effect of IL-17 was abrogated (11.41 ± 0.99 vs. 53.30 ± 3.87, \(P = 0.0005\)), in contrast, the suppressive roles of IL-27 was augmented furtherly (10.94 ± 0.45 vs. 18.33 ± 0.21, \(P = 0.0001\)). Meanwhile, the addition of anti-IL-27 (32.85 ± 1.31 vs. 25.14 ± 2.35, \(P = 0.046\)), but not anti-IFN-\(\gamma\) mAb (30.67 ± 2.99 ± 0.64, \(P = 0.046\)), but not anti-IFN-\(\gamma\) mAb (30.67 ± 2.99 ± 0.64, \(P = 0.046\)), but not anti-IFN-\(\gamma\) mAb (30.67 ± 2.99 ± 0.64, \(P = 0.046\)), but not anti-IFN-\(\gamma\) mAb (30.67 ± 2.99 ± 0.64, \(P = 0.046\)), but not anti-IFN-\(\gamma\) mAb (30.67 ± 2.99 ± 0.64, \(P = 0.046\)).

**Figure 2:** Signal transductions of interleukin (IL)-27, interferon (IFN)-\(\gamma\), and IL-17 in A549 cells. The data of percentages of T-cell cytokine receptor-WSX-1\(^+\) (IL-27R), IFN-\(\gamma\)R1\(^+\) and IL-17R\(^+\) A549 cells of five independent experiments were evaluated by flow cytometry (a), and the representative flow cytometric histograms are shown (b). The data show the expressions of the phosphorylated signal transducer and activator of transcription signalings in A549 cells determined by flow cytometry (c), and the representative flow cytometric histograms were shown (d). The results are reported as mean ± standard error of mean from five independent experiments. *\(P < 0.05\) compared with the corresponding controls.
Thus, we speculated that IL-27 might be and IL-27 activated STAT1 and β
+ LFA-1, especially majority PMCs expressed ICAM-1, A549 cells and PMCs. As shown in Figure 6a and b, some A549 cells and PMCs expressed ICAM-1, VCAM-1, and LFA-1, especially majority PMCs expressed ICAM-1, as well as almost all A549 cells and PMCs expressed integrin-β1. We further evaluated effects of IL-27, IFN-γ, and IL-17 on the expression levels of ICAM-1, VCAM-1, and LFA-1, and found that except for the expressions of ICAM-1 stimulated by IL-27 and LFA-1 stimulated by IFN-γ on A549 cells, IL-27 and IFN-γ elevated the expressions of ICAM-1, VCAM-1, and LFA-1, both on A549 cells and PMCs. Simultaneously, the promotive effects of IL-27 were abrogated by STAT1 inhibitor both on A549 cells and PMCs, however, the inhibition of STAT3 signaling further enhanced the expressions of cellular adhesion molecules without ICAM-1 and VCAM-1 expressed on A549 cells. Similarly, STAT1 inhibitor also could abolish the inductive effects of IFN-γ on cellular adhesion molecules except VCAM-1 on A549 cells and LFA-1 on PMCs. Interestingly, though IL-17 had no effects on cellular adhesion molecules as IL-27 or IFN-γ, IL-17 could elevate the expressions of VCAM-1 on A549 cells and PMCs and LFA-1 on PMCs by blocking STAT3 signaling [Figure 6a and b].

We analyzed the numbers of A549 cells marked with CFSE labeling adhered to PMC monolayer within 12 h by flow cytometry. IL-27 (62.61 ± 2.04 vs. 56.38 ± 0.86, P = 0.03) and IFN-γ (67.90 ± 3.45 vs. 56.38 ± 0.86, P = 0.02), but not IL-17 (54.20 ± 0.48 vs. 56.38 ± 0.86, P = 0.07), substantially enhanced the adhesion of A549 cells. As anti-ICAM-1 and/or anti-VCAM-1 mAb abrogated the adhesion of A549 cells significantly, STAT1 inhibitor could abolish the promotive effects of IL-27 (44.32 ± 4.16 vs. 62.61 ± 2.04, P = 0.01) and IFN-γ (49.09 ± 4.80 vs. 67.90 ± 3.45, P = 0.02), however, STAT3 inhibitor could further augment the adhesion induced by IL-27 (75.46 ± 2.84 vs. 62.61 ± 2.04, P = 0.01) [Figure 6c and d].

**Discussion**

Interleukin-27, a novel member of IL-12 family, is identified as a potential anti-tumor agent instead of IL-12. In several tumor models, IL-27 has been reported to have anti-proliferative and anti-angiogenic activities on cancer cells. However, it has never been linked to MPE, thus, besides proliferative and migratory effects of IL-27, we demonstrated that IL-27 regulated apoptosis and adhesive activities on lung cancer cells depending on STAT signalings.

In the present study, we found that the concentration of IL-27 and the percentage of IL-27-secreting CD4+ T-cells in MPE were higher than those in the blood which prompted us that IL-27 might exert an immune regulation in MPE environment. And then, we observed that the medium level of IL-27 was expressed on A549 cells. Simultaneously, both STAT1 and STAT3 signaling were activated in A549 cells. A number of researches had demonstrated that STAT1 signaling inhibited oncogenic development while STAT3 signaling promotes oncogenic development, and IL-27 activated STAT1 and STAT3 signaling via combining with different subunits of IL-27R. Thus, we speculated that IL-27 might be...
Figure 3: Effects of interleukin (IL)-27, interferon (IFN)-γ, and IL-17 on A549 cells proliferation. (a) Comparisons of Ki-67+ A549 cells tested by flow cytometry in each group. The representative flow cytometric histograms of Ki-67+ A549 cells are shown. (b) The results are reported as mean ± standard error of mean from five independent experiments. *P < 0.001, compared with the Control group; †P < 0.01, compared with the IL-27 group; ‡P = 0.02, compared with the IFN-γ group; §P < 0.001, compared with the IL-17 group; ||P < 0.05, compared with the malignant pleural effusion + IgG group.
involved in immune regulation on lung cancer cells in MPE environment.

Interleukin-27, the suppression of tumor growth, has been reported in several cancers, such as melanoma, leukemia.\cite{13,14,28} We also noted that IL-27 and IFN-γ, but not IL-17, both suppressed A549 cells growth, and these effects were abolished by blocking STAT1 signaling. However, the proliferation of A549 cells was further suppressed by blocking STAT3 pathway. Importantly, anti-IL-27 mAb could promote A549 cells proliferation in MPE supernatants. Furthermore, IL-27 had a similar effect to IFN-γ which oppose to IL-17 that promoted apoptosis of A549 cells, and this effect was also reversed by STAT1 inhibitor and enhanced by inhibition of STAT3 signaling. Simultaneously, the addition of anti-IL-27 and anti-IFN-γ mAb into the MPE supernatants could prevent A549 cells from apoptosis. Therefore, these results implied that IL-27 could suppress proliferation and promote apoptosis of lung cancer cells in MPE by activating STAT1 pathway, and the inhibition of STAT3 signaling might augment the anti-tumor effects of IL-27 in MPE environment.

Metastasis is one of the characteristics of malignant tumor which largely shorten the survival time of cancer patients. In our research, by activating STAT1 pathway, IL-27 and IFN-γ both suppressed the migratory activity of A549 cells in response to PMC supernatants, whereas IL-17 promoted the migration of A549 cells by activating STAT3 pathway. Then these findings were proved by the addition of anti-IL-27, anti-IFN-γ, and anti-IL-17 mAb into MPE supernatants, and the migratory effects above were reversed. However,
STAT3 signaling activated by IL-27 was not associated with the migration of A549 cells. Hence, IL-27 might prevent the migration of lung cancer cells into the pleural space of the patients by activating STAT1 signaling. It was reported that adhesion molecules had closely relationship with the adhesion of cancer cells to host cells, and ICAM-1 was elucidated to reinforce the metastasis of malignant. In the current study, ICAM-1 and VCAM-1, as well as their ligands LFA-1 and integrin-β1, expressed on A549 cells and PMCs isolated from MPE. Except for IL-17, IL-27, and IFN-γ substantially augmented the expressions of ICAM-1, VCAM-1, and LFA-1 on both two cells, which induced the promotive adherent activity of A549 cells to PMCs. Simultaneously, STAT1 inhibitor could reverse the promotive activity of IL-27 and IFN-γ, whereas blocking STAT3 signaling could further enhance this effect of IL-27. Overall, inhibiting STAT1 signaling and activating STAT3 signaling which induced by IL-27 would suppress the adhesion effects of lung cancer cells to pleural cells.

In conclusion, IL-27, the same as IFN-γ, suppressed proliferation and augmented apoptosis of lung cancer cells, as well as strongly restrained migratory activity of lung cancer cells in responding to MPE. Interestingly, these functions of IL-27 were abrogated by STAT1 inhibitor,
but enhanced by STAT3 inhibitor. Thus, IL-27 exerted anti-tumor effects depending on STAT signaling in MPE. Furthermore, though IL-27 initiated intercellular adhesion of lung cancer cells to PMCs, IL-27–STAT pathway could probably regulate the pleural metastasis of lung cancer cells. Therefore, this investigation might provide a novel notion.
of targeting IL‑27 to develop more effective anti-tumor immunotherapy.

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