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Impairment of Bleomycin-Induced Lung Fibrosis in CD28-Deficient Mice

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Lung fibrosis is an important pulmonary disease with a high mortality rate, but its pathophysiological mechanism has not been fully clarified. Various types of cells have been implicated in the development of lung fibrosis, including T cells. However, the contribution of functional molecules expressed on T cells to the development of lung fibrosis remains largely unknown. In this study, we determined whether costimulation via CD28 on T cells was crucial for the development of lung fibrosis by intratracheally administering bleomycin into CD28-deficient mice. Compared with wild-type mice, the CD28-deficient mice showed markedly impaired lung fibrosis after injection with low doses of bleomycin, as judged by histological changes and hydroxyproline content in the lungs. In addition, bleomycin-induced T cell infiltration into the airways and production of several cytokines and chemokines including IL-5 were also impaired in the CD28-deficient mice. Furthermore, adoptive transfer of CD28-positive T cells from wild-type mice recovered the impaired bleomycin-induced lung fibrosis in CD28-deficient mice. These findings suggest that the CD28-mediated T cell costimulation plays a critical role in the development of lung fibrosis, possibly by regulating the production of cytokines and chemokines in the lung. Thus, manipulation of the CD28-mediated costimulation could be a potential therapeutic strategy for the prevention of lung fibrosis. The Journal of Immunology, 2001, 167: 1977–1981.

Materials and Methods

Induction of lung fibrosis
Female 7- to 10-wk-old C57BL/6 wild-type mice and C57BL/6 CD28-deficient mice were purchased from Charles River Japan (Atsugi, Japan) and The Jackson Laboratory (Bar Harbor, ME), respectively. The animals received an intratracheal injection of 1 mg/kg (low-dose) or 3 mg/kg (high-dose) BLM hydrochloride (Nippon Kayaku, Tokyo, Japan) dissolved in 50 μl saline on day 0. Mice were sacrificed, and lung tissues were excised on day 17 for histological examination.

Histological scoring of lung fibrosis
The right lungs of each mouse were resected, fixed in 10% formalin, embedded in paraffin, sectioned, stained with H&E solution, and examined by light microscopy for histological changes. Morphological evaluation of BLM-induced lung inflammation and fibrosis was performed using a semi-quantitative scoring method as previously described (11). The pathological scores were defined as follows: 0, no lung abnormality; 1, presence of inflammation and fibrosis involving <25% of the lung; 2, lesions involving 25–50% of the lung; and 3, lesions involving >50% of the lung. The mean of the pathological scores for three sections was determined for individual mice.

Hydroxyproline assay
Whole collagen content in the left lung was evaluated by determining hydroxyproline content as previously described (12). Briefly, after acid hydrolysis of the lung with 6 N HCl at 110°C for 16 h in a sealed glass tube, hydroxyproline content was determined by HPLC.

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2 Address correspondence and reprint requests to Dr. Ko Okumura, Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail address: kokumua@med.juntendo.ac.jp

3 Abbreviations used in this paper: BLM, bleomycin; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; MIP, macrophage inflammatory protein; MCP-1, monocyte chemoattractant protein-1.
Bronchialalveolar lavage (BAL)

BAL was performed as previously described (13) with some modification. Briefly, the mice were sacrificed by the abdominal aorta dissection, the trachea was cannulated, and the airway lumen was washed ten times with 0.7 ml ice-cold PBS. The bronchoalveolar lavage fluid (BALF) was centrifuged at 400×g for 5 min at 4°C, and total leukocyte count was determined. The concentration of IL-5 in the supernatant was measured by ELISA as described below. For differential cell counts, cells were spun onto glass slides, air-dried, fixed with ethanol, and stained with Wright-Giemsa solution. The number of eosinophils, neutrophils, lymphocytes, and macrophages in 200 cells was counted based on morphology.

Cytokine ELISA

The concentration of IL-5 in the BALF on day 4 after instillation of BLM was determined using a murine IL-5 ELISA kit (BD PharMingen, San Diego, CA) according to the manufacturer’s recommendation.

RNase protection assays

RNase protection assays were performed using the RiboQuant MultiProbe RNase protection assay system (BD PharMingen) according to the manufacturer’s recommendation. Total RNA was isolated from the lung tissues on days 0 (without BLM treatment), 7, and 17 after the BLM administration, and 7 μg of RNA samples were subjected to the RNase protection assay system using mouse chemokine template set mCK-5b (catalog number 45026P). Autoradiograms were visualized and quantified by using an image analyzer (Fuji BAS2500; Fuji, Tokyo, Japan).

Flow cytometry

FITC-labeled anti-CD3, CD4, and CD11b mAbs, PE-labeled anti-CD80 and CD86 mAbs, PerCP-labeled anti-CD8 mAb, and control rat IgG2b were purchased from BD PharMingen. BALF cells (1×10⁶) suspended in PBS were first incubated with unlabeled anti-CD16/CD32 mAb (BD PharMingen) to block nonspecific binding to FcγR. After washing, the cells were then incubated on ice with a mixture of FITC-, PE-, or PerCP-labeled mAbs. After washing again, the cells were subjected to flow cytometry on a FACScan (BD Biosciences, San Jose, CA), and the data were analyzed with CellQuest software (BD Biosciences). Alveolar macrophages collected from BALF-treated mice were gated on the basis of forward and side scatter profiles and identified by FITC-labeled anti-CD11b. For all samples, dead cells were excluded from the analysis by propidium iodide staining.

FIGURE 1. BLM-induced lung fibrosis in CD28⁺/⁺ and CD28⁻/⁻ mice. Lungs were removed from CD28⁺/⁺ (A–C) or CD28⁻/⁻ (D–F) mice on day 17 after the injection of a low dose (1 mg/kg) of BLM, and paraffin sections were stained with H&E. Original magnifications, ×16 (A and D), ×40 (B and E), and ×80 (C and F). Pictures represent one of six mice in each group.

Table 1. Histological evaluation of BLM-induced lung fibrosis in CD28⁺/⁺ or CD28⁻/⁻ mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pathological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD28⁺/⁺ mice</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>BLM (low dose)</td>
<td>2.05 ± 0.43</td>
</tr>
<tr>
<td>BLM (high dose)</td>
<td>2.50 ± 0.84</td>
</tr>
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</table>

*Results are shown as mean ± SD of six mice in each group. In the low-dose BLM-treated groups, the difference between CD28⁺/⁺ and CD28⁻/⁻ mice was statistically significant (*, p < 0.05).

Adoptive transfer of wild-type T cells

T cells were isolated from the spleen and lymph nodes of C57BL/6 wild-type mice by passage through a nylon wool column (Wako, Osaka, Japan). The purity of the T cell population was >83% CD3⁺ as determined by flow cytometry. Forty million cells per mouse were injected i.v. into CD28-deficient mice 2 h before intratracheal injection of BLM.

Data analysis

Statistical analysis of the results was performed using Fisher’s least significant difference test for multiple comparisons. Values of p < 0.05 were considered to be significant.

Results

Effect of CD28 deficiency on BLM-induced lung fibrosis in mice

To determine whether the CD28-mediated T cell costimulation contributed to the development of lung fibrosis, we induced lung fibrosis in CD28-deficient mice by intratracheal injection of BLM as described in Materials and Methods.

Because we thought that the dosage of BLM might affect the final response, we first examined the effect of two doses of BLM (low-dose, 1 mg/kg; and high-dose, 3 mg/kg).

Injection of BLM either at a low or high dose induced focal fibrotic lesions with thickened intraalveolar septa, collapse of alveolar septa, and massive infiltration of lymphocytes in the lung interstitium in wild-type mice as histologically estimated on day 17 after the injection (Fig. 1, A–C, and data not shown). As shown in Table 1, histological scoring of the fibrotic lesions revealed that

FIGURE 2. Hydroxyproline content in lung after BLM treatment. Lungs were removed from CD28⁺/⁺ (●) and CD28⁻/⁻ (□) mice with (+) or without (−) low-dose (1 mg/kg) or high-dose (3 mg/kg) BLM treatment on day 17, and the hydroxyproline content was determined as described in Materials and Methods. Results are indicated as mean ± SD of six mice in each group. The difference in lung hydroxyproline content between CD28⁺/⁺ and CD28⁻/⁻ mice was statistically significant after the low-dose BLM treatment (+, p < 0.05).
the high-dose (3 mg/kg) administration of BLM induced lung fibrosis to a comparable level in wild-type and CD28-deficient mice. In contrast, the low-dose (1 mg/kg) administration of BLM showed significant suppression of BLM-induced lung fibrosis in CD28-deficient mice compared with wild-type mice (Table I and Fig. 1, D–F).

This observation was confirmed by measuring hydroxyproline content in the lungs of BLM-treated mice. Hydroxyproline content in the lung is a good marker for lung collagen content (12). We assessed hydroxyproline content in the left lungs on day 17 after the injection of BLM and found that lung hydroxyproline content was significantly lower in CD28-deficient mice than that in wild-type mice at the low dose of BLM (Fig. 2). No significant reduction of hydroxyproline content was observed in CD28-deficient mice at the high dose of BLM (Fig. 2). These results indicated that BLM-induced lung fibrosis was significantly impaired in CD28-deficient mice when a low dose of BLM was injected. Thus, we performed the following experiments using the low dose (1 mg/kg) of BLM unless otherwise noted.

**CD28 deficiency attenuates BLM-induced infiltration of lymphocytes and neutrophils into the airway**

Infiltration of inflammatory cells consisting of macrophages, lymphocytes, and neutrophils into the lungs was reported to precede lung fibrotic changes after instillation of BLM (14). To determine whether the CD28 deficiency affected the BLM-induced infiltration of inflammatory cells into the lungs, we differentially counted the inflammatory cells in BALF on day 7 after the injection of BLM. As shown in Table II, there was no significant difference in the numbers of macrophages in the BALF of wild-type and CD28-deficient mice. However, the numbers of lymphocytes and neutrophils were significantly decreased in the BALF of CD28-deficient mice (Table II). To further characterize the lymphocyte subpopulations in the BALF, we performed FACS analysis on day 7 after the instillation of BLM. As shown in Fig. 3, the ratio of CD4/CD8-positive T cells in the BALF from CD28-deficient mice was decreased compared with that in wild-type mice, suggesting that the population of CD4+ T cells was markedly decreased in the BALF from CD28-deficient mice. In detail, the proportion of CD4+ T cells in the BALF from wild-type mice was 42.9 ± 5.4%, and in that from CD28-deficient mice it was 27.1 ± 2.8%, although the proportion of CD8+ T cells was 36 ± 2.3% from wild-type mice and 43.0 ± 5.0% from CD28-deficient mice (mean ± SD of five mice in each group). Actually, the absolute number of CD4+ T cells was significantly decreased in the BALF from CD28-deficient mice (Table III). These results indicated that BLM-induced infiltration of T cells, especially CD4+ T cells, was impaired in CD28-deficient mice.

**Induction of CD86 expression on alveolar macrophages after BLM treatment**

To further address the possible contribution of CD28-mediated T cell costimulation to the BLM-induced lung fibrosis, we determined by FACS analysis whether the CD28 ligands, CD80 and CD86 (15), were expressed on alveolar macrophages. As shown in Fig. 4A, the proportion of CD80 and CD86 expression on alveolar macrophages was significantly impaired in CD28-deficient mice compared with wild-type mice. However, the proportion of CD8+ T cells in the BALF from CD28-deficient mice was significantly decreased compared with that in wild-type mice, suggesting that the population of CD4+ T cells was markedly decreased in the BALF from CD28-deficient mice. In detail, the proportion of CD4+ T cells in the BALF from wild-type mice was 42.9 ± 5.4%, and in that from CD28-deficient mice it was 27.1 ± 2.8%, although the proportion of CD8+ T cells was 36 ± 2.3% from wild-type mice and 43.0 ± 5.0% from CD28-deficient mice (mean ± SD of five mice in each group). Actually, the absolute number of CD4+ T cells was significantly decreased in the BALF from CD28-deficient mice (Table III). These results indicated that BLM-induced infiltration of T cells, especially CD4+ T cells, was impaired in CD28-deficient mice.

**Effect of CD28 deficiency on production of cytokines and chemokines in BLM-treated lungs**

To explore the possible mechanisms for the impaired lung inflammation and fibrosis in CD28-deficient mice, we examined BLM-induced cytokine and chemokine production in the lung. Previous studies have suggested that several cytokines and chemokines including IL-5, macrophage-inflammatory protein (MIP)-1α, MIP-2, monocyte chemotactic protein (MCP)-1, and RANTES were involved in the development of BLM-induced lung fibrosis (14, 16–19).

**Table II. Leukocyte counts in BALF**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD28+/+ mice</th>
<th>CD28−/− mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>22.4 ± 7.2 × 10⁴</td>
<td>18.3 ± 4.9 × 10⁴</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.2 ± 0.1 × 10⁴</td>
<td>0.7 ± 0.5 × 10⁴</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>12.5 ± 6.3 × 10⁴</td>
<td>12.5 ± 6.3 × 10⁴</td>
</tr>
</tbody>
</table>

*Results are shown as mean ± SD of six mice in each group. After the BLM treatment, the differences in the numbers of lymphocytes and neutrophils between CD28+/+ and CD28−/− mice were statistically significant (*, p < 0.05).*

**Table III. Absolute numbers of CD4+ or CD8+ T cells in BALF**

<table>
<thead>
<tr>
<th>CD28+/+ Mice</th>
<th>CD28−/− Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>14.5 ± 5.6 × 10⁴</td>
</tr>
<tr>
<td>CD8+</td>
<td>12.5 ± 5.1 × 10⁴</td>
</tr>
</tbody>
</table>

*Results are shown as mean ± SD of six mice in each group. The difference in the numbers of CD4+ T cells between CD28+/+ and CD28−/− mice was statistically significant (*, p < 0.05).*

![CD28 expression](image-url)

**FIGURE 3.** Infiltration of CD4+ or CD8+ T cells in BALF after injection of BLM. Leukocytes in BALF were prepared from CD28+/+ or CD28−/− mice on day 7 after the injection of low-dose BLM and stained with mAbs against CD4 and CD8. Numbers represent percentages in each quadrant. Five mice were examined for each experimental group, and the data presented are from one representative of three independent experiments.
BLM treatment induced significant IL-5 production in the BALF from wild-type mice on day 4, whereas it was not detectable in the BALF from CD28-deficient mice (Fig. 5A). In addition, RNase protection assays showed that induction of mRNAs for MIP-1α and MCP-1 were significantly reduced in CD28-deficient mice (Fig. 5B and C), although there were no significant differences in the induction of mRNAs for RANTES and MIP-2 between wild-type and CD28-deficient mice (Fig. 5B and data not shown). These findings indicated that the production of IL-5, MIP-1α, and MCP-1 involved in the BLM-induced lung fibrosis were impaired in CD28-deficient mice.

Effect of CD28-positive T cell transfer into CD28-deficient mice on BLM-induced lung fibrosis

To further determine whether the absence of CD28-positive T cells was critically involved in the impairment of BLM-induced lung fibrosis in CD28-deficient mice, we examined the effect of CD28-positive T cell transfer into CD28-deficient mice on BLM-induced lung fibrosis. T cells isolated from the spleen and lymph nodes of wild-type C57BL/6 mice were injected i.v. into CD28-deficient mice 2 h before injection of BLM. As shown in Table IV, histological scoring of the fibrotic lesions revealed that the transfer of wild-type T cells significantly recovered the impaired BLM-induced lung fibrosis in CD28-deficient mice. As described above, lung hydroxyproline content was significantly lower in CD28-deficient mice than that in wild-type mice on day 17 after injection of BLM (Fig. 6). However, this impairment was totally restored after the transfer of wild-type T cells into CD28-deficient mice (Fig. 6). These results indicated that the absence of CD28-positive T cells was critically involved in the impairment of BLM-induced lung fibrosis in CD28-deficient mice.

Discussion

In this study, we demonstrated that lung fibrosis induced by a low dose (1 mg/kg) of BLM was attenuated in CD28-deficient mice as estimated by lung histology and hydroxyproline content (Figs. 1 and 2 and Table I). The critical role of CD28-positive T cells in developing BLM-induced lung fibrosis was further substantiated.

Table IV. Histological evaluation of BLM-induced lung fibrosis in CD28−/− mice with or without wild-type T cell transfer

<table>
<thead>
<tr>
<th>CD28</th>
<th>T Cell Transfer</th>
<th>Pathological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>−</td>
<td>2.17 ± 0.75</td>
</tr>
<tr>
<td>−/−</td>
<td>−</td>
<td>1.00 ± 0.00*</td>
</tr>
<tr>
<td>−/+</td>
<td>+</td>
<td>1.40 ± 0.55</td>
</tr>
</tbody>
</table>

* Results are shown as mean ± SD of five mice in each group. *, p < 0.05 compared with CD28+/+ mice.
BLM-induced lung inflammation appears to be at least partly responsible for the suppression of T cell infiltration and cytokine/chemokine production (Tables II and III). These findings suggest that the CD28-mediated T cell costimulation plays a critical role in the development of BLM-induced lung fibrosis. Thus, the administration of BLM at a low dose appeared to be critical for the T cell dependence of BLM-induced lung fibrosis. The results suggest that the blockade of the CD28-mediated costimulatory pathway could have therapeutic potential to prevent lung fibrosis associated with pulmonary diseases such as idiopathic pulmonary fibrosis and connective tissue diseases.

References


