

Involvement of NK 1.1-Positive $\gamma\delta$ T Cells in Interleukin-18 Plus Interleukin-2-Induced Interstitial Lung Disease

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Interstitial lung disease (ILD) is induced by various factors in humans. However, the exact mechanism of ILD remains elusive. This study sought to determine the role of natural killer (NK) 1.1⁺ $\gamma\delta$ T cells in ILD. The injection of IL-18 plus IL-2 (IL-18/IL-2) into C57BL/6 (B6) mice induced acute ILD that resembled early-stage human ILD. An accumulation of NK1.1⁺ $\gamma\delta$ T cells similar to NK cells was evident in the lungs. The T Cell Receptor (TCR) V γ and V δ repertoires of NK1.1⁺ $\gamma\delta$ T cells indicated polyclonal expansion. The expression of IL-2 receptor β (R β) and IL-18R β in NK1.1⁺ $\gamma\delta$ T cells was higher than in NK1.1⁻ $\gamma\delta$ T cells. IL-18/IL-2 stimulated the proliferation of NK1.1⁺ $\gamma\delta$ T cells, but not NK1.1⁻ $\gamma\delta$ T cells. The IL-18/IL-2-stimulated NK1.1⁺ $\gamma\delta$ T cells produced higher concentrations of IFN- γ than did NK1.1⁻ $\gamma\delta$ T cells. Moreover, NK1.1⁺ $\gamma\delta$ T and NK1.1⁻ $\gamma\delta$ T cells constituted completely different cell populations. The IL-18/IL-2-induced ILD was milder in TCR $\delta^{-/-}$ and IFN- $\gamma^{-/-}$ mice, compared with B6 mice. Furthermore, cell-transfer experiments demonstrated that NK1.1⁺ $\gamma\delta$ T cells could induce the expansion of NK cells and IFN- γ mRNA in the lung by IL-18/IL-2. Our results suggest that NK1.1⁺ $\gamma\delta$ T cells function as inflammatory mediators in the early phase of IL-18/IL-2-induced ILD.

Keywords: interstitial lung disease; NK1.1; $\gamma\delta$ T cell; interleukin-18; interferon- γ

Interstitial lung disease (ILD) is intractable, and is induced by various factors such as autoimmune diseases, drugs, and occupational and environmental exposures (1). For example, chemotherapy with bleomycin (BLM) and busulphan was reported to cause lung fibrosis in some patients (2). Histopathologically, the diffuse infiltration of mononuclear and polymorphonuclear leukocytes is evident in the lung during early stages of human ILD. After interstitial inflammation, florid fibroblast proliferation within both the interstitium and the alveolar space is often detected. The same pathology is observed in murine models of BLM-induced ILD (1).

IL-18, a member of the IL-1 family, is a proinflammatory cytokine (3, 4). IL-18 is known to induce the synergistic secretion of IFN- γ with IL-12, IL-2, or antigens. On the other hand, previous studies reported that IL-18 induced the secretion of Th2 cytokines from T cells, natural killer (NK) cells, NK T cells, basophils, and mast cells (4–9). Thus, IL-18 can act as a cofactor

CLINICAL RELEVANCE

This is the first report, to the best of our knowledge, on the functional analysis of NK 1.1⁺ $\gamma\delta$ T cells in murine models of lung disease.

for the development of both Th1 and Th2 cells. Recently, Okamoto and colleagues (10) reported on a new murine model of ILD induced by IL-18 plus IL-2 (IL-18/IL-2). Daily administration of IL-18 with IL-2, but not IL-18 or IL-2 alone, induced ILD in mice. In this murine model, a low dose of IL-18/IL-2 caused inflammation in several cells, with congestion and severe alveolar wall thickening, increased lung weight, and hydroxyproline (10). Unlike BLM-induced ILD, lung fibrosis was not caused by IL-18/IL-2-induced ILD. The pathological condition of BLM-induced ILD mainly involved fibroblastic proliferation (11). However, little fibroblastic proliferation was evident in IL-18/IL-2-induced ILD. This model of ILD is characterized by a severe infiltration of NK cells, mononuclear cells, and polymorphonuclear leukocytes in the lung. Furthermore, severe cell infiltration proceeded rapidly after the injection of IL-18/IL-2. Whereas almost all BLM-induced ILD mice had died 30 days after treatment with BLM, almost all of the IL-18/IL-2-induced ILD mice died 7 days after the injection of IL-18/IL-2 (12). Because of the rapid and severe cell infiltration in the lungs of mice with IL-18/IL-2-induced ILD, the mouse is considered a suitable model for studying the early phase of human ILD.

$\gamma\delta$ T cells exercise nonredundant functions in protecting against infectious agents. Unlike $\alpha\beta$ T cells and B cells, $\gamma\delta$ T cells preferentially colonize nonlymphoid tissue (e.g., epithelial or mucosal) (13). $\gamma\delta$ T cells produce a wide variety of cytokines, chemokines, and growth factors. Furthermore, $\gamma\delta$ T cells play an important role in regulating the initial immune response to several pathogens by influencing the migration and activity of neutrophils, macrophages, and T and NK cells (14). Previously, $\gamma\delta$ T cells were reported to play a crucial role in lung disease in several murine models. In the airway hyperresponsiveness (AHR) murine model, $\gamma\delta$ T cells regulated AHR (15–18). By contrast, in the BLM-induced ILD murine model, $\gamma\delta$ T cells played a protective role against the pathological condition (19). As already described, $\gamma\delta$ T cells play an effective or suppressive role in lung disease by secreting a variety of cytokines. We recently discovered an increase in $\gamma\delta$ T cells that expressed one of the NK cell markers, NK1.1, in IL-18/IL-2-induced ILD mice. Haas and colleagues (20) reported that NK1.1⁺ $\gamma\delta$ T cells could secrete large amounts of IFN- γ compared with NK1.1⁻ $\gamma\delta$ T cells in the presence of IL-18/IL-12. On the other hand, NK cells possess the well-known potential to secrete IFN- γ in the presence of IL-18/IL-12 or IL-18/IL-2 (21). Thus, IL-18/IL-2-induced ILD is thought to be caused by IFN- γ -producing cells at the local site of inflammation. Whereas NK cells and NK1.1⁺ $\gamma\delta$ T cells can

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produce large amounts of IFN- γ against IL-18/IL-2, the role of NK1.1 $^{+}$ γ δ T cells in IL-18/IL-2-induced ILD remains elusive.

The present study is an extension of our previous work, and seeks to analyze the pathogenesis of ILD, with a special focus on the NK1.1 $^{+}$ γ δ T cell subset. The results provide evidence for NK1.1 $^{+}$ γ δ T cell subset accumulation in the lungs of mice with IL-18/IL-2-induced ILD. NK1.1 $^{+}$ γ δ T cells produced IFN- γ and exacerbated ILD. Furthermore, IFN- $\gamma^{-/-}$ mice showed a resistance to IL-18/IL-2-induced ILD. In T Cell Receptor (TCR) $\delta^{-/-}$ mice, the severity of ILD, the production of IFN- γ , and the number of NK cells in the involved lungs were significantly low. Cell-transfer experiments demonstrated that NK1.1 $^{+}$ γ δ T cells could induce the expansion of NK cells in the lungs by IL-18/IL-2. These results suggest that NK1.1 $^{+}$ γ δ T cells accelerate ILD through the up-regulation of IFN- γ and NK-cell infiltration of the lung. Thus, NK1.1 $^{+}$ γ δ T cells may play a crucial role in the pathogenesis of IL-18/IL-2-induced ILD. We also discuss the functional role of NK1.1 $^{+}$ γ δ T cells in the generation of ILD.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) and IFN- γ -deficient (IFN- $\gamma^{-/-}$) mice with a C57BL/6 background were purchased from Charles River Japan, Inc. (Tokyo, Japan). TCR δ -deficient (TCR $\delta^{-/-}$) mice (22) with the C57BL/6 background were provided by Rikagakukenkyujo Bio Resource Center (RIKEN BRC), a participant in the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. Only female mice were used in this study. The animals were kept under specific pathogen-free conditions, and studied at age 4–5 weeks. In the present study, we used young mice (aged 4–5 weeks) for experiments because we observed more severe inflammation in the lungs of young mice than older mice (aged 8–9 weeks). The Committee on Institutional Animal Care and Use at Tsukuba University approved all experimental protocols.

Induction of ILD with IL-18 plus IL-2

Recombinant human IL-2 (rhIL-2) and recombinant mouse IL-18 (rmIL-18) were obtained from MBL (Nagoya, Japan). Mice were treated once a day for 3 days with an intraperitoneal injection of rhIL-2 (100,000 U) and rmIL-18 (1 μ g). In the present study, we used 100,000 U of rhIL-2, whereas Okamoto and colleagues used 50,000 U of rhIL-2 in the same experiments (10). We used the higher amount because we could not obtain satisfying results with 50,000 U of rhIL-2.

Sorting of NK1.1 $^{-}$ and NK1.1 $^{+}$ γ δ T Cells from the Lung, Spleen, and Liver

Lymphocytes from the lung, spleen, and liver were isolated with a TCR γ δ^{+} isolation kit (Miltenyi Biotec KK, Auburn, CA). Pre-enriched cells were stained with anti-NK1.1 and anti-TCR δ monoclonal antibodies (mAbs), and stained cells were sorted using FACS Vantage (Becton Dickinson, Franklin Lakes, NJ). The purity of γ δ T cells in this experiment was greater than 92% (NK1.1 $^{-}$ γ δ T cells) and greater than 93% (NK1.1 $^{+}$ γ δ T cells).

Cell Purification and Adoptive Transfer of NK1.1 $^{+}$ or NK1.1 $^{-}$ γ δ T cells

NK1.1 $^{+}$ or NK1.1 $^{-}$ γ δ T cells from splenocytes were harvested by the method already described. These splenic NK1.1 $^{+}$ or NK1.1 $^{-}$ γ δ T cells were washed in PBS and resuspended in PBS at 2×10^6 cell/ml. The suspended cells were transferred at 2×10^5 cells in a total volume of 100 μ l via the tail vein into TCR $\delta^{-/-}$ mice. At 24 hours after the adoptive transfer of NK1.1 $^{+}$ or NK1.1 $^{-}$ γ δ T cells, IL-18 plus IL-2 were injected and induced ILD.

Statistical Analysis

Data are expressed as median or mean $\pm$ SEM. Data were analyzed using the software package Stat View version 5.0 (SAS Institute, Cary, NC). Differences between groups were examined for statistical significance using the Student *t* test. For multiple group comparisons, one-way

ANOVA was performed, followed by the Dunnett test. $P < 0.05$ denoted a statistically significant difference.

Further experimental details, the RT-PCR for TCR-V γ and TCR-V δ profiling, the quantification of gene expression, staining and flow cytometry, intracellular cytokine staining, the measurement of γ δ T-cell responses to IL-18 plus IL-2 *in vitro*, and the measurement of cytokines in culture supernatant or lung tissues are described in the online supplement.

RESULTS

NK1.1 $^{+}$ γ δ T Cells Increased in the Lungs, Spleens, and Draining Lymph Nodes of Mice with IL-18 plus IL-2-Induced ILD

As reported previously, we were able to induce ILD with the administration of IL-18/IL-2, which was limited to the lungs, and did not involve other tissues, such as the lacrimal gland, salivary gland, liver, stomach, kidney, adrenal gland, small intestine, and large intestine. A large increase was evident not only in CD3 $^{-}$ NK1.1 $^{+}$ cells but also in CD3 $^{+}$ NK1.1 $^{+}$ cells in the lungs of mice with IL-18/IL-2-induced ILD (Figure 1A). We analyzed the TCR population of CD3 $^{+}$ NK1.1 $^{+}$ cells increased by the administration of IL-18/IL-2. Interestingly, NK1.1 $^{+}$ γ δ T cells were increased in the lungs, but NK1.1 $^{+}$ α β T cells were not (Figure 1B). Furthermore, we confirmed that most NK1.1 $^{-}$ and NK1.1 $^{+}$ γ δ T cells were CD4 $^{-}$ CD8 $^{-}$ dominant (Figure 1C). The number of NK1.1 $^{+}$ γ δ T cells among the pulmonary lymphocytes of IL-18/IL-2-treated mice was significantly higher than in control mice ($P < 0.001$; Figure 2A). In contrast, the number of NK1.1 $^{-}$ γ δ T cells was similar in both groups. In the spleens and draining lymph nodes (DLNs), the numbers of NK1.1 $^{+}$ γ δ T cells increased after the administration of IL-18/IL-2 (Figure 2B). However, the administration of IL-18/IL-2 did not increase the numbers of NK1.1 $^{+}$ γ δ T cells in the liver and intraepithelial lymphocytes (IELs).

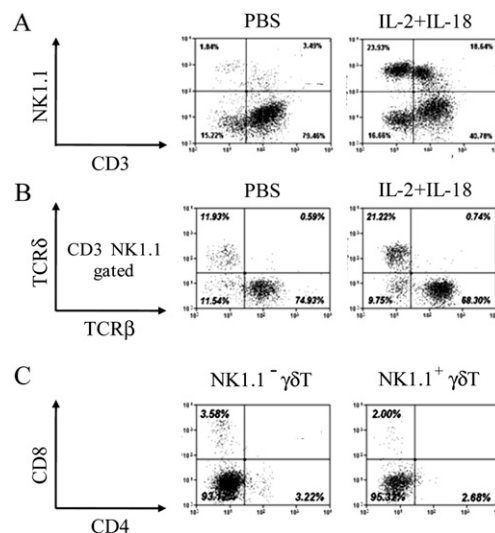


Figure 1. Accumulation of natural killer (NK) 1.1 $^{+}$ γ δ T cells in addition to NK cells in the lungs of mice with IL-18 plus IL-2-induced interstitial lung disease (ILD). (A) Pulmonary lymphocytes were harvested from B6 mice treated with PBS or IL-18/IL-2, as described in MATERIALS AND METHODS. Cells were stained with anti-NK1.1 and anti-CD3 ϵ monoclonal antibodies (mAbs). (B) Pulmonary lymphocytes from B6 mice treated with IL-18/IL-2 or PBS were stained with anti-NK1.1, anti-CD3 ϵ , anti-T Cell Receptor (TCR) β , and anti-TCR δ mAbs. (C) Pulmonary lymphocytes from B6 mice treated with IL-18/IL-2 were stained with anti-NK1.1, anti-CD3 ϵ , anti-TCR δ , anti-CD4, and anti-CD8 mAbs. Data are representative of more than three independent experiments.

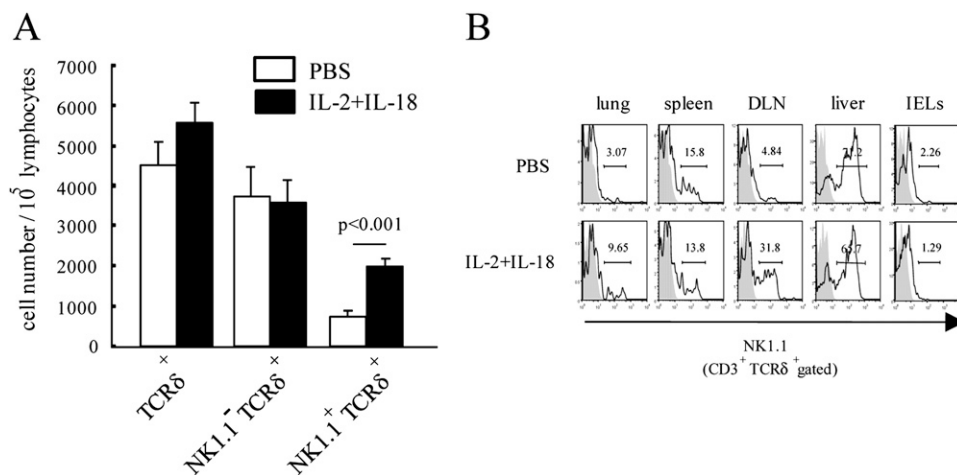


Figure 2. Increased proportion of NK1.1⁺ $\gamma\delta$ T cells in lungs, spleens, and draining lymph nodes (DLNs) of mice with IL-18 plus IL-2-induced ILD. (A) Pulmonary lymphocytes were harvested from B6 mice treated with IL-18/IL-2 or PBS, as described in MATERIALS AND METHODS. Cells were stained with anti-NK1.1, anti-CD3 ϵ , and anti-TCR δ mAbs. Data represent mean $\pm$ SEM. $P < 0.05$. (B) Lymphocytes included in lungs, spleens, DLNs, livers, and intraepithelial lymphocytes (IELs) were harvested from B6 mice treated with IL-18/IL-2 or PBS, as described in MATERIALS AND METHODS. Each group of lymphocytes was stained with anti-NK1.1, anti-CD3 ϵ , and anti-TCR δ mAbs and isotype-matched immunoglobulin, and used for FACS analysis. Data are representative of at least two independent experiments.

TCR V γ and V δ Repertoire of NK1.1⁺ $\gamma\delta$ T Cells Infiltrating the Lungs of IL-18 plus IL-2-Induced-ILD Mice

To determine the TCR repertoire of NK1.1⁻ and NK1.1⁺ $\gamma\delta$ T cells, we examined the RT-PCR analysis. NK1.1⁻ and NK1.1⁺ $\gamma\delta$ T cells in the lung, spleen, and liver were sorted from PBS-treated or IL-2/IL-18-treated mice. In PBS-treated mice, NK1.1⁺ $\gamma\delta$ T cells from each tissue contained a repertoire of V γ 1, 2, and 4, and of V δ 1–8 (Figure 3). Moreover, NK1.1⁻ $\gamma\delta$ T cells from each tissue contained a repertoire of V γ 1, 2, 4, 5, and 6, and of V δ 1–8 (Figure 3). Furthermore, in IL-18/IL-2-treated mice, NK1.1⁺ $\gamma\delta$ T cells from each tissue contained a repertoire of V γ 1, 2, and 4, and of V δ 1–8 (Figure 3). In addition, NK1.1⁻ $\gamma\delta$ T cells from each tissue contained a repertoire of V γ 1, 2, 4, 5, and 6, and V δ 1–8 (Figure 3). These results indicate that NK1.1⁺ and NK1.1⁻ $\gamma\delta$ T cells contained a polyclonal TCR repertoire.

NK1.1⁺ $\gamma\delta$ T Cells Secrete IFN- γ in the Presence of IL-18 plus IL-2

To determine whether NK1.1⁺ $\gamma\delta$ T cells can secrete various cytokines, cells were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin and stained for intracellular cytokines. The results indicated that NK1.1⁺ $\gamma\delta$ T cells can produce large amounts of IFN- γ and TNF- α (Figure 4A). Furthermore, the ability of NK1.1⁺ $\gamma\delta$ T cells to secrete IFN- γ , TNF- α , and IL-4 was significantly higher than that of NK1.1⁻ $\gamma\delta$ T cells (Figure 4B). Conversely, the ability of NK1.1⁺ $\gamma\delta$ T cells to secrete IL-17 was significantly lower than that of NK1.1⁻ $\gamma\delta$ T cells. Furthermore, we analyzed the production of cytokines from NK1.1⁺ and NK1.1⁻ $\gamma\delta$ T cells when stimulated with IL-18 and IL-2. NK1.1⁺ $\gamma\delta$ T cells secreted IFN- γ and TNF- α in the presence of IL-18/IL-2 (Figure 4C). IL-4 and IL-17 were not detected under each condition. Both NK1.1⁺ and NK1.1⁻ $\gamma\delta$ T cells secreted IFN- γ in the presence of IL-18/IL-2, and the ability of NK1.1⁺ $\gamma\delta$ T cells to secrete IFN- γ was significantly higher than that of NK1.1⁻ $\gamma\delta$ T cells ($P < 0.05$). However, no significant difference was evident in the secretion of TNF- α between the two types of cells.

Previously, IFN- γ was thought to play a crucial role in mice with IL-18/IL-2-induced ILD (10). However, the specific mechanisms were unknown. To clarify the role of IFN- γ in IL-18/IL-2-induced ILD, we examined IFN- γ ^{-/-} mice.

Histological examination indicated milder cell infiltration in the lungs of IFN- γ ^{-/-} mice compared with B6 mice (Figure

4D). Furthermore, pulmonary NK cells in IFN- γ ^{-/-} mice (10.7% $\pm$ 0.52%) were significantly reduced, compared with control mice (29.3% $\pm$ 3.79%, $P < 0.05$; Figure 4E).

NK1.1⁺ $\gamma\delta$ T Cells Express High Concentrations of IL-2 Receptor β and IL-18 Receptor β , and Proliferate in Response to Stimulation with IL-18 plus IL-2

To determine whether $\gamma\delta$ T cells react to IL-18 and IL-2, we analyzed the expression of IL-2 receptor β (IL-2R β) and IL-18 receptor β (IL-18R β) in $\gamma\delta$ T cells of splenocytes from B6 mice. As shown in Figure 5A, NK1.1⁺ $\gamma\delta$ T cells expressed more IL-2R β and IL-18R β than did NK1.1⁻ $\gamma\delta$ T cells. To examine the response of NK1.1⁺ and NK1.1⁻ $\gamma\delta$ T cells to IL-18/IL-2 *in vitro*, splenocytes from naive B6 mice were cocultured with IL-18/IL-2 for 96 hours. The reproduction rate of NK1.1⁺ $\gamma\delta$ T cells against IL-18/IL-2 was significantly higher than that of NK1.1⁻ $\gamma\delta$ T cells ($P < 0.05$; Figure 5B).

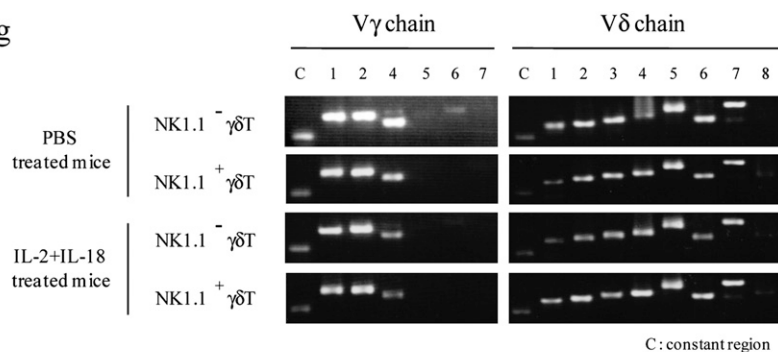
Next, the expression of NK1.1 in $\gamma\delta$ T cells was examined at 0, 24, 48, 72, and 96 hours (Figure 5C). Interestingly, 24 and 48 hours after coculturing with IL-18/IL-2, the expression of NK1.1 in $\gamma\delta$ T cells was significantly lower than in naive $\gamma\delta$ T cells (at 0 hour) ($P < 0.005$ and $P < 0.05$, respectively; Figure 5C). After 96 hours, the expression of NK1.1 in $\gamma\delta$ T cells was significantly higher than in naive $\gamma\delta$ T cells ($P < 0.01$). This biphasic response suggests that NK1.1 in $\gamma\delta$ T cells was first down-regulated by IL-18/IL-2, but showed subsequent recovery.

We also examined the expression of NK1.1 in $\gamma\delta$ T cells cocultured with PBS or IL-18/IL-2. No difference was evident between the expression of NK1.1 in NK1.1⁺ and NK1.1⁻ $\gamma\delta$ T cells after coculturing with IL-18/IL-2 (Figure 5D). These results indicate that NK1.1⁺ $\gamma\delta$ T cells should be completely different for cell populations from NK1.1⁻ $\gamma\delta$ T cells.

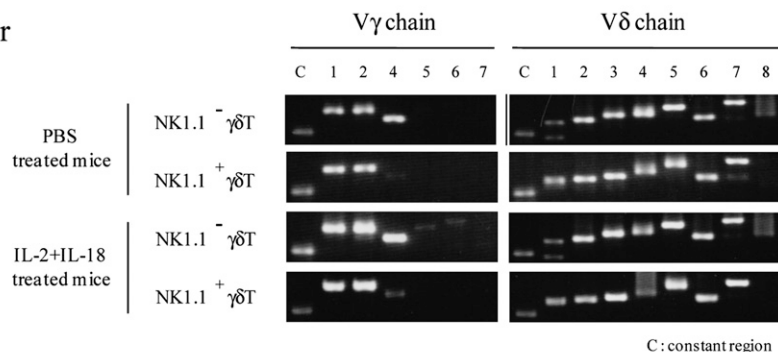
Reduction of Severity of IL-18 plus IL-2-Induced ILD and Number of NK Cells in the Lungs of TCR δ ^{-/-} Mice

To clarify the role of NK1.1⁺ $\gamma\delta$ T cells in IL-18/IL-2-induced ILD, we examined TCR δ ^{-/-} mice. Histological examination indicated milder cell infiltration in the lungs of TCR δ ^{-/-} mice compared with B6 mice (Figure 6A). Furthermore, flow cytometric analysis demonstrated a significant reduction in pulmonary NK cells in TCR δ ^{-/-} mice treated with IL-18/IL-2 (25.3% $\pm$ 0.92%), compared with control mice (38.3% $\pm$

Lung



Liver



Spleen

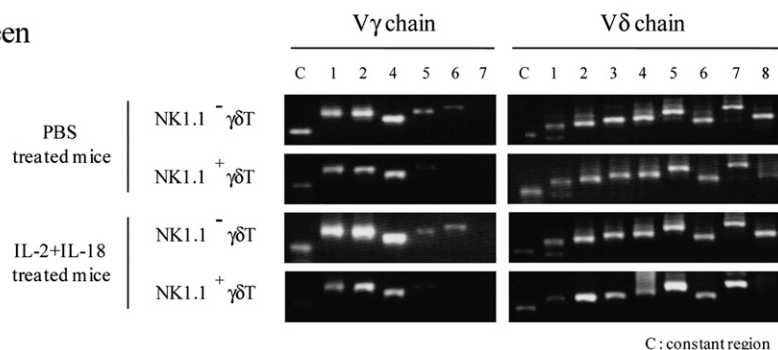


Figure 3. TCR-V γ and TCR-V δ repertoire of NK1.1⁺ $\gamma\delta$ T cells from mice with IL-18 plus IL-2-induced ILD. TCR-V γ and TCR-V δ gene expression in NK1.1⁻ and NK1.1⁺ $\gamma\delta$ T cells were purified from pulmonary lymphocytes, splenocytes, and liver lymphocytes of PBS-treated or IL-18/IL-2-treated mice, and were analyzed by RT-PCR, as described in MATERIALS AND METHODS. (C) Constant region. Data are representative of at least two independent experiments.

2.56%, $P < 0.05$; Figure 6B). The ratio of IFN- γ production in the lungs of TCR $\delta^{-/-}$ mice treated with IL-18/IL-2 (0.68 ± 0.080) was significantly lower compared with B6 mice (1.0 ± 0.047 , $P < 0.005$; Figure 6C).

NK1.1⁺ $\gamma\delta$ T Cells Accelerate the Severity of IL-18/IL-2-Induced ILD through the Number of NK Cells and the Expression of IFN- γ mRNA in the Lung

To examine the role of NK1.1⁺ $\gamma\delta$ T cells in IL-18/IL-2-induced ILD, we transferred NK1.1⁺ or NK1.1⁻ $\gamma\delta$ T cells to TCR $\delta^{-/-}$ mice, and then induced ILD (Figure 7A). The ratio of pulmonary NK cells was increased in TCR $\delta^{-/-}$ mice after the transfer of NK1.1⁺ $\gamma\delta$ T cells (0.81 ± 0.04), compared with NK1.1⁻ $\gamma\delta$ T cells (0.63 ± 0.02 , $P < 0.05$; Figure 7B). The expression of IFN- γ mRNA was increased in TCR $\delta^{-/-}$ mice with the transfer of NK1.1⁺ $\gamma\delta$ T cells (0.34 ± 0.03), compared with control TCR $\delta^{-/-}$ mice (0.14 ± 0.03 , $P < 0.005$; Figure 7C). These results suggest that NK1.1⁺ $\gamma\delta$ T cells affect the proliferation of NK cells, and increase IFN- γ mRNA expression in IL-18/IL-2-induced ILD.

DISCUSSION

In our IL-18/IL-2-induced ILD murine model, NK cells and IFN- γ play important roles in the generation of ILD (10). Here, we demonstrated an abundance of NK cells and NK1.1⁺ $\gamma\delta$ T cells, but not NK1.1⁻ $\gamma\delta$ T cells, in the lungs of mice with IL-18/IL-2-induced ILD. These findings suggest that not only NK but also NK1.1⁺ $\gamma\delta$ T cells are effector cells in the early phase of ILD.

The NK1.1 molecule is a specific marker for murine NK cells, and is expressed in NK cells and $\alpha\beta$ T cells. Most NK1.1⁺ $\alpha\beta$ T cells, termed NK T cells, use an invariant T-cell receptor (TCR) such as TCR V α 14 with various TCR V β genes. NK1.1⁺ $\alpha\beta$ T cells recognize glycolipid antigens presented by the non-polymorphic CD1d molecules (23, 24). Recently, Rosemary and colleagues (25) reported the presence of NK1.1⁺ $\gamma\delta$ T cells in naive thymus, liver, spleen, and bone marrow. The proportion of NK1.1⁺ $\gamma\delta$ T cells relative to $\gamma\delta$ T cells varied from 10–15% in the spleen, to greater than 50–70% in the liver. In the present study, NK1.1⁺ $\gamma\delta$ T cells formed 3–4% of the cells in naive lung and DLNs, and a few percent of the cells in IELs, the spleen, and the liver. Nishimura and colleagues (26) reported that the

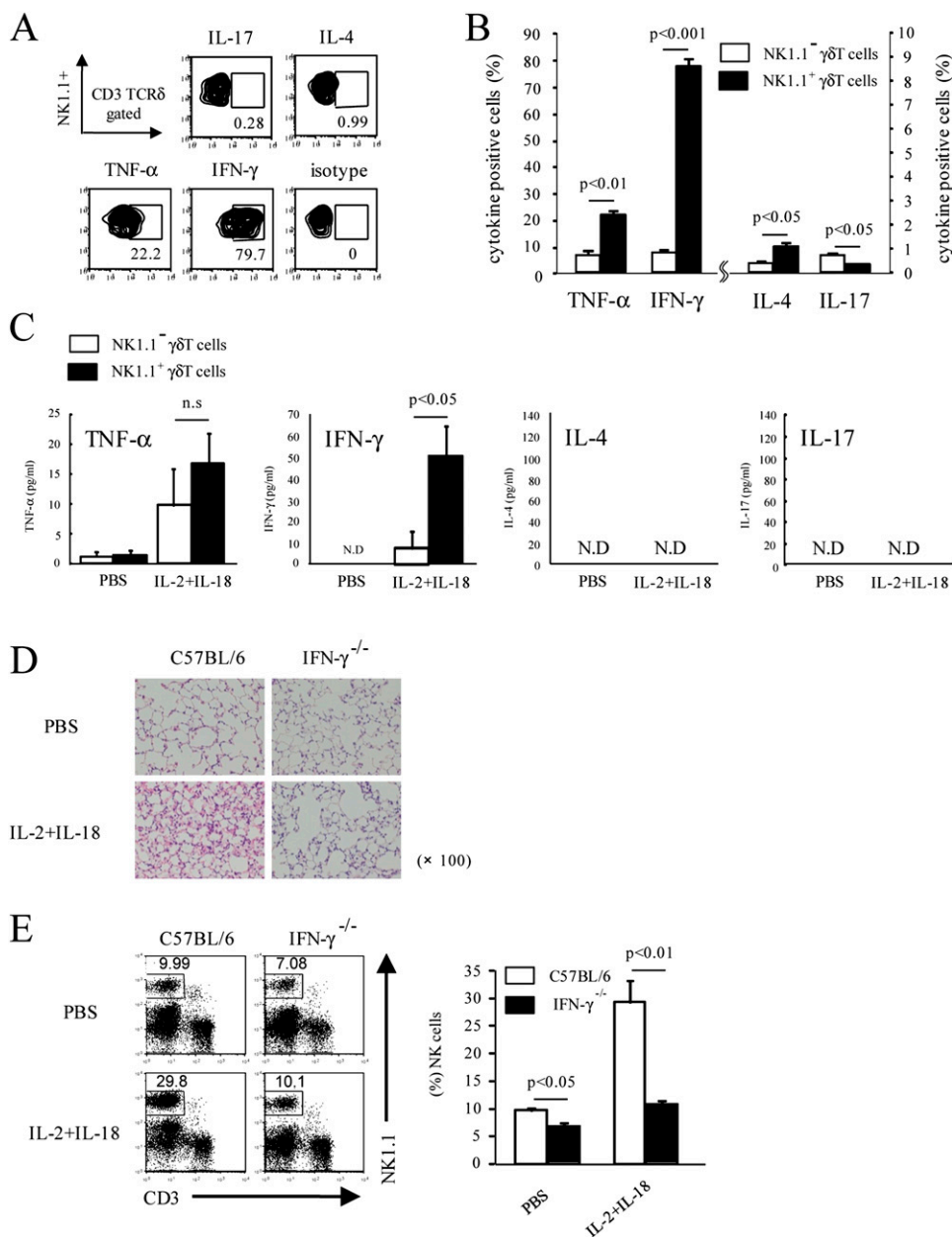


Figure 4. Cytokine profile of NK1.1⁻ $\gamma\delta$ T cells and NK1.1⁺ $\gamma\delta$ T cells. (A) Intracellular staining of TNF- α , IFN- γ , IL-4, and IL-17 was performed after the *in vitro* stimulation of splenocytes from naive B6 mice by phorbol 12-myristate 13-acetate (PMA)/ionomycin for 6 hours, and analyzed among gated CD3⁺ TCR δ ⁺ NK1.1⁺ cells by flow cytometry. Data are representative of at least two independent experiments. (B) Comparison of cytokine (TNF- α , IFN- γ , IL-4, and IL-17) secretion capacity by NK1.1⁺ and NK1.1⁻ $\gamma\delta$ T cells. (C) Sorted NK1.1⁻ $\gamma\delta$ T cells and NK1.1⁺ $\gamma\delta$ T cells from naive B6 mice were cocultured with PBS or IL-18/IL-2 for 96 hours, as described in MATERIALS AND METHODS. TNF- α , IFN- γ , IL-4, and IL-17 in the culture supernatant were measured using ELISA. Data represent mean $\pm$ SEM. $P < 0.05$. N.D., not detected. Data are representative of at least two independent experiments. (D) Lung tissues were harvested from B6 and IFN- γ ^{-/-} mice at 24 hours after treatment with IL-18/IL-2 for 3 days. Lung tissues were stained with hematoxylin and eosin. Original magnification, $\times 100$. (E) Lung tissues were harvested from B6 and IFN- γ ^{-/-} mice at 24 hours after treatment with IL-18/IL-2 for 3 days. Pulmonary lymphocytes were isolated as described in MATERIALS AND METHODS. Pulmonary lymphocytes were analyzed by flow cytometry. Data are representative of at least three independent experiments. Data are mean $\pm$ SEM. $P < 0.05$.

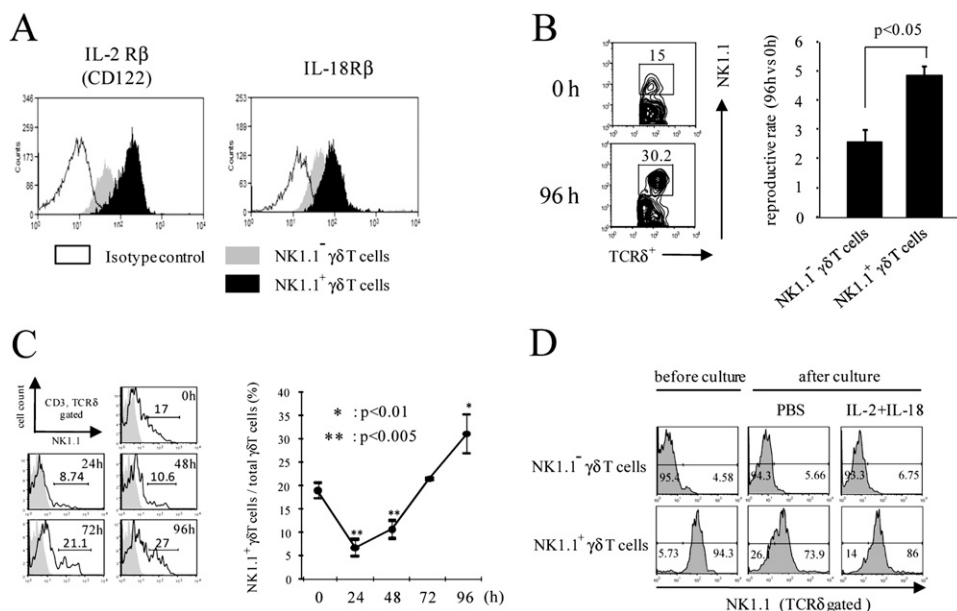
expression of major histocompatibility complex (MHC) class II is essential for the development and activation of NK1.1⁺ $\gamma\delta$ T cells in the thymus and periphery.

In the present study, histological analysis demonstrated that pulmonary inflammation in mice with IL-18/IL-2-induced ILD is similar to that in the early stages of human interstitial pneumonitis, suggesting that the mouse constitutes a suitable model for human ILD. The mechanism responsible for NK1.1⁺ $\gamma\delta$ T and NK cell accumulation in the lung may involve the high expression of IL-18R β and IL-2R β in NK1.1⁺ $\gamma\delta$ T cells compared with NK1.1⁻ $\gamma\delta$ T cells.

NK1.1⁺ $\gamma\delta$ T cells were reported to secrete several cytokines, including IL-4, IL-10, IL-13, and TNF- α (20, 27). We showed that NK1.1⁺ $\gamma\delta$ T cells were able to secrete large amounts of TNF- α , IFN- γ , and IL-4 after stimulation with PMA/ionomycin, compared with NK1.1⁻ $\gamma\delta$ T cells. However, under conditions where IL-18/IL-2 was present, NK1.1⁺ $\gamma\delta$ T cells were able to secrete large amount of IFN- γ compared with NK1.1⁻ $\gamma\delta$ T cells, but not TNF- α and IL-4. Haas and colleagues (20) showed that NK1.1⁻ $\gamma\delta$ T cells

could produce IL-17. In the present study, we also showed that NK1.1⁻ $\gamma\delta$ T cells could produce more IL-17 compared with NK1.1⁺ $\gamma\delta$ T cells via stimulation with PMA/ionomycin. However, when the stimulus involved IL-18/IL-2, we could not detect IL-17-producing $\gamma\delta$ T (NK1.1⁻ and NK1.1⁺) cells. Similar results were obtained with IL-4. These differences were thought to be attributable to the biological properties of IL-18. The combination of IL-18 and IL-2 is known for strongly inducing the production of IFN- γ by NK and $\gamma\delta$ T cells (18). Indeed, IFN- γ ^{-/-} mice showed a significant amelioration of IL-18/IL-2-induced ILD. Thus, NK and NK1.1⁺ $\gamma\delta$ T cells with their high potential for producing IFN- γ may act as inflammatory mediators for ILD in IL-18/IL-2-induced ILD mice. In addition, experiments using TCR δ ^{-/-} mice clearly showed a significant amelioration of IL-18/IL-2-induced ILD in comparison with B6 mice. These results indicate that NK1.1⁺ $\gamma\delta$ T cells and NK cells may play a crucial role in the generation of IL-18/IL-2-mediated ILD.

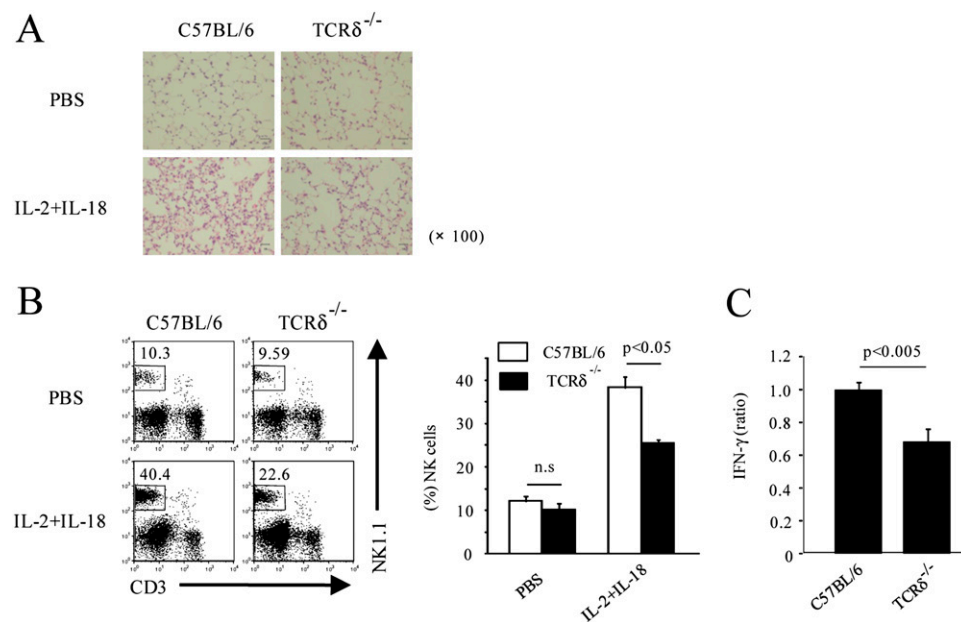
Carding and Egan (28) reported that the majority of $\gamma\delta$ T cells used TCR γ 1, 4, 5, 6, and 7 genes. TCR V γ 1⁺ and TCR



mean $\pm$ SEM; $P < 0.05$. (C) Splenocytes from C57BL/6 mice were cocultured with IL-18/IL-2 for 0, 24, 48, 72, and 96 hours. (Left) The expression of NK1.1 in γδT cells in each group of cultured cells was determined by flow cytometry. Representative flow cytometry demonstrates the expression of NK1.1 in cultured γδT cells with IL-18/IL-2 for 0, 24, 48, 72, and 96 hours. (Right) Proportion of NK1.1⁺ γδT cells among total γδT cells at 0, 24, 48, 72, and 96 hours of culture conditions. Data represent mean $\pm$ SEM; $P < 0.05$. Each group of cultured cells was stained with anti-CD3ε, anti-TCRδ, and anti-NK1.1 mAbs. Data are representative of at least two independent experiments. $*P < 0.01$. $**P < 0.005$. (D) Splenocytes from C57BL/6 mice were cocultured with IL-18/IL-2 for 0, 24, 48, 72, and 96 hours. Culture cells were analyzed by flow cytometry. Data are representative of at least two independent experiments. (E) Sorted NK1.1⁻ γδT cells and NK1.1⁺ γδT cells were cocultured with PBS or IL-18/IL-2 for 96 hours, as described in MATERIALS AND METHODS. At 96 hours after coculturing with PBS or IL-18/IL-2, the expression of NK1.1 in γδT cells was analyzed by flow cytometry. Data are representative of at least two independent experiments.

Vγ4⁺ γδT cells are detected in the blood, lung, liver, spleen, and lymph nodes, whereas TCR Vγ5⁺ γδT cells are present in the skin, TCR Vγ6⁺ γδT cells are present in the lung, tongue, and uterus, and TCR Vγ7⁺ γδT cell are present in IELs. Interestingly, TCR Vγ1⁺ and TCR Vγ7⁺ γδT cells produce predom-

inantly Th1 cytokines, although TCR Vγ4⁺ γδT cells secrete Th1 cytokines (29). Our results showed that NK1.1⁺ γδT cells as well as NK1.1⁻ γδT cells infiltrating the lungs of mice with IL-18/IL-2-induced ILD contained all TCR Vγ (Vγ 1, 2, 4, 5, 6, and 7) and TCR Vδ (Vδ 1–8) repertoires, suggesting polyclonal



production in the lungs of B6 and TCRδ^{-/-} mice were calculated relative to those of B6 mice. IFN-γ production in the lungs of B6 mice was assumed to be 1.0. Data are representative of at least three independent experiments, and graph shows the pooled data of three experiments. Data are mean $\pm$ SEM. $P < 0.05$.

Figure 6. Amelioration of IL-18 plus IL-2-induced ILD, and reduced number of pulmonary NK cells and production of IFN-γ, in TCRδ^{-/-} mice. (A) Lung tissues were harvested from B6 and TCRδ^{-/-} mice at 6 hours after treatment with IL-18/IL-2 for 4 days. Lung tissues were stained with hematoxylin and eosin. Original magnification, $\times 100$. (B) Lung tissues were harvested from B6 and TCRδ^{-/-} mice at 6 hours after treatment with IL-18/IL-2 for 4 days. Pulmonary lymphocytes were isolated as described in MATERIALS AND METHODS. Pulmonary lymphocytes were analyzed by flow cytometry. Data are representative of at least three independent experiments, and graph shows the pooled data of three experiments. Data represent mean $\pm$ SEM; $P < 0.05$. (C) Lung tissues were harvested from B6 and TCRδ^{-/-} mice at 6 hours after injection with IL-18/IL-2 for 4 days. IFN-γ in lung supernatants was measured by ELISA. The ratios of IFN-γ

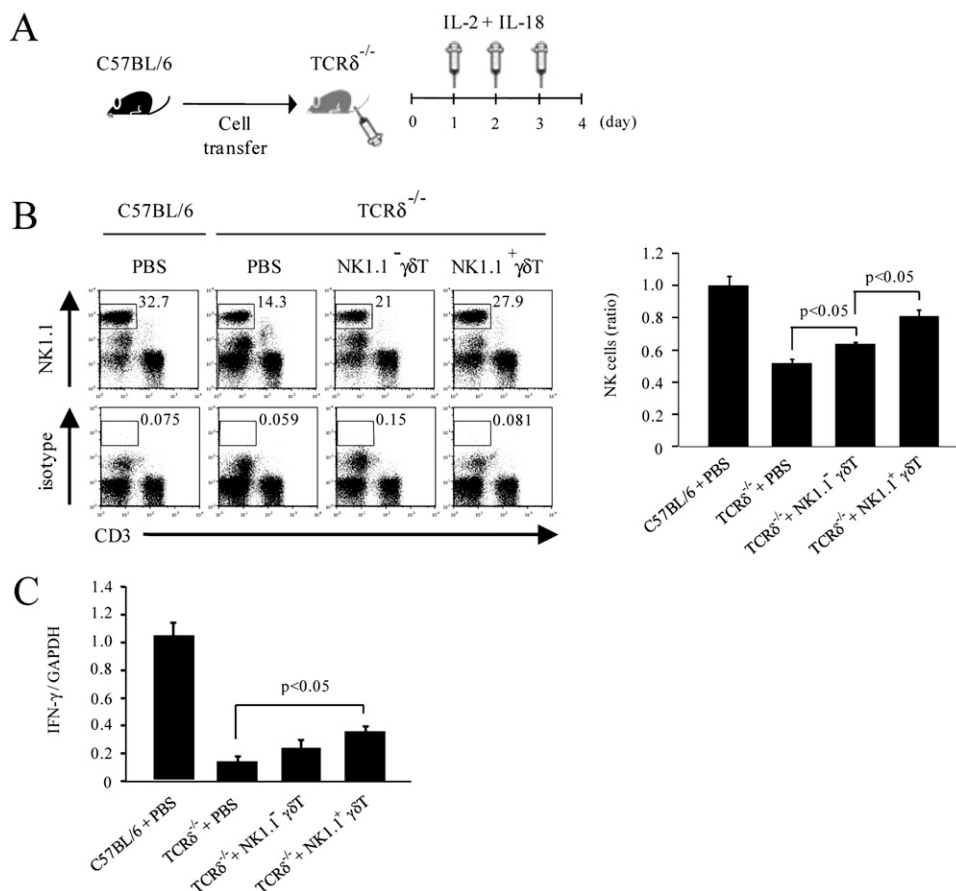


Figure 7. NK1.1⁺ $\gamma\delta$ T cells accelerate the severity of IL-18 plus IL-2-induced ILD. (A) Naive B6 mice were harvested, and NK1.1⁻ $\gamma\delta$ T and NK1.1⁺ $\gamma\delta$ T cells were purified from splenocytes, as described in MATERIALS AND METHODS. These cells were transferred into TCRδ^{-/-} mice (2×10^5 /mouse). At 24 hours after the transfer, TCRδ^{-/-} recipient mice were treated with IL-18/IL-2 for 3 days. At 24 hours after the last injection, lung tissues were analyzed. (B) Three B6 mice received transfers with PBS, and three TCRδ^{-/-} mice received transfers with PBS, NK1.1⁻ $\gamma\delta$ T, or NK1.1⁺ $\gamma\delta$ T cells. At 24 hours after cell transfer, these mice were treated with IL-18/IL-2 for 3 days. Pulmonary lymphocytes were isolated as described in MATERIALS AND METHODS. Pulmonary lymphocytes were analyzed by flow cytometry. Data are shown as a ratio of NK cells compared with those in control mice. The value of control mice is indicated by the mean of three independent experiments as 1.0. Other values represent mean $\pm$ SEM of three independent mice, using the average value of the control mouse. The values as mean $\pm$ SEM for B6 mice + PBS were 1 ± 0.061 ; for TCRδ^{-/-} mice + PBS, 0.514 ± 0.030 ; for TCRδ^{-/-} mice + NK1.1⁻ $\gamma\delta$ T cells, 0.636 ± 0.015 ; and for TCRδ^{-/-} mice + NK1.1⁺ $\gamma\delta$ T cells, 0.809 ± 0.043 ; $P < 0.05$. (C) Three B6

mice received transfers with PBS, and three TCRδ^{-/-} mice received transfers with PBS, NK1.1⁺, or NK1.1⁻ $\gamma\delta$ T cells. At 24 hours after cell transfer, these mice were treated with IL-18/IL-2 for 3 days. Lung mRNA was extracted, and the expression of IFN- γ mRNA was analyzed by RT-PCR. Data are shown as a ratio of IFN- γ -producing cells, compared with those in control mice. The value of control mice was shown by the mean of three independent experiments as 1.0. Other values represent the mean $\pm$ SEM of three independent mice, using the average of the control mouse. Values as mean $\pm$ SEM for B6 mice + PBS were 1 ± 0.094 ; for TCRδ^{-/-} mice + PBS, 0.132 ± 0.041 ; for TCRδ^{-/-} mice + NK1.1⁻ $\gamma\delta$ T cells, 0.219 ± 0.059 ; and for TCRδ^{-/-} mice + NK1.1⁺ $\gamma\delta$ T cells, 0.327 ± 0.048 ; $P < 0.05$.

$\gamma\delta$ T cell expansion in the lung. These results indicate that NK1.1⁺ $\gamma\delta$ T cells may recognize a wide variety of antigens, and thus are unlikely invariant NK T cells. However, whether NK1.1⁺ $\gamma\delta$ T cells recognize any antigens in IL-18/IL-2-induced ILD remains unknown.

Crowe and colleagues (29) reported that the expression of NK1.1 in TCR $\alpha\beta$ NK T cells was down-regulated by stimulation with TCR within 24 hours, and it returned to the naive level within 6 days *in vivo*. In our study, we examined the expression level of the NK1.1 molecule in NK1.1⁺ $\gamma\delta$ T cells after stimulation with IL-18/IL-2. The expression of NK1.1 in $\gamma\delta$ T cells was also down-regulated within 24 hours and 48 hours, but it increased to the 1.5 times level at 96 hours, similar to TCR $\alpha\beta$ NK T cells. Moreover, NK1.1⁻ $\gamma\delta$ T cells did not express the NK1.1 molecule, even after culturing with IL-18/IL-2 for 96 hours, indicating that NK1.1⁺ $\gamma\delta$ T cells may have different cell populations than NK1.1⁺ $\gamma\delta$ T cells. Further examination will be necessary to clarify whether NK1.1⁺ $\gamma\delta$ T cells develop from immature NK1.1⁻ $\gamma\delta$ T cells or NK1.1⁺ $\gamma\delta$ T cells.

NK cells and IFN- γ are believed to play important roles in the pathogenesis of IL-18/IL-2-induced ILD mice. Label and colleagues (30) and Gardner and colleagues (31) emphasized the role of $\gamma\delta$ T cells in the regulation of NK cell functions such as proliferation and cytokine expression. We showed that the infiltration of NK cells in the lung after injection with IL-18/

IL-2 was significantly lower in TCRδ^{-/-} mice than in B6 mice. Lung tissue staining clearly showed that cell infiltration was inhibited in TCRδ^{-/-} mice compared with B6 mice. Although the rate of pulmonary NK cells was low, the histological result showed a clear difference between TCRδ^{-/-} and B6 mice. The histological findings may be attributable to effects not only by NK cells but also by $\gamma\delta$ T cells. The results demonstrated that $\gamma\delta$ T cells participated in the proliferation of NK cells after injection with IL-18/IL-2. Furthermore, the production of IFN- γ in lung tissue from TCRδ^{-/-} mice treated with IL-18/IL-2 was significantly lower compared with that in B6 mice. In addition, we observed evidence that the number of pulmonary NK cells was significantly increased and the expression of IFN- γ mRNA tended to be higher via the adoptive cell transfer of NK1.1⁺ $\gamma\delta$ T cells into TCRδ^{-/-} mice compared with NK1.1⁻ $\gamma\delta$ T cells. Therefore, these findings in TCRδ^{-/-} mice support the notion that NK1.1⁺ $\gamma\delta$ T cells may enhance the accumulation of NK cells in the lung in IL-18/IL-2-induced ILD.

The CD161 molecule in humans is homologous with murine NK1.1. Previous studies reported the presence of CD161⁺ $\gamma\delta$ T cells in peripheral blood mononuclear cells in patients with HIV (32) and multiple sclerosis (33). However, no reports on CD161⁺ $\gamma\delta$ T cells in patients with interstitial pneumonitis are available. Thus, further experiments on human $\gamma\delta$ T cells in lungs from patients with ILD are necessary for a better understanding of the pathogenesis of ILD.

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