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Enhancement of CD147 on M1 macrophages induces differentiation of Th17 cells in the lung interstitial fibrosis

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ABSTRACT

Lung interstitial fibrosis is a chronic lung disease, and few effective therapies are available to halt or reverse the progression of the disease. In murine and human lung fibrosis, the expression of CD147 is increased. However, the role of CD147 in lung fibrosis has not been identified, and it remains to be determined whether lung fibrosis would be improved by decreasing the expression of CD147. A murine bleomycin-induced lung interstitial fibrosis model was used in the experiments, and HAb18 mAbs and CsA were administered during the induction of lung fibrosis. In our study, we found that the HAb18 mAbs markedly reduced the collagen score and down-regulated M1 macrophages and Th17 cells. In vitro, flow cytometry analysis showed that M1 macrophages induced higher Th17 differentiation than M2 macrophages. After treatment with HAb18 mAbs or after reducing the expression of CD147 by lentivirus interference in M1 macrophages, the level of Th17 cells were significantly inhibited. In conclusion, HAb18 mAbs or CsA treatment ameliorates lung interstitial fibrosis. CD147 promoted M1 macrophage and induced the differentiation of Th17 cells in lung interstitial fibrosis, perhaps by regulating some cytokines such as IL-6, IL-1β, IL-12 and IL-23. These results indicated that CD147 may play an important role in the development of lung interstitial fibrosis.

1. Introduction

Lung interstitial fibrosis is a chronic lung disease that is characterized by the excessive accumulation of extracellular matrix (ECM), remodeling of the lung architecture and serious complications in a variety of rheumatic autoimmune diseases. Most patients with lung interstitial fibrosis experience progressive respiratory failure within 3–8 years of the onset of the symptoms [1]. Few effective therapies are available to halt or reverse the progression of this disease with a high mortality rate. Thus, there is a large and unmet medical need for the development of new therapeutic strategies [2].

Th17 cells, which have been reported to represent a completely distinct subset of $CD4^+$ T cells that produce IL-17A and IL-17F and express the IL-23 receptor (R) and retinoic acid orphan receptor (RORγt) [3], play an important role in pulmonary fibrosis [4–6]. Although the differentiation of Th17 cells in lung fibrosis has not been reported, it has been described in autoimmune syndromes such as EAE (experimental autoimmune encephalomyelitis) and collagen-induced arthritis [7,8]. The differentiation of Th17 cells is influenced by cytokines such as TGF-β, IL-1β, IL-12, IL-23 [9–13]. In addition, macrophages involved in local inflammation induced the differentiation of Th17 cells in response to Th17-promoting cytokines [14,15]. Macrophages are widely distributed immune system cells [16] that can be phenotypically polarized by the microenvironment to mount specific functional programs. Polarized macrophages can be broadly classified in two main groups: classically activated macrophages (or M1) that exhibit potent microbicidal properties and promote strong IL-12-mediated Th1 responses [17] and alternatively activated macrophages (or M2) that support Th2-associated effector functions [18]. The relationship between different types of macrophages and the differentiation of Th17 cells has not been elucidated.

CD147 is a cell surface glycoprotein that belongs to the immunoglobulin superfamily [19]. Several studies have shown that CD147 is related to lung interstitial fibrosis. For example, high levels of CD147 expression have been found in BALF and lung tissues [20]; Betsuyaku T and Guillot S have reported in their respective studies that lung fibrosis is associated with increased basigin/CD147 expression in mice and humans [21,22].

Abbreviations: BLM, bleomycin; CsA, Cyclosporin A; HE, hematoxilin and eosin; BALF, bronchoalveolar lavage fluid; ECM, extracellular matrix; RORγt, retinoic acid orphan receptor; EAE, experimental autoimmune encephalomyelitis

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These results indicated that CD147 might play an important role in lung interstitial fibrosis. It has also been observed that in UIP-IPF (usual interstitial pneumonia-idiopathic pulmonary fibrosis) patients, CD147 expression was increased in fibrotic lesions, especially in macrophages and alveolar epithelial cells [23]. The role of CD147 on macrophages in pulmonary fibrosis has not been elucidated, and an antibody treatment against CD147 in lung fibrosis has still not been developed.

Although macrophages can induce the differentiation of Th17 cells in local inflammation [24], the regulatory effect of macrophage subtypes on Th17 cell differentiation in bleomycin-induced lung fibrosis is not clear. High levels of CD147 expression can be found on macrophages [23]; however, the role of CD147 on macrophages during the disease process remains to be investigated. In this study, we explored the changes in lung fibrosis in mice after HAb18G/CD147 mAbs treatment, the relationship between macrophage subtypes and the differentiation of Th17 cells in lung interstitial fibrosis, and the role of CD147 during differentiation.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice were obtained from the Fourth Military Medical University, Laboratory Animal Co. Ltd. All of the mice were at least 8 weeks old and weighed 18–23 g at the time of experimentation. The animal experiments were approved by the Animal Experiment Administration Committee of the University.

The experimental mice were divided into five groups: a control group that received a saline injection, a bleomycin group (BLM), a bleomycin + saline group, a bleomycin + HAb18 mAbs group, and a bleomycin + CsA group. Each group contained 36 mice.

2.2. Experimental model of bleomycin-induced lung interstitial fibrosis

Lung interstitial fibrosis was induced as previously described [25]. Briefly, after the body weight was recorded, the mice were anesthetized via intraperitoneal injection of 30 mg/kg pentobarbital sodium. A midline incision was made in the neck, and the trachea was exposed by blunt dissection. Bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) was dissolved in 0.1 ml saline and injected into the animals' lungs via 0.25 ml syringes at a dose of 7.5 mg/kg body weight. The control group received an equal volume of sterile saline. After the bleomycin or saline was injected into the trachea, the animal operating plate was shaken to facilitate distribution of the solution throughout the lungs. The day of bleomycin injection was considered to be d 0. The mice were then injected with saline or 10 mg/kg HAb18 mAbs (prepared in our laboratory) or 50 mg/kg CsA (purchased from Enzo company) beginning on d 1 and then once a day at the same time point. At 4 d, 7 d, 14 d, and 28 d after bleomycin injection, three mice from each group were sacrificed by cervical dislocation so that a bronchoalveolar lavage could be performed and to collect lung samples for histologic and cellular analysis.

2.3. Histologic and immunohistochemical analysis

Mice were killed at d 4, 7, 14 or 28 following treatment with bleomycin. The left lung was removed and fixed in 10% neutral phosphate-buffered formalin (pH 7.4). The tissues were dehydrated gradually in ethanol, embedded in paraffin, cut into 4-μm sections, stained with hematoxylin and eosin (H&E) or Masson staining, and examined by light microscopy.

Each successive field was individually assessed for the severity of interstitial fibrosis using the semi-quantitative grading system described by Ashcroft [26]. The grade of pulmonary fibrosis was scored in a blinded fashion by examining 30 randomly chosen regions per sample at a magnification of 100×. A score ranging from 0 (normal lung) to 8 (total fibrosis) was assigned. The major criteria for grading pulmonary fibrosis included inflammatory cell infiltration, edema, interstitial thickening of alveolar or bronchiolar walls, and collagen deposition, as follows: grade 0 = normal lung, grade 1 = minimal fibrous thickening of the alveolar or bronchial walls, grades 2–3 = moderate thickening of walls without obvious damage to the lung architecture, grades 4–5 =increased fibrosis with definite damage to the lung architecture and the formation of fibrous bands or small fibrous masses, grades 6–7 = severe distortion of the structure and large fibrous areas ('honeycomb lung' was placed in this category), grade 8 = total fibrous obliteration of the field. The mean score of all fields was taken as the fibrosis score of the lung section [27].

The antibody used for immunohistochemical analyses was anti-mouse CD147 antibody (Abcam Ltd, CA). The images from each slide were analyzed by a pathologist blinded to the experiment. We supposed a score ranging from 1 (normal lung) to 3 (total fibrosis). The following intensity scores were used: −, positive cell ratio < 5%, grade 1; +, ratio of 5% to 50%, grade 2; and ++, ratio > 50%, grade 3. The mean scores of 8 mice were taken as the score of CD147 expression for the lung section.

2.4. Analysis of bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage (BAL) was performed by washing the lungs three times with three different 1-ml aliquots of phosphate-buffered saline (PBS). The BALF was centrifuged at 450 *g* for 10 min, the pellet was resuspended in 100 μl PBS, and the total number of cells was counted using a cell counting board. The number of lymphocytes, monocytes and neutrophils were counted by Giemsa staining.

2.5. Flow cytometry (FCM)

Lung tissues from mice at d 4, 7, and 14 following the bleomycin administration were minced and digested with collagenase and hyaluronidase, respectively. After lysis of the RBCs, the dissociated cells were underlain with 5 ml of lymphocyte separation solution (Mediatech) and centrifuged at 2000 rpm for 20 min. The mononuclear cells in the middle layer were collected for flow cytometry. The cells homogenized from the lungs of the mice were incubated with 1 μg/10^6 cells of an Fc receptor-blocking Ab (clone 24G2; American Type Culture Collection, Manassas, VA) and then were stained with the following 5 antibodies: PE-conjugated anti-mouse IL-17 (Clone TC11-18H10, BD Pharmingen), PE-conjugated anti-mouse CD206 (Clone C068SC2, Biolegend), FITC-conjugated anti-mouse CD16/32 (Clone 2.4G2, BD Pharmingen), APC-conjugated anti-mouse F4/80 (Clone BM8, Biolegend), and PerCP-conjugated anti-mouse CD4 (Clone GK1.5, Biolegend). For intracellular cytokine staining, the cells were cultured with 1 μg/ml phorbol myfismte acetate (PMA), 1 μg/ml ionomycin (Ion) and 1 μM monensin for 4 h, stained for surface markers and further processed using a BD Cytofix/Cytoperm and BD Perm/Wash kit (BD Biosciences, North Ryde, New South Wales, Australia). The cells were then stained with PE-conjugated anti-mouse CCR6 (Clone 140706, R&D), FITC-conjugated anti-mouse IFN-γ (Clone XMG1.2, BD Pharmingen) and PerCP-conjugated anti-mouse RORγt (Clone 600380, R&D).

Analysis was performed on a FACScan flow cytometer using CellQuest software (Becton-Dickinson).

2.6. Isolation and co-culture of $CD4^+$ T cells and macrophage subsets

The lung tissues of mice at d 14 following bleomycin instillation were prepared to isolate the mononuclear cells according to the above method. For cell sorting experiments, a MoFlo (Beckman Coulter) instrument was used.

The M1 macrophages, defined as $F4/80^+CD16/32^+$ cells [12], and the M2 macrophages, defined as $F4/80^+CD206^+$ cells [14], and $CD4^+$ T cells were sorted by a fluorescence-activated cell sorter. Then, the

M1 macrophages and M2 macrophages were incubated in a 6-well plate for 2 h. The $CD4^+$ T cells 5 μg/ml anti-CD3 and 10 μg/ml anti-CD28 (purchased from BD Pharmingen) were co-cultured, respectively, with M1 macrophages or M2 macrophages in 6-well plates. Three days later, the suspending $CD4^+$ T cells were collected for flow cytometry analysis.

2.7. Polymerase chain reaction (PCR) analysis

The total RNA was isolated from the mononuclear cells collected in the lymphocyte separation solution and the purified M1, CD147-deficient M1 macrophages and M2 macrophages homogenized from the lung tissues of the mice following bleomycin instillation. Complementary DNA was

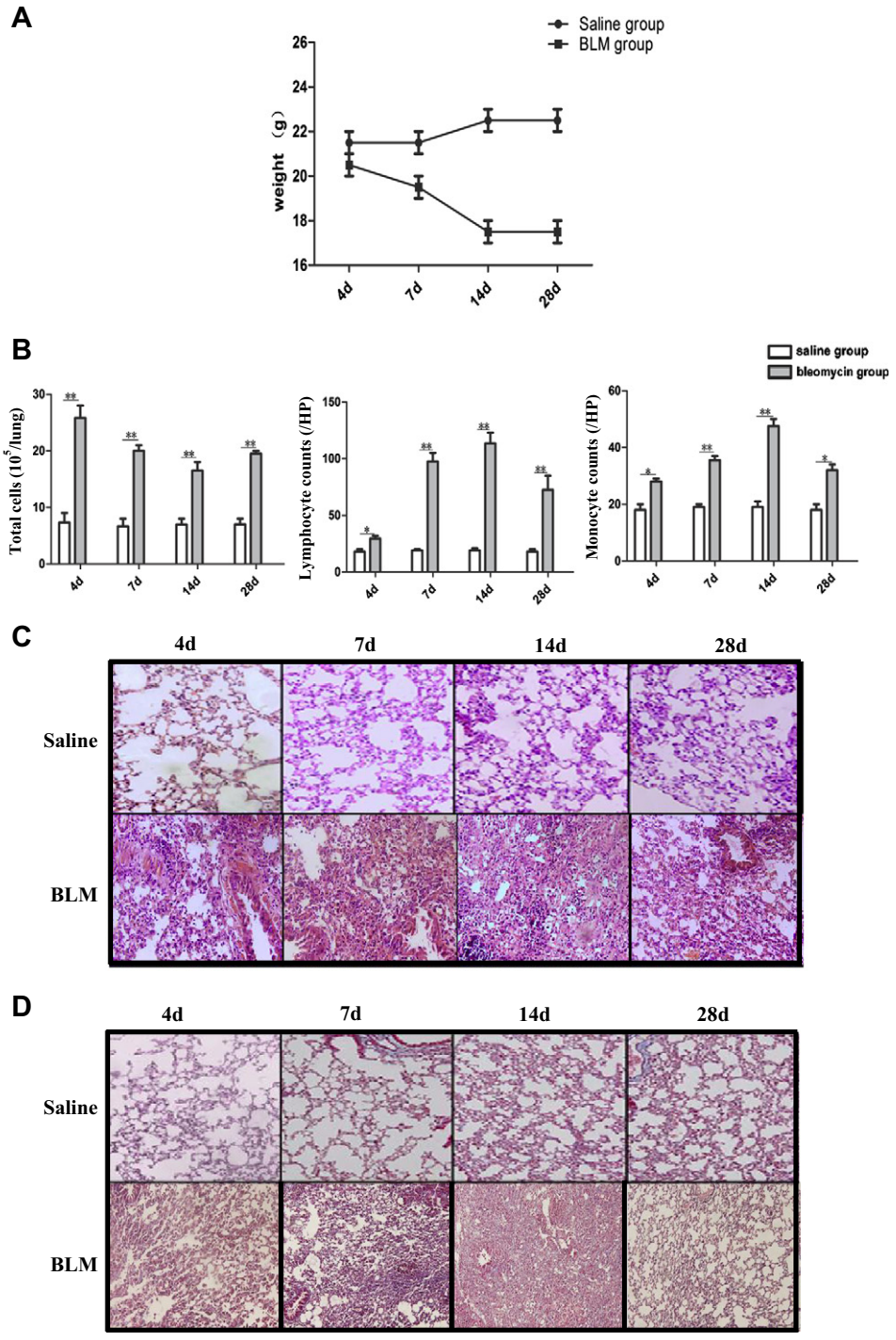

Fig. 1 The changes of mice instilled with bleomycin or saline. Following experiments were carried out at d 4, 7, 14, or 28 after instillation with bleomycin or saline. A. Changes in the body weight of the studied mice. Mice in the bleomycin group reached the minimum at d 14 (* = $P < 0.05$). B. Evaluation of the number of infiltrating cells (total cells, lymphocytes and monocytes) in BALF. Compared with the saline group, the total cells, lymphocytes and monocytes were increased in the bleomycin group (**$P < 0.01$, *$P < 0.05$). C. HE staining (×400) for histopathological changes in lung tissues. In the bleomycin group, multifocal diffuse changes were apparent at d 4, 7, and 14. D. Masson staining (×200) for histopathological changes in the lung tissues. E. Quantitative analysis of the fibrotic grades induced by bleomycin using the Ashcroft scale. Collagen fibrosis significantly increased at d 14. (** = $P < 0.01$, * = $P < 0.05$). F. Immunohistochemistry staining (×400) for CD147 in lung tissues. The expression of CD147 on lung tissues at d 14 exceeded that at d 4 (** = $P < 0.01$, * = $P < 0.05$).

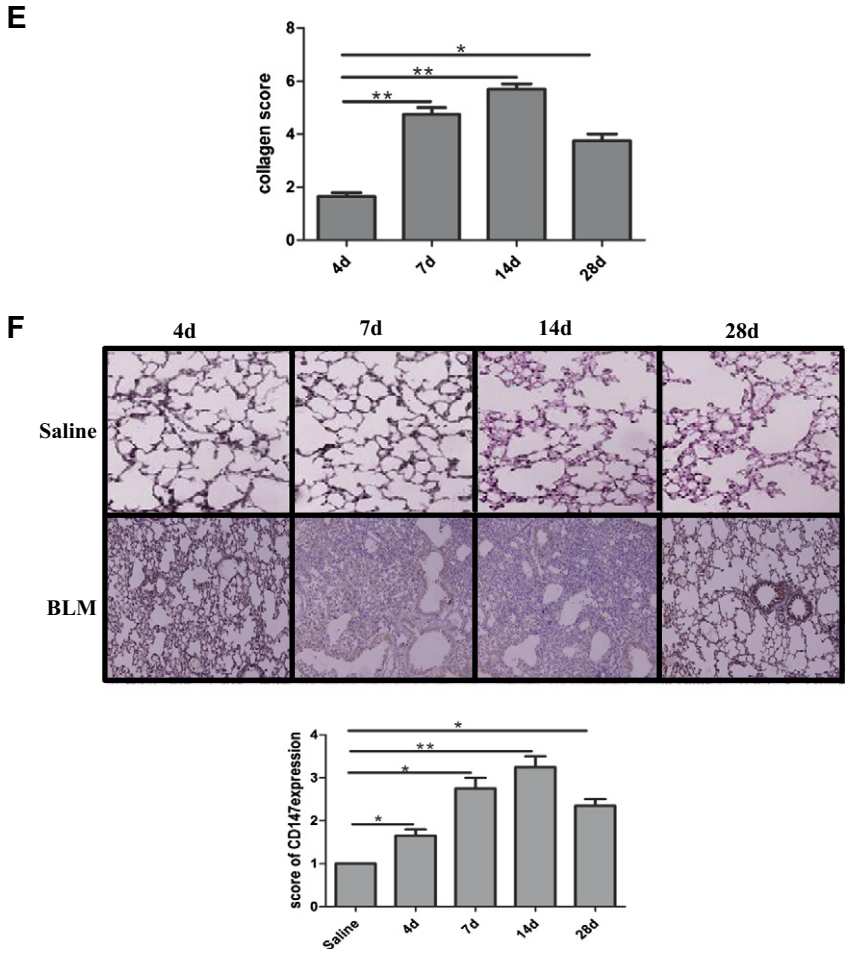

Fig. 1 (*continued*).

prepared by reverse transcription, and the RNA expression levels were determined by real-time quantitative PCR using a LightCycler (Mx3005P QPCR System) instrument and SYBR Green reagent. The expression levels were normalized for GADPH. The following primer sequences were used: for GADPH, 5′-TCA-ACG-GCA-CAG-TCA-AGG-3′ (forward) and 5′-ACT-CCA-CGA-CAT-ACT-CAG-C-3′ (reverse); for iNOS, 5′-CCCTTCCGAAGTTT CTGGCAGCAGC-3′ (forward) and 5′-CCAAAGCCACGAGGCTCTGACAGCC-3′ (reverse); for CD86, 5′-TCAGTCAGGATGGGAGTGGTA-3′ (forward) and 5′-ATCCAAGAGCCATTCCTACCT-3′ (reverse); for CD206, 5′CATGAG GCTTCTCTTGCTTCTG-3′ (forward) and 5′-TTGCCGTCTGAACTGAGATGG-3′ (reverse); for ArgI, 5′-CAGAAGAATGGAAGAGTCAG-3′ (forward) and 5′-GGTGACTCCCTGCATATCTG-3′ (reverse); for TGF-β,5′-GAT-CCC-CCT-AGC-CTC-TCA-TC-3′(forward), and 5′-GCA-CAA-GCA-GGA-T TG-AGT-GA-3′ (reverse); for IL-6, 5′-AGT-TGC-CTT-CTT-GGG-ACT-GA-3′(forward) and 5′-CAG-AAT-TGC-CAT-TGC-ACA-AC-3′ (reverse); for IL-1β, 5′-GCG-TCC-AAA-GCA-GTT-CCC-AT T-AGA-CAA-CTG-CA-3′ (forward) and 5′-CCG-GAA-TTC-CCA-GC C-CAT-ACT-TTA-GGA-AGA-3′ (reverse); for IFN-γ, 5′-CAG-CTC-TT C-CTC-ATG-GCT-GTT-C-3′ (forward) and 5′-GTC-ACC-ATC-CTT-TTG-CCA-GTT-C-3′ (reverse); and for IL-4, 5′-GCA-ACG-AAG-AAC-AC C-ACA-GAG-A-3′ (forward) and 5′-GAT-GAA-TCC-AGG-CAT-CGA-AAA-G-3′ (reverse).

2.8. Cell culture and transduction with shRNA lentivirus vectors

The 293T cells and M1 macrophages from the lungs of the mice were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FBS). All of the recombinant lentiviruses were produced by transient transfection of 293T cells. Briefly, sub-confluent 293T cells were co-transfected with 10 μl of liposome2000, 2.25 μg psPAX2 (packaging plasmid), 0.25 μg pMD2G (envelope plasmid) and 2.5 μg of pLKO-A6 (containing CD147 shRNA insert) or 2.5 μg of pLKO-NC (empty lentiviral vector as a control). All of the plasmids were purchased from Open Biosystems. After 18 h, the media was changed, and the recombinant lentivirus vectors were harvested 24 h later. For transduction, M1 macrophages from the lungs of the mice were plated in 24-well plates (3×10^5 cells/well), and after 24 h, media containing the recombinant lentivirus vectors were added. Following 16 h of incubation, the cells were washed and split, and puromycin was added to half of the transduced cells at a final concentration of 4 μg/ml. Three days later, the cells were harvested and analyzed by Western blotting. Then, the harvested CD147-deficient M1 macrophages were prepared for the following study.

2.9. Bio-Plex analysis of cytokines in the co-culture medium of $CD4^+$ T cells with M1, M2 or CD147-deficient M1 macrophages

To quantify the cytokines that can induce the generation of Th17 cells in the conditioned medium of M1, M2 and CD147-deficient M1 macrophages, a multiplex suspension protein array was performed using the Bio-Plex protein array system and multiplexed (IL-6, IL-1β, IFN-γ, IL-12, IL-23 and IL-4) bead-based immunoassay kits (Bio-Rad). $CD4^+$ T cells were isolated from lymphocytes and were co-cultured with M1, M2 or CD147-deficient M1 macrophages for 24 h. The media from the co-cultured cells was collected and prepared for a multiplex

suspension protein array. A sample of 0.05% FBS medium alone was also run to account for the background levels of secreted proteins. The data were expressed as pg/ml of conditioned medium (mean ± SEM) with an N = 3 for each group.

2.10. Statistical analysis

The experiments were performed independently at least three times. The data were expressed as the mean value ± SEM. The statistical

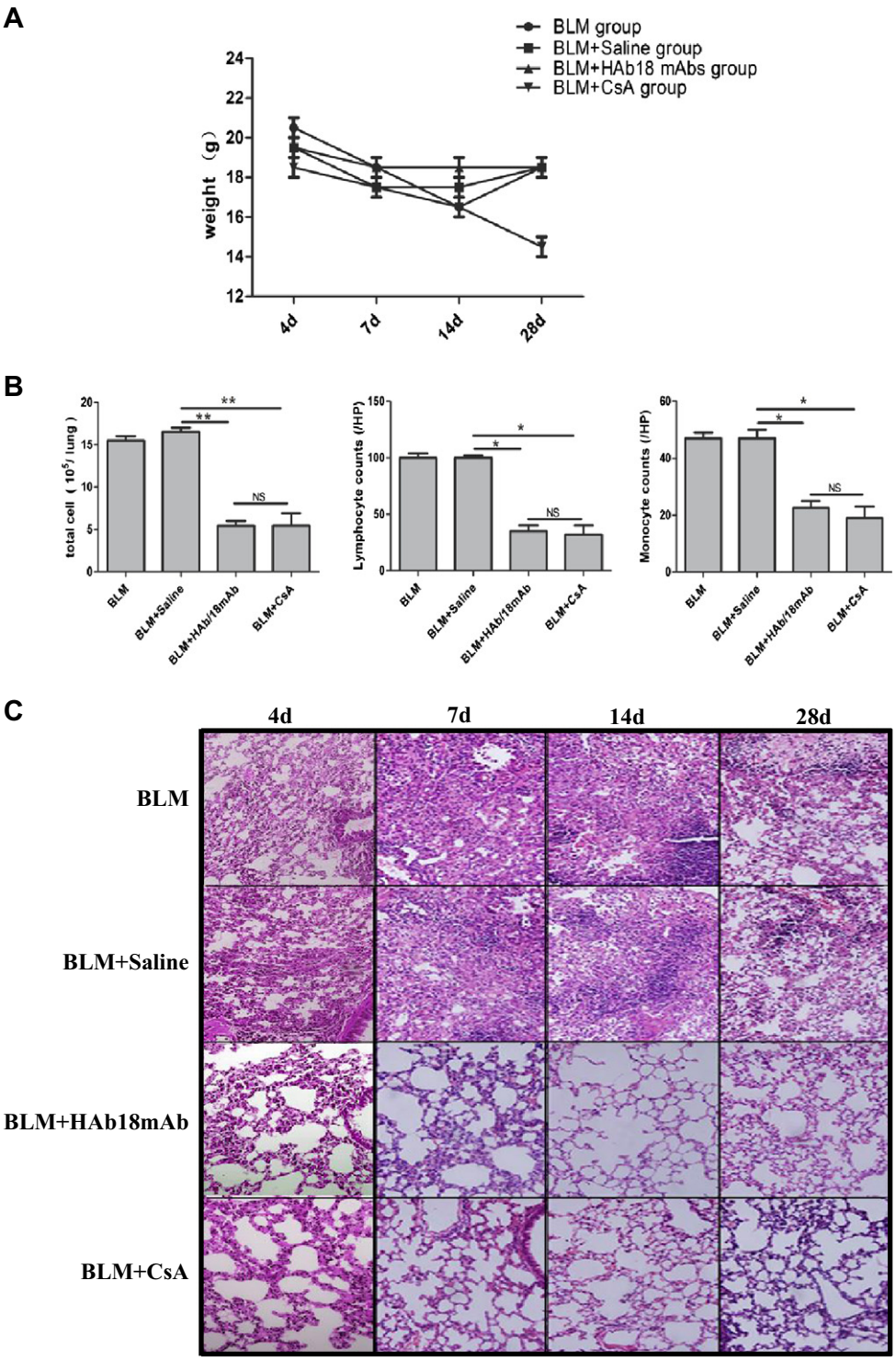

Fig. 2 The changes in bleomycin-induced mice treated with saline, HAb18G/CD147 mAbs or CsA. A. Changes in the body weight of the studied mice after instillation with bleomycin when treated with HAb18G/CD147 mAbs or CsA. The body weight was maintained in mice treated with HAb18G/CD147 mAbs but reduced in the group that received CsA (* = $P < 0.05$). B. Evaluation of the number of infiltrating cells (total cells, lymphocytes, and monocytes) in the BALF at d 14 after treatment with bleomycin. HAb18G/CD147 mAbs or CsA prevented total cells, lymphocytes, and monocytes from reduction (** = $P < 0.01$). C. HE staining (×400) for histopathological changes in lung tissues at days 4, 7, 14, 28. Collagen deposition decreased in the mice treated with HAb18G/CD147 mAbs or CsA. D. Masson staining (×200) for histopathological changes in lung tissues at days 4, 7, 14, and 28. Inflammation and fibrosis decreased in mice treated with HAb18G/CD147 mAbs or CsA. E. Quantitative analysis of fibrotic grades using the Ashcroft scale. (** = $P < 0.01$). F. Immunohistochemistry staining (×400) for CD147 in lung tissues at days 4, 7, 14, and 28. The expression of CD147 in the lung tissues of mice treated with HAb18G/CD147mAbs or CsA was less than that observed for the bleomycin group (* = $P < 0.05$).

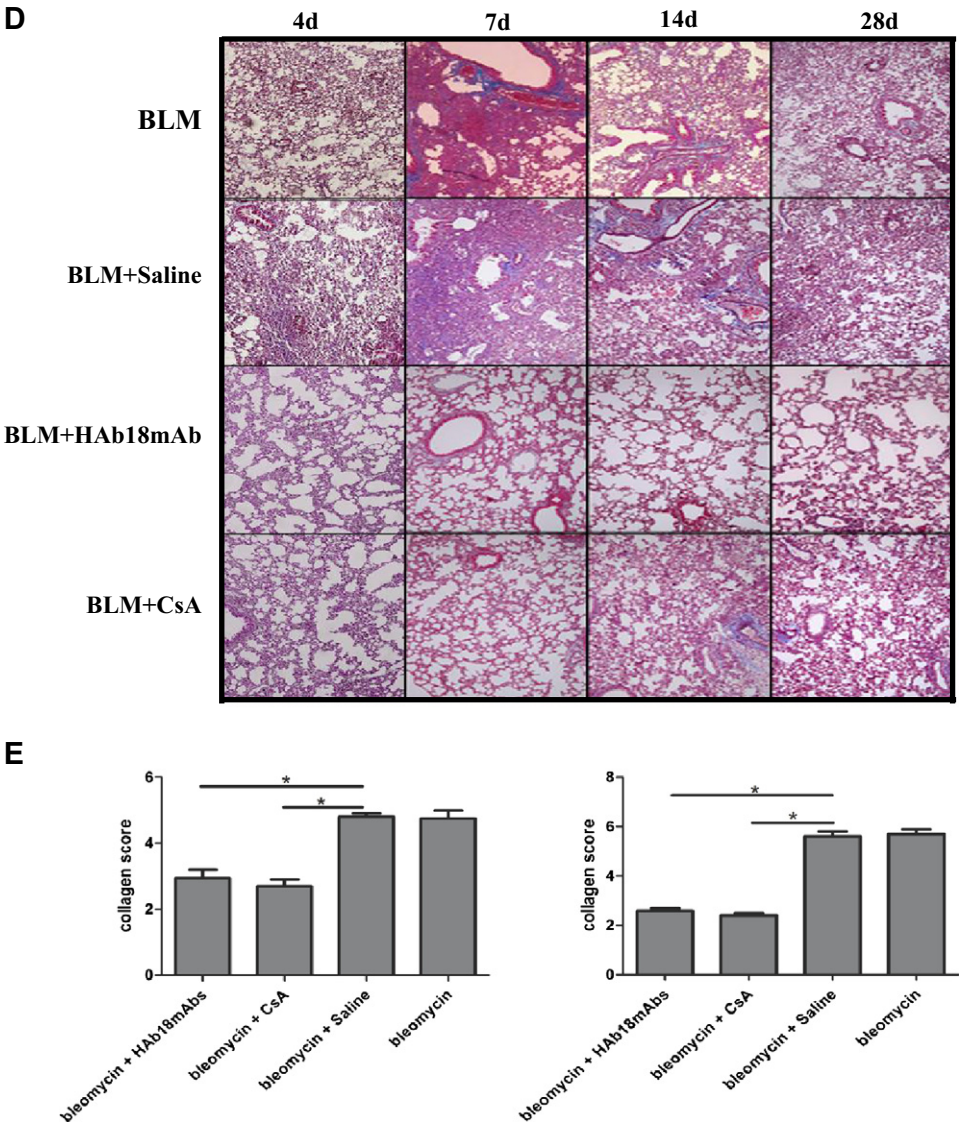

Fig. 2 (*continued*).

analyses used were an analysis of variance (ANOVA) followed by the appropriate post hoc tests including multiple comparison tests (LSD). All of the analyses were made using the SPSS 11.5 statistical software package. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. The levels of bleomycin-induced lung interstitial fibrosis changed at d 4, 7, 14, 28

After the instillation of bleomycin, the body weight of the mice gradually reduced and reached a minimum at d 14 ($P < 0.05$, compared with saline group) (Fig. 1A). The BALF analysis showed that compared with the saline group, the number of total cells, lymphocytes and monocytes was increased in the mice that received bleomycin ($P < 0.01$). The numbers of lymphocytes and monocytes increased after bleomycin instillation and peaked at d 14 ($P < 0.05$). The total cell number decreased gradually from d 4 to d 14 and improved slightly at d 28 ($P < 0.05$) (Fig. 1B). The neutrophils gradually decreased from d 4 to d 14 (Sup. 1). HE staining and Masson staining showed that the lung tissue sections from the saline group displayed normal structure and no pathologic changes under a light microscope. On the 4th, 7th, 14th and 28th day after bleomycin instillation, collagen fibers and large numbers of inflammatory cells were found, and small fibrous areas emerged in the bleomycin group mice (Fig. 1C and D). According to the Ashcroft scale, the most serious fibrosis was found at d 14 ($P < 0.05$, compared with d 4) when the collagen score was 5.4 ± 0.52. The level of fibrosis at d 28 (the collagen score was 3.6 ± 0.47) was less severe when compared with d 14 (Fig. 1E). By histochemical staining, the expression of CD147 on monocytes, neutrophils and lymphocytes at d 14 was greater than that at d 4 ($P < 0.05$) (Fig. 1F, Sup. 3).

3.2. HAb18 mAbs or CsA treatment of lung interstitial fibrosis

Treatment with HAb18 mAbs protected the mice from losing body weight ($P < 0.05$, compared with bleomycin group). However, mice treated with CsA had a greater reduction in body weight than the mice treated with HAb18 mAbs after d 14 ($P < 0.05$) (Fig. 2A). BALF analysis showed that after treatment with HAb18 mAbs or CsA, the counts of total cells, lymphocytes, and monocytes were reduced compared

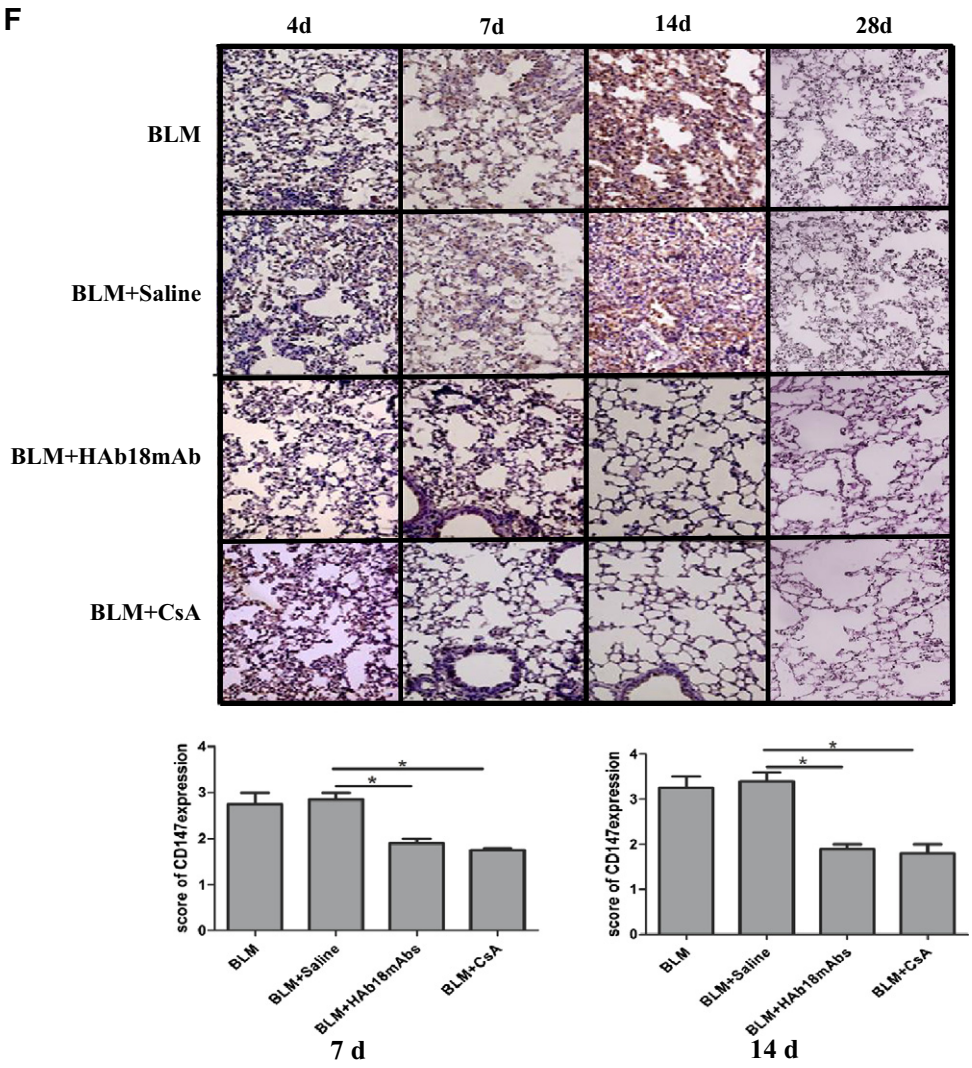

Fig. 2 *(continued).*

with the bleomycin group or the bleomycin + saline group ($P < 0.05$) (Fig. 2B and Sup. 1).

The histological examination and Masson trichrome staining showed that when the bleomycin-induced mice were treated with HAb18 mAbs or CsA, the lung tissues presented less inflammation and less fibrosis at d 4, 7, 14, 28 (Fig. 2C, D). Compared with the bleomycin group, the collagen score was reduced in mice treated with HAb18 mAbs or CsA ($P < 0.05$) (Fig. 2E, Sup. 2). The expression of CD147 in the lung tissues of mice treated with HAb18 mAbs or CsA was less than that of the bleomycin group ($P < 0.05$) (Fig. 2F, Sup. 3).

3.3. Changes in the M1, M2 macrophages and Th17 cells in the lung tissues after bleomycin instillation

M1, M2 macrophages and Th17 cells from the lung tissues of the bleomycin-treated group at d 4, 7 and 14 were detected by flow cytometry and real-time PCR. Flow cytometry showed that the expression of CD16/32, CD206 and IL-17 increased gradually and peaked at d 14 ($P < 0.05$) (Fig. 3A). The primary markers of M1 macrophages (CD16/32, CD86, iNOS) and M2 macrophages (CD206, ArgI) also increased gradually and peaked at d 14 (Fig. 3B).

M1 and M2 macrophages and Th17 cells from the lung tissues of the bleomycin group and the HAb18 mAbs-treated group were also detected by flow cytometry and real-time PCR. At d 14, the results showed that the levels of IL-17 in the HAb18 mAbs-treated group decreased significantly compared with the bleomycin group (2.56% ± 0.45% to 4.24% ± 0.63%, $P < 0.05$), and the number of $CD16/32^+$ macrophages decreased slightly (5.96% ± 0.35% to 7.53% ± 0.47%, $P < 0.05$). No obvious changes were observed in the $CD206^+$ M2 macrophages (8.06% ± 0.67% to 7.83% ± 0.54%) (Fig. 3C). The levels of CD16/32, CD86, and iNOS decreased, but no changes in CD206 or ArgI were detected after treatment with HAb18 mAbs (Fig. 3D).

3.4. Macrophages induced differentiation of the Th17 cells

M1 and M2 macrophages and $CD4^+$ T cells from lung tissues in the bleomycin-instilled group at d 14 were sorted with a fluorescence-activated cell sorter. The M1 and M2 macrophages were co-cultured with $CD4^+$ T cells for three days. The control group used in this experiment was $CD4^+$ T cells cultured without macrophages. After incubation, the $CD4^+$ T cells were collected and analyzed by flow cytometry. The results showed that the expression levels of IL-17, CCR6 and RORγt after co-culture with M1 macrophages were

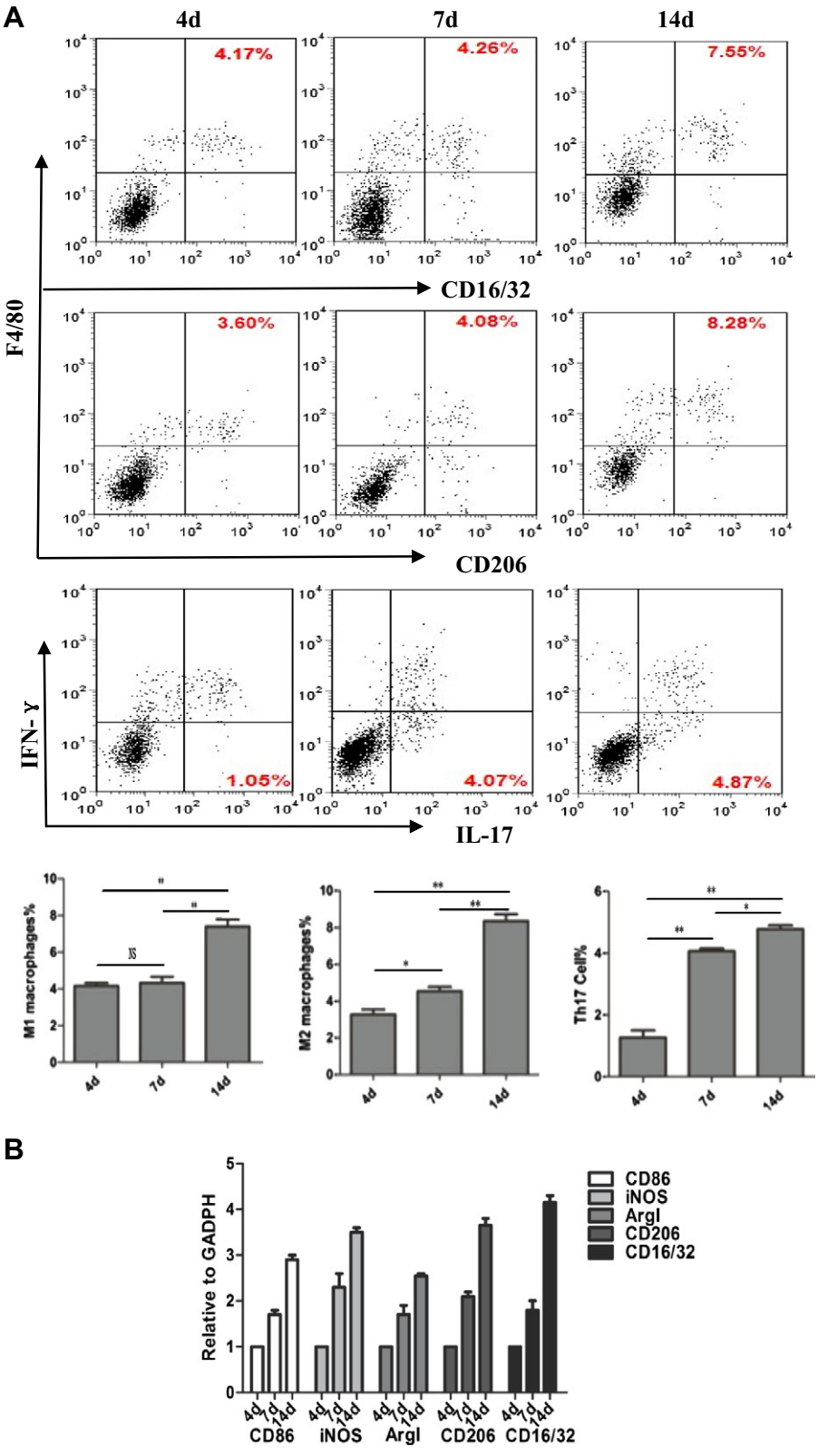

Fig. 3 Analysis of M1 and M2 macrophages and Th17 cells. A. M1 and M2 macrophages and Th17 cells in the lung tissues of the studied mice were analyzed by flow cytometry at d 4, 7, and 14 after treatment with bleomycin. The quantity of M1 and M2 macrophages and Th17 cells increased gradually and peaked at d 14 (** = $P < 0.01$, * = $P < 0.05$). B. Real-time PCR for CD16/32, CD86, iNOS, CD206 and Arg1 in the bleomycin-treated group at d 4, 7 and 14. C. Detection of M1 and M2 macrophages and Th17 cells in fibrotic lungs at d 14 after treatment with HAb18G/CD147 mAbs. M1 macrophages and Th17 cells in the HAb18G/CD147 mAbs-treated group decreased (* = $P < 0.05$, compared with the bleomycin group). D. Real-time PCR for CD16/32, CD86, iNOS, CD206 and Arg1 in the bleomycin group and the HAb18G/CD147 mAbs-treated group. The following 3 groups were studied: C, control, the normal group; B, the bleomycin treated group; and B + H, the bleomycin and HAb18G/CD147-treated group. The detection of Th17 cells was gated on CD4.

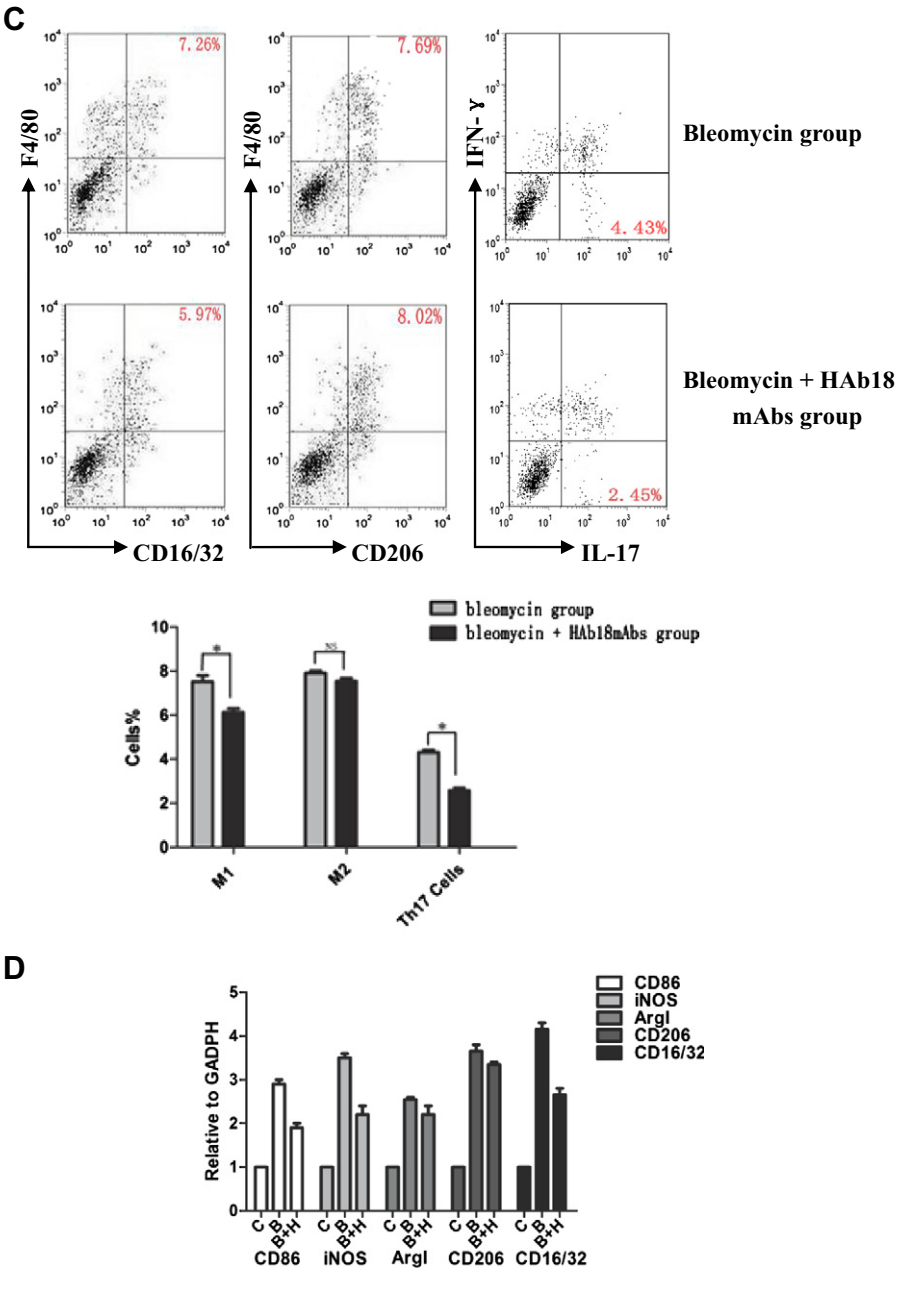

Fig. 3 (*continued*).

approximately 3- (3.78% ±0.28% to 1.24% ± 0.15%), 4- (4.87% ± 0.32% to 1.38% ± 0.23%) and 2-fold (2.36% ± 0.46% to 0.82% ± 0.21%) higher, respectively, than the expression levels activated by the M2 macrophages ($P < 0.05$) (Fig. 4A).

The total RNA was isolated from the M1 and M2 macrophages. Real-time quantitative PCR analysis for TGF-β, IL-6, IL-1β, IFN-γ, and IL-4 revealed that the levels of IL-6 and IL-1β were higher in M1 macrophages ($P < 0.01$), and the levels of IFN-γ IL-4 and TGF-β were higher in M2 macrophages ($P < 0.01$) (Fig. 4B).

$CD4^+$ T cells were co-cultured with M1 or M2 macrophages, and the culture media was collected and subjected to cytokine analysis. The culture media in the control group was collected from $CD4^+$ T cells that were cultured without macrophages. The Bio-Plex suspension array system results showed that the levels of IL-6, IL-1β, IL-23 and IL-12 in the M1 co-culture media were higher than those in M2 co-culture media ($P < 0.05$) (Fig. 4C).

3.5. CD147 on M1 macrophages induced Th17 cells

Western blotting showed that compared with the control group, the expression of CD147 on macrophages from the lungs of mice was suppressed after transfection with shRNA lentivirus vectors (Fig. 5A).

The levels of IL-17 in the group treated with HAb18 mAbs were lower than those in the normal M1 macrophages group (1.84% ±0.45% to 4.55% ± 0.68%, $P < 0.05$), and there was no difference between the $CD4^+$ T cells cultured in the presence or absence of the HAb18 mAbs (Fig. 5B). When the expression of CD147 was knocked down using shRNA lentivirus interference, the levels of Th17 cells in the CD147-deficient group were lower compared with those of the normal M1 macrophages group (2.06% ± 0.48% to 4.84% ± 0.72%, $P < 0.05$) (Fig. 5C). The control group was $CD4^+$ T cells cultured in the absence of macrophages.

$CD4^+$ T cells were co-cultured with M1 or CD147-deficient M1 macrophages, and the culture media was collected for cytokine analysis. The culture media collected from $CD4^+$ T cells cultured in the absence of macrophages served as a control. The Bio-Plex suspension array system results showed that when CD147 was knocked down, the levels of IL-6 and IL-1β were lower ($P < 0.05$, compared with M1 macrophages) (Fig. 5D), and the levels of IL-23 and IL-12 were below the limit of detection (data not shown).

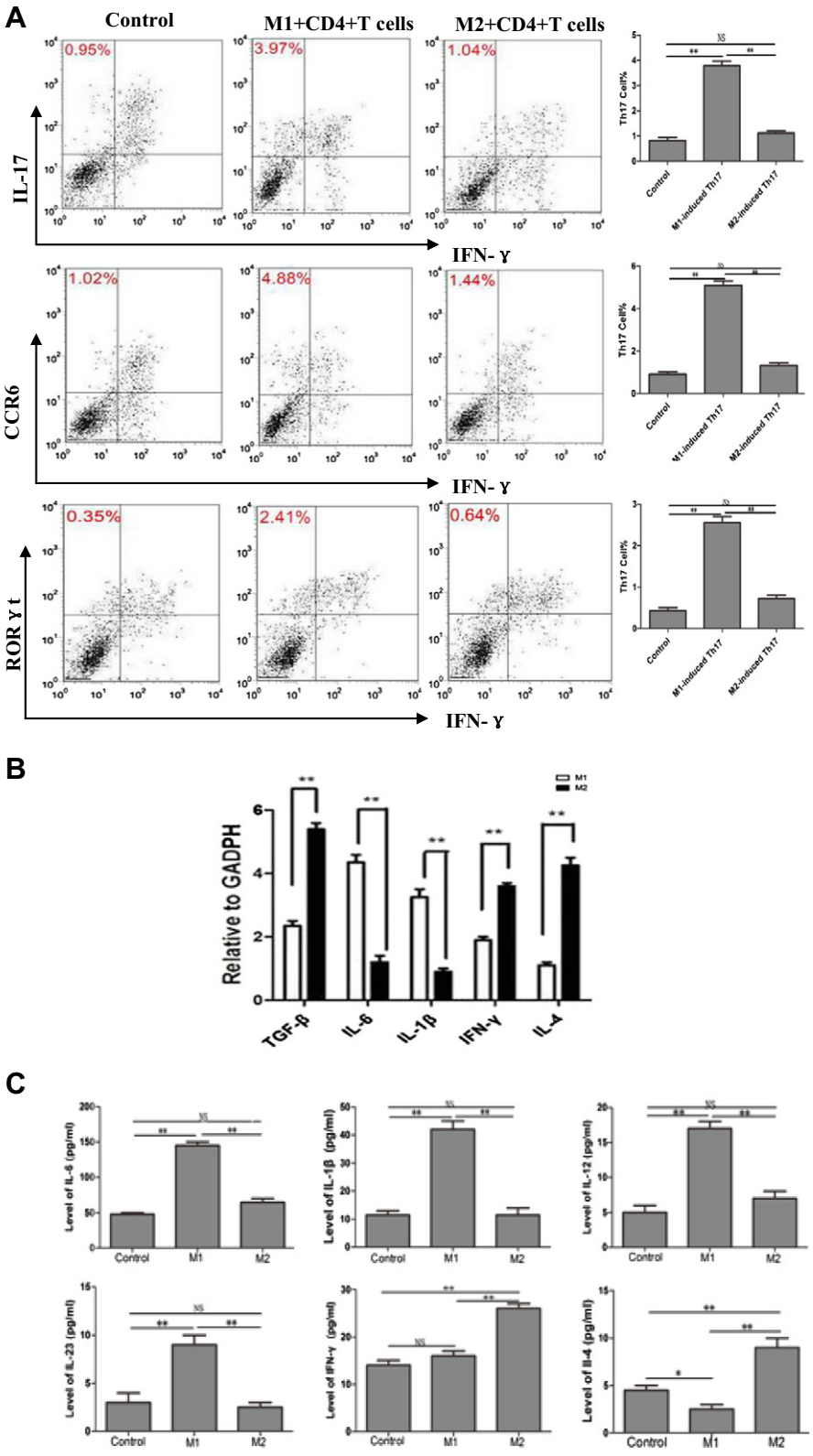

Fig. 4. Induction of Th17 cell differentiation by M1 or M2 macrophages. A. The expression levels of IL-17, CCR6 and RORγt induced by M1 macrophages were approximately 3-fold, 4-fold and 2-fold higher, respectively, than those induced by M2 macrophages (** = $P < 0.01$, * = $P < 0.05$). B. Analysis of TGF-β, IL-6, IL-1β, IFN-γ and IL-4 levels using real-time quantitative polymerase chain reaction (PCR). The results were normalized against the expression of GADPH (** = $P < 0.01$). C. Bio-Plex analysis of secreted cytokines. Presented in this panel are the 6 cytokines that showed significant differences between the M1 and M2 macrophages, including interleukin (IL)-6, IL-1β, IL-12, IL-23, interferon-γ (IFN-γ), IL-4 (** = $P < 0.01$, * = $P < 0.05$).

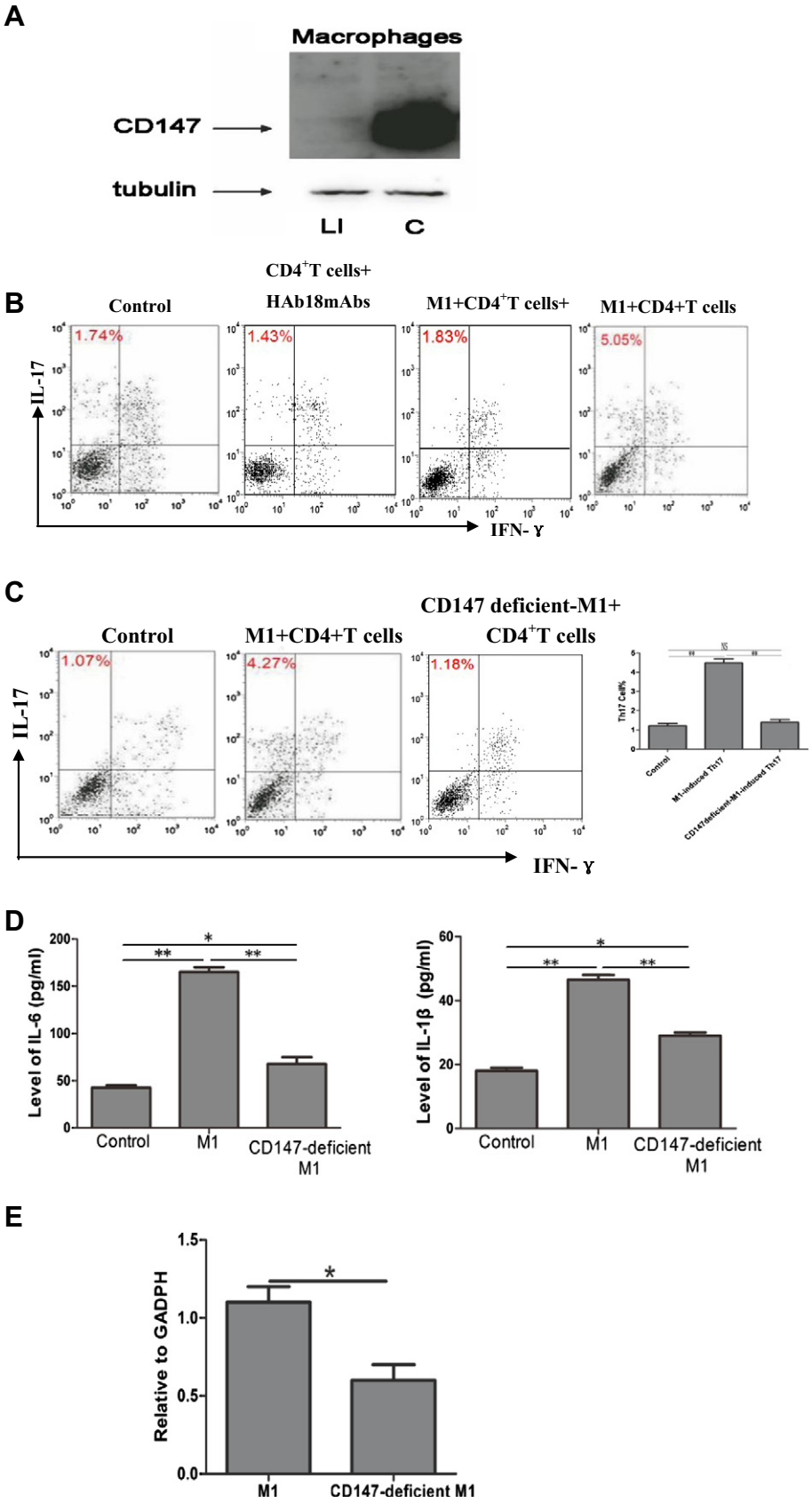

Fig. 5. The role of CD147 on M1 macrophages in inducing the differentiation of Th17 cells. A. Analyses of CD147 using Western blotting. LI represents CD147 shRNA lentivirus interference, and C represents the control group with the empty lentivirus vector. B. The effect of HAb18G/CD147 mAbs on Th17 cell differentiation. The level of Th17 cell differentiation was lower when treated with HAb18G/CD147 mAbs (* = $P < 0.05$). C. Effect of CD147 knock-down on Th17 cells differentiation. The level of Th17 cell differentiation was lower when the expression of CD147 was down-regulated using lentivirus interference (* = $P < 0.05$). D. Bio-Plex analysis of secreted cytokines. When CD147 was knocked down, the levels of IL-6 and IL-1β were lower (** = $P < 0.01$, * = $P < 0.05$), and the levels of IL-23 and IL-12 were below the limit of detection. E. Analysis of TGF-β in M1 or CD147-deficient M1 macrophages using real-time PCR. The results were normalized against the expression of GADPH (** = $P < 0.01$).

The total RNA was isolated from M1 macrophages or CD147-deficient M1 macrophages in the co-culture system. Real-time quantitative PCR analysis for TGF-β showed that the TGF-β expression on M1 macrophages was markedly reduced when CD147 was knocked down ($P < 0.05$) (Fig. 5E).

4. Discussion

In this study, we found that treatment with HAb18 mAbs or CsA ameliorated lung interstitial fibrosis, and the presence of M1 macrophages and Th17 cells was reduced when treated with HAb18 mAbs. M1 macrophages had a positive effect on the differentiation of Th17 cells, and CD147 might promote this process by regulating Th17-promoting cytokines, including TGF-β, IL-6, IL-1β, IL-12 and IL-23. High expression levels of CD147 was found in lung fibrosis, and we also found that CD147 expression increased as the degree of pulmonary fibrosis increased in bleomycin-induced lung interstitial fibrosis. Together, these data indicated that CD147 might play an important role in lung interstitial fibrosis. The HAb18 mAbs block the CD147 molecule and then inhibit the functions of CD147 [28]. CsA is an immunosuppressive regimen in use for many years [29]. Both CsA and HAb18 mAbs have an antagonistic peptide AP-9 against CD147 [30]. Therefore, we used CsA treatment as the positive control and contrasted it with the antibody treatment. Both HAb18 mAbs and the CsA treatment ameliorated lung interstitial fibrosis. The immunohistochemical analyses showed a gradual reduction of CD147 expression in the lung tissues when treated with HAb18 mAbs or CsA; therefore, we hypothesized that the HAb18 mAbs or CsA might improve lung interstitial fibrosis by inhibiting the functions of CD147. However, the HAb18 mAbs seem to privilege over CsA. For example, the body weight was conserved by treatment with HAb18 mAbs but was reduced rapidly in mice treated with CsA; this may have resulted from a side effect of CsA [31].

In these experiments, the most important phenomenon observed was that the number of M1 macrophages, M2 macrophages and Th17 cells increased as the lung inflammation was aggravated, and treatment with HAb18 mAbs resulted in a reduction of M1 macrophages and Th17 cells at d 14. These results indicated that the presence of CD147 might have an important role in maintaining the quantity of M1 macrophages and Th17 cells. The correlation of the changes in M1 macrophages and Th17 cells suggested that M1 macrophages might have some relationship with the Th17 cells. Therefore, we performed experiments in vitro to search for evidence of the relationship between M1 macrophages and Th17 cells.

In our present study, we found that of the two primary macrophage subtypes (M1 and M2), M1 macrophages induced a higher level of Th17 differentiation than the M2 macrophages. The M1 macrophages secrete important cytokines, such as IL-6 and IL-1β, both of which can promote Th17 cell differentiation [32–34]. In addition, IL-4 and IFN-γ have been reported to be inhibitors of the differentiation of Th17 cells [36]. In our study, we found that IL-6 and IL-1β expression levels were higher in M1 macrophages than in M2 macrophages and that IL-4 and IFN-γ were more highly expressed in M2 macrophages. Therefore, the M1 macrophages induced a higher level of Th17 cell differentiation than the M2 macrophages. In addition, TGF-β, an important cytokine in Th17 cell differentiation [35], was expressed at a higher level in M2 macrophages, which seemed to induce more Th17 cell differentiation. However, the higher levels of IL-4 and IFN-γ in M2 macrophages may inhibit the function of TGF-β. Although, M2 macrophages had more TGF-β than M1 macrophages, because of the higher level of IL-4 and IFN-γ, M2 macrophages are less significant to Th17 cells differentiation.

We assessed the role of CD147 in several in vitro experiments. The results of flow cytometry in this study showed that both M1 and M2 macrophages expressed high levels of CD147, and no significant difference in the positive rate or the fluorescence intensity was observed between the two subtypes of macrophages [Sup. 4], despite the observation that M1 macrophages induced a higher level of Th17 differentiation. How does CD147 on M1 macrophages affect the differentiation of Th17 cells? Experiments in vitro indicated that CD147 might have a significant role in the induction of Th17 cell differentiation by M1 macrophages. To define this point, we studied the effect of CD147 knock-down on related cytokines in M1 macrophages. According to the results of other studies, CD147 levels were positively correlated with the expression of TGF-β and IL-1β [37–39]; these observations agree with our findings. We also found that IL-6, IL-12 and IL-23 were reduced when CD147 expression was knocked down in M1 macrophages. The presence of the cytokine IL-6 induces the expression of the IL-21 and IL-23 receptor, which then activates the positive IL-21 autocrine regulation of Th17 cell differentiation [40–42]. The positively regulating effects of CD147 on IL-1β, IL-6, IL-12 and IL-23 suggested that CD147 promoted the differentiation of Th17 cells by regulating cytokines. However, the role of CD147 on M2 macrophages in the pathogenesis of lung fibrosis has not been explored and will be the subject of future studies.

Bleomycin-induced lung interstitial fibrosis in mice has been widely used in scientific research because its histological features are similar to those observed in people. In recent years, this model has made significant contributions to the study of Th17 cells [43]. Although bleomycin-induced lung interstitial fibrosis in mice is a commonly used model, it has limitations. Bleomycin-induced lung interstitial fibrosis in mice can be reversed without any interference [44]. This is in agreement with our observation that the symptoms of lung fibrosis improved around d 28 and showed a decrease in inflammatory cells infiltration and a reduction in collagen deposition. Because of the reversal of bleomycin-induced lung fibrosis at d 28, the data regarding M1 macrophages, M2 macrophages and Th17 cells at d 28 were not shown in this manuscript.

5. Conclusions

In summary, the data presented in this study show that HAb18 mAbs had a therapeutic effect on lung interstitial fibrosis. M1 macrophages induced a higher level of Th17 cell differentiation than M2 macrophages, and CD147 promoted this progress. These results indicate that CD147 might play an important role in the process of lung interstitial fibrosis, and an antibody treatment against CD147 might provide a new therapy for the treatment of lung interstitial fibrosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.06.008.

References

[1] The immunology of fibrosis, Annu. Rev. Immunol. 31 (2013) 107–135.
[2] Tasha E. Fingerlin, Elissa Murphy, Weiming Zhang, Anna L. Peljto, et al., Genome-wide association study identifies multiple susceptibility loci for pulmonary fibrosis, Nat. Genet. 45 (2013) 613–620.
[3] I.I. Ivanov, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, et al., The orphan nuclear receptor RORct directs the differentiation program of proinflammatory IL-17+ T helper cells, Cell 126 (2006) 1121–1133.
[4] N.C. Mishra, J. Rir-sima-ah, G.R. Grotendorst, R.J. Langley, S.P. Singh, S. Gundavarapu, W.M. Weber, J.C. Pena-Philippides, M.R. Duncan, M.L. Sopori, Inhalation of sulfur mustard causes long-term T cell-dependent inflammation: possible role of Th17 cells in chronic lung pathology, Int. Immunopharmacol. 13 (1) (2012 May) 101–108.
[5] P.L. Simonian, C.L. Roark, F. Wehrmann, et al., Th17-polarized immune response in a murine model of hypersensitivity pneumonitis and lung fibrosis, J. Immunol. (2009) 1550–6606.
[6] J. Bakowska, I.Y. Adamson, Collagenase and gelatinase activities in bronchoalveolar lavage fluids during bleomycin-induced lung injury, J. Pathol. 185 (1998) 319–323.

[7] X. Wang, C. Ma, J. Wu, J. Zhu, Roles of T helper 17 cells and interleukin-17 in neuroautoimmune diseases with emphasis on multiple sclerosis and Guillain-Barré syndrome as well as their animal models, J. Neurosci. Res. 91 (7) (2013) 871–881.
[8] E.B. Samoilova, J.L. Horton, B. Hilliard, T.S. Liu, Y. Chen, IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells, J. Immunol. 161 (1998) 6480–6486.
[9] S. Lee, H.J. Hwang, Y. Kim, Modeling the role of TGF-β in regulation of the Th17 phenotype in the LPS-driven immune system, Bull. Math Biol. 76 (5) (2014) 1045–1080.
[10] Y. Chung, S.H. Chang, G.J. Martinez, X.O. Yang, R. Nurieva, H.S. Kang, et al., Critical regulation of early Th17 differentiation by interleukin-1 signaling, Immunity 30 (2009) 576–587.
[11] J.M. Wang, L. Shi, C.J. Ma, X.J. Ji, R.S. Ying, X.Y. Wu, K.S. Wang, G. Li, J.P. Moorman, Z.Q. Yao, Differential regulation of interleukin-12 (IL-12)/IL-23 by Tim-3 drives T(H)17 cell development during hepatitis C virus infection, J. Virol. 87 (8) (2013) 4372–4383.
[12] B.R. Marks, H.N. Nowyhed, J.Y. Choi, A.C. poholek, J.M. Odegard, R.A. Flavell, et al., Thymic Th17 cells in wild-type mice that directly migrate to lung, gut and liver, Nat. Immunol. 10 (2009) 1125–1132.
[13] I. Gutcher, M.K. Donkor, Q. Ma, A.Y. Rudensky, R.A. Flavell, M.O. Li, Autocrine transforming growth factor-b1 promotes in vivo Th17 cell differentiation, Immunity 34 (2011) 396–408.
[14] Paul J. Egan, Annemarie van Nieuwenhuijze, Ian K. Campbell, Ian P. Wicks, Promotion of the local differentiation of murine th17 cells by synovial macrophages during acute inflammatory arthritis, Arthritis Rheum. 58 (2008) 3720–3729.
[15] Arpita Mondal, Deepali Sawant, Alexander L. Dent, Transcriptional repressor BCL6 controls Th17 Responses by controlling gene expression in both T cells and macrophages, J. Immunol. 184 (2010) 4123–4132.
[16] A. Mantovani, A. Sica, M. Locati, Macrophage polarization comes of age, Immunity 23 (4) (2005) 344–346.
[17] R. Tomasini, V. Secq, L. Pouyet, A.K. Thakur, TAp73 is required for macrophage-mediated innate immunity and the resolution of inflammatory responses, Cell Death Differ. (2013) 293–301.
[18] Antonio Sica, Alberto Mantovani, Macrophage plasticity and polarization: in vivo veritas, J. Clin. Invest. 122 (2012) 3.
[19] Kazuki Nabeshima, Hiroshi Iwasaki, Kaori Koga, Hironobu Hojo, Junji Suzumiya, Masahiro Kikuchi, Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression, Pathol. Int. 56 (2006) 7.
[20] T. Betsuyaku, K. Kadomatsu, G.L. Griffin, T. Muramatsu, R.M. Senior, Increased basigin in bleomycin-induced lung injury, Am. J. Respir. Cell Mol. Biol. 28 (5) (2003) 600–606.
[21] D.M. Mosser, et al., Receptor mediated subversion of macrophage cytokine production by intracellular pathogens, Curr. Opin. Immunol. 11 (1999) 406.
[22] J.S. Duffield, et al., Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair, J. Clin. Invest. 115 (2005) 56.
[23] V. Yurchenko, S. Constant, M. Bukrinsky, Dealing with the family: CD147 interactions with cyclophilins, Immunology 117 (3) (2006) 301–309.
[24] Y. Tatano, T. Shimizu, H. Tomioka, Unique macrophages different from M1/M2 macrophages inhibit T cell mitogenesis while upregulating Th17 polarization, Sci. Rep. 20 (4) (2014) 4146.
[25] S. Kremer, R. Brever, I.S. Lossos, Effect of immunomodulators on bleomycin-induced lung injury, Respiration 66 (1999) 455–462.
[26] T. Ashcroft, Judy M. Simpson, V. Timbrelli, Simple method of estimating severity of pulmonary fibrosis on a numerical scale, J. Clin. Pathol. 41 (1988) 467–470.
[27] M. He, H. Kubo, K. Ishizawa, A.E. Hegab, The role of the receptor for advanced glycation end-products in lung fibrosis, Am. J. Physiol. 293 (2007) 1427–1436.
[28] Jing Xu, Hui-Yun Xu, Qing Zhang, Fei Song, Jian-Li Jiang, Xiang-Min Yang, Li Mi, Zhi-Nan Chen, HAb18G/CD147 functions in invasion and metastasis of hepatocellular carcinoma, Mol. Cancer Res. 5 (2007) 605.
[29] Henrik Ekberg, Calcineurin inhibitor sparing in renal transplantation, Transplantation 86 (2008) 6.
[30] Y. Yang, N. Lu, J. Zhou, Z.N. Chen, P. Zhu, Cyclophilin A up-regulates MMP-9 expression and adhesion of monocytes/macrophages via CD147 signalling pathway in rheumatoid arthritis, Rheumatology (Oxford) 47 (9) (2008 Sep) 1299–1310.
[31] M. Jiang, C. Wang, Q. Meng, F. Li, K. Li, L. Lu, Cyclosporin A attenuates weight gain and improves glucose tolerance in diet-induced obese mice, Mol. Cell. Endocrinol. 370 (2013) 96–102.
[32] L. Zhou II, R. Spolski Ivanov, R. Min, K. Shenderov, IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways, Nat. Immunol. 8 (2007) 967–974.
[33] Y. Chung, S.H. Chang, G.J. Martinez, X.O. Yang, R. Nurieva, H.S. Kang, et al., Critical regulation of early Th17 differentiation by interleukin-1 signaling, Immunity 30 (2009) 576–587.
[34] J. Das, G. Ren, L. Zhang, A.I. Robers, X. Zhao, A.L. Both well, et al., Transforming growth factor-beta is dispensable for the molecular orchestration of Th17 cell differentiation, J. Exp. Med. 206 (2009) 2407–2416.
[35] P.R. Mangan, L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, et al., Transforming growth factor-beta induced development of the T(H)17 lineage, Nature 441 (2006) 231–234.
[36] Hwang Eun Sook, Transcriptional regulation of T helper 17 cell differentiation, Yonsei Med. J. 51 (4) (2010) 484–491.
[37] S. Xue, S.X. Li, Z.S. Wu, X.N. Wang, Q. Wu, F. Yang, Expression of CD147, matrix metalloproteinases and transforming growth factor beta1 in breast cancer, Zhonghua Bing Li Xue Za Zhi 38 (8) (2009) 524–528.
[38] N.R. Dean, J.R. Newman, E.E. Helman, W. Zhang, S. Safavy, D.M. Weeks, Anti-EMMPRIN monoclonal antibody as a novel agent for therapy of head and neck cancer, Clin. Cancer Res. 15 (12) (2009) 4058–4065.
[39] Y. Saijo, M. Tanaka, M. Miki, et al., Proinflammatory cytokine IL-1 beta promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction, J. Immunol. 169 (2002) 469–475.
[40] T. Korn, E. Bettelli, W. Gao, A. Awasthi, A. Jager, T.B. Strom, et al., IL-21 initiates an alternative pathway to induce proinflammatory Th17 cells, Nature 448 (2007) 484–487.
[41] R. Nurieva, X.O. Yang, G. Martinez, Y. Zhang, A.D. Panopoulos, L. Ma, et al., Essential autocrine regulation by IL-21 in the generation of inflammatory T cells, Nature 448 (2007) 480–483.
[42] Chi-Keung Wan, Oh. Jangsuk, Peng Li, Erin E. West, Elizabeth A. Wong, et al., The cytokines IL-21 and GM-CSF have opposing regulatory roles in the apoptosis of conventional dendritic cells, Immunity 38 (2013) 1081–1284.
[43] Gregory F. Sonnenberg, Meera G. Nair, Thomas J. Kirn, Colby Zaph, Lynette A. Fouser, David Artis, Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A, J. Exp. Med. 207 (6) (Jun 7 2010) 1293–1305.
[44] S. Ohshima, Y. Saeki, T. Mima, M. Sasai, K. Nishioka, S. Nomura, M. Kopf, Y. Katada, T. Tanaka, M. Suemura, et al., Interleukin 6 plays a key role in the development of antigen-induced arthritis, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8222–8226.