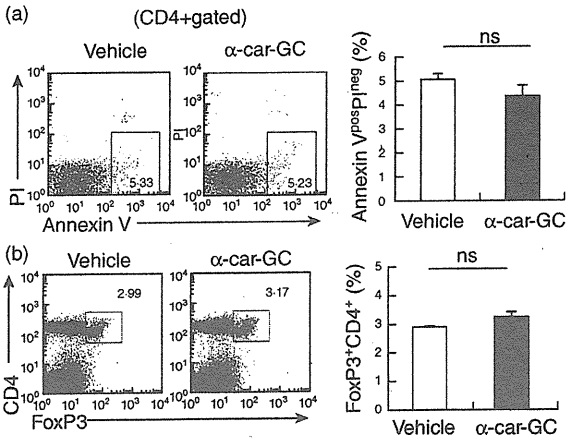

Fig. 5. α-carba-GalCer does not alter the number of forkhead box P3 (FoxP3)$^+$ regulatory T cells (T_{regs}) or apoptotic T cells. DBA/1 mice were immunized with type II collagen (CII) in complete Freund's adjuvant (CFA) and 2 μg of α-carba-GalCer ($n = 3$) or vehicle ($n = 3$). Twelve days after CII/glycolipid immunization, draining lymph node (DLN) cells were collected. The frequency of (a) apoptotic T cells [annexin V-positive and propidium iodide (PI)-negative cells represented early apoptotic cells, while annexin V-positive and PI-positive cells represented late apoptotic or necrotic cells] and (b) T_{regs} (FoxP3$^+$CD4$^+$) and in DLN cells was determined by fluorescence activated cell sorter. Data are representative of at least two experiments. Values represent mean ± standard error of the mean of three mice per group (n.s.: not significant).

upper panel). The smaller proportion of IL-17-producing cells was observed in both the CD4$^+$ and CD4$^-$ (DN, CD8$^+$) T cell population (Fig. 6a,b, middle and lower panels). In contrast, the proportion of IFN-γ-producing T cells was larger in α-carba-GalCer-treated mice (Fig. 6a,b, upper panel). The larger proportion of IFN-γ-producing T cells was observed only in the CD4$^-$ (DN, CD8$^+$) T cell population (Fig. 6a,b, lower panel). The proportion of IL-17-producing T cells was also lower in α-GalCer-treated mice, but the effect was smaller than that in α-carba-GalCer-treated mice. In addition, the ratio of IFN-γ-/IL-17-producing T cells was significantly higher in α-carba-GalCer-treated mice (Fig. 6c). The Th1 polarization was evident in both the CD4$^+$ T cell and CD4$^-$ T cell populations (Fig. 6d,e). These findings suggest that treatment with α-carba-GalCer polarizes the systemic T cell response towards Th1 and suppresses Th17 cell differentiation or activation.

α-carba-GC-treatment attenuates IL-6 and IL-23 gene expression in the initial phase of CIA

It has been suggested that TGF-β and IL-6 induce Th17 differentiation and that IL-23 is required for expansion and maintenance of Th17 cells. Thus, we determined whether this Th17-related gene expression occurs in CII/α-carba-GalCer-immunized mice. RNA was purified from DLN cells 3 days and 10 days after immunization and the expression of Th17-related cytokine transcripts was determined by quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis. At day 3, the expression of IL-6 and IL-23p19 transcripts was significantly more reduced in mice treated with α-carba-GalCer than in mice treated with vehicle, while the expression of TGF-β transcripts in mice treated with α-carba-GalCer was comparable to that in control mice (Fig. 7a). α-GalCer treatment had no effect on these gene expressions at day 3 (Fig. 7a). In addition, all the Th17-related gene expressions were not significantly different among vehicle-, α-GalCer- and α-carba-GalCer-treated mice at day 10 (Fig. 7b). These results suggested that α-carba-GalCer suppresses IL-6 and IL-23p19 expression in the initial phase of CIA.

α-carba-GalCer treatment enhanced NK T cell activation and IFN-γ production in the initial phase of CIA

To analyse the activation state of NK T cells in the initial phase of CIA, we determined the frequency and cytokine production of NK T cells in the liver, spleen and DLN 3 days after CII/glycolipid immunization. The frequency of NK T cells in α-carba-GalCer-treated mice was comparable to that in the vehicle-treated mice (Fig. 8a). It has been reported that TCR down-modulation of NK T cells was observed when NK T cells were activated with glycolipid [35]. Notably, in the current study, a lower expression of CD1d-tetramers that bind to the invariant TCR of NK T cells was observed in α-carba-GalCer-treated mice, suggesting that NK T cells were activated at this time (Fig. 8a). The TCR down-modulation was also observed in α-GalCer-treated mice, but only partially (Fig. 8a). Moreover, intracellular cytokine staining showed that IFN-γ production by liver, splenic and DLN NK T cells in α-carba-GalCer-treated mice was higher than that in vehicle-treated mice (Fig. 8b,c). The α-GalCer teatment also induced higher IFN-γ production by NK T cells, but was lower than α-carba-GalCer treatment (Fig. 8b,c). Although we observed abundant IL-17-producing NK T cells in the peripheral lymph nodes but not in the liver and spleen, as reported previously [36], α-carba-GalCer treatment had no effect on IL-17 production by NK T cells in the DLN (Fig. 8b). These findings suggest that α-carba-GalCer treatment enhanced the activation and IFN-γ production of NK T cells in the initial phase of CIA. Thus, α-carba-GalCer treatment could regulate Th17-mediated autoimmune diseases negatively through NK T cell-derived IFN-γ in the initial phase of CIA.

Discussion

NK T cells are unconventional T cells that recognize glycolipid antigens and secrete several types of proinflammatory and anti-inflammatory cytokines [15,16,29]. Although

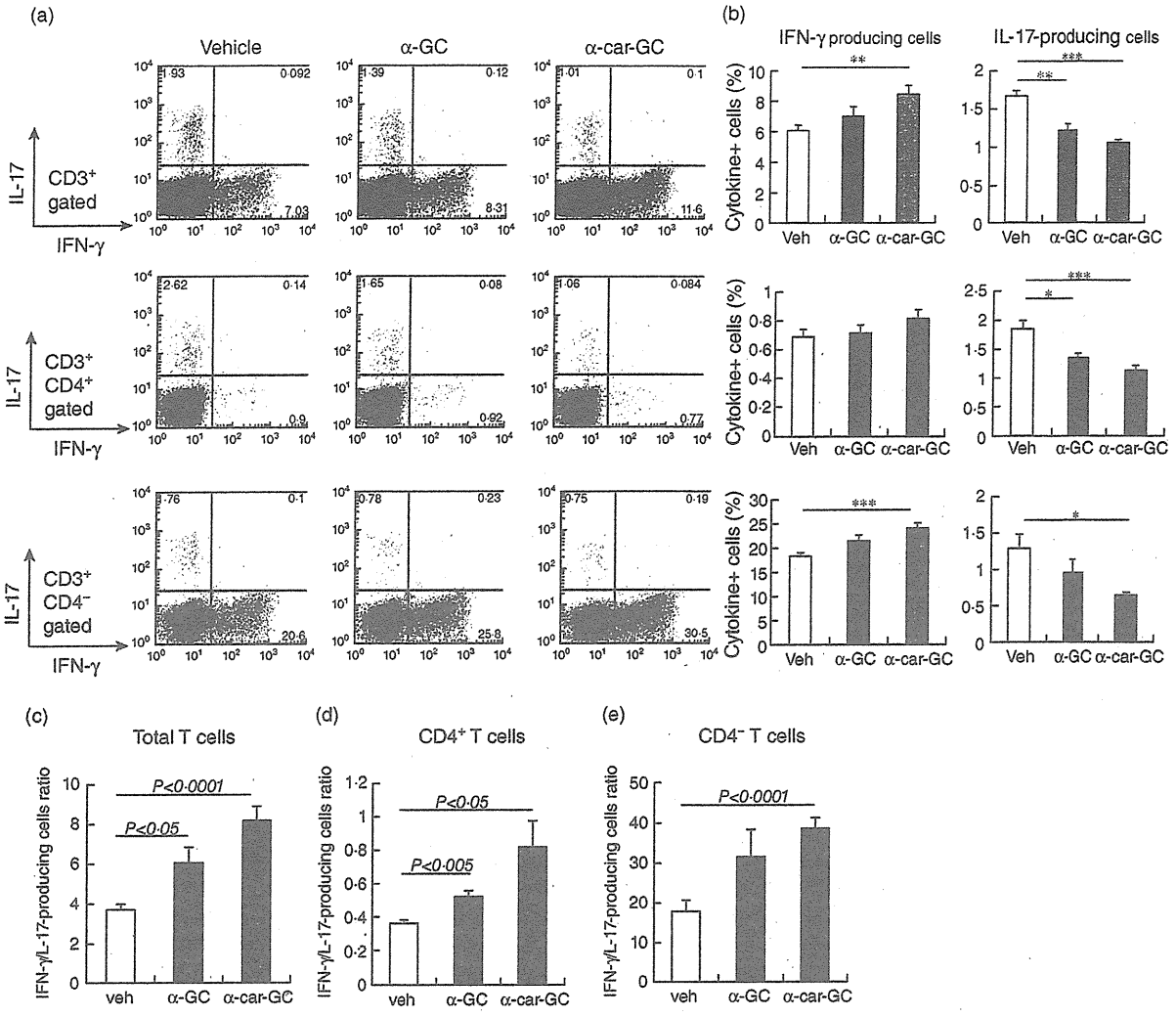

Fig. 6. α-carba-GalCer treatment polarizes T cell response towards T helper type 1 (Th1) and suppresses Th17 cell activation or differentiation. Ten days after type II collagen (CII) and vehicle, α-galactosylceramide (α-GalCer) or α-carba-GalCer immunization, draining lymph node (DLN) cells were stimulated with phorbol myristate acetate/ionomycin for 6 h and intracellular cytokine [interferon (IFN)-γ, interleukin (IL)-17] concentrations were measured by flow cytometry. (a) Representative flow cytometry demonstrating IFN-γ and IL-17 expression in total T cells ($CD3^+$ population: top panel), $CD4^+$T cells ($CD3^+CD4^+$ population: middle panel), and $CD4^-$ T cells ($CD3^+CD4^-$ population: bottom panel) of DLN cells from each group. (b) Proportion of IFN-γ- and IL-17-producing cells among total T cells (top), $CD4^+$ T cells (middle) and $CD4^-$ T cells (bottom) in each group. (c) The ratio of IFN-γ-producing cells (%) to IL-17-producing cells (%) in (c) total T cells (d) $CD4^+$ T cells and (e) $CD4^-$ T cells in each group. Data represent mean ± standard error of the mean of nine mice per group from three independent experiments ($n = 3$ mice per experiment).*$P < 0{\cdot}01$; **$P < 0{\cdot}005$; ***$P < 0{\cdot}0001$.

endogenous ligands for NK T cells have not yet been identified, several synthetic ligands have been used in immunotherapy for cancer and autoimmune disease models [16]. The present study demonstrated that treatment with α-carba-GalCer, a novel synthetic NK T cell ligand, suppressed the development of CIA and that this effect was mediated by IFN-γ.

In the present study, we first showed the biological function of α-carba-GalCer in CIA-susceptible DBA/1 mice, because several reports suggested that there were some differences in NK T cell response among mouse strains [22]. We demonstrated that i.v. injection of α-carba-GalCer selectively induced serum Th1 cytokines in DBA/1 mice, similar to a previous report on C57BL6 mice, although the concentration of cytokines in DBA/1 mice was lower than that in C57BL/6 mice (data not shown). Therefore, we concluded that α-carba-GalCer could be used as a Th1-type glycolipid ligand for therapy of CIA in DBA/1 mice.

Interestingly, s.c. injection, unlike i.v. injection, of α-GalCer or α-carba-GalCer with CFA did not lead to

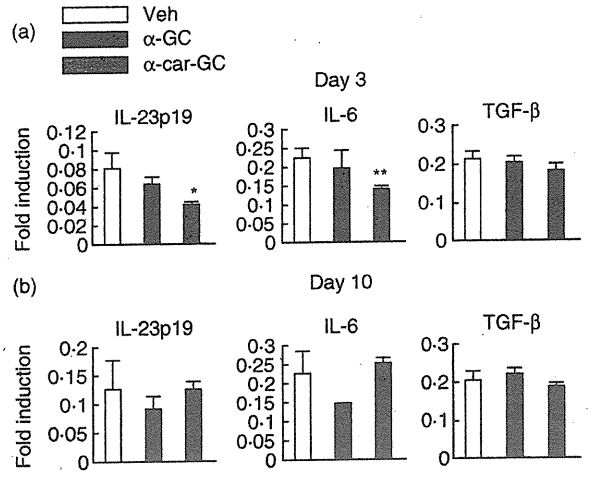

Fig. 7. α-carba-GalCer-treatment attenuates T helper type 17 (Th17)-related gene expression in the initial phase of collagen-induced arthritis (CIA). RNA was purified from draining lymph node (DLN) cells of each mouse (a) 3 days and (b) 10 days after glycolipid and type II collagen (CII) co-immunization. The levels of gene expression were evaluated by quantitative reverse transcription–polymerase chain reaction for the indicated cytokines. Results are reported as group means ± standard deviation, with $n = 3$ for each group. Data are representative of two independent experiments. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

detectable IFN-γ in serum (data not shown), although intracellular staining for IFN-γ showed clearly that α-carba-GalCer induced strong IFN-γ production by NK T cells. We believe that the activation profile of NK T cell *in vivo* is dependent on the route of administration of NK T cell ligand. It is well known that i.v. (or i.p.) injection of α-GalCer or its analogues rapidly induce several cytokines in serum and TCR down-modulation on NK T cells within 24 h after stimulation. TCR expression levels recover gradually and NK T cells then proliferate rapidly and the expansion of NK T cells peaks at about 72 h after stimulation. In contrast, our data of s.c. injection of NK T cell ligand show that NK T cell expansion is not so strong compared with that in i.v. injection reported previously, and TCR down-modulation is still observed at 72 h after stimulation in addition to the loss of induction of serum IFN-γ. Based on these observations, we speculate that the effect of s.c. injection of glycolipid might be weaker, but it sustained longer than that of i.v. injection. It might also be possible that emulsifying with adjuvant (CFA) made glycolipid remain longer in the injected site.

IFN-γ is an important proinflammatory cytokine in infection and tumour rejection. Conversely, IFN-γ also exhibits anti-inflammatory properties in autoimmune diseases, acting as an inducer of apoptosis and T_{regs} [12–14]. In our study, neither apoptotic T cells nor FoxP3$^+$ T_{regs} were identified in α-carba-GalCer-treated mice. These results indicate that apoptosis and FoxP3$^+$ T_{regs} did not mediate the suppressive effect of α-carba-GalCer on CIA.

The results also showed an increased proportion of IFN-γ-producing T cells and a decreased proportion of IL-17-producing T cells in α-carba-GalCer-treated mice, suggesting that this NK T ligand polarizes the Th1/Th17 cytokine balance to Th1. Chu *et al.* [11] reported that IFN-γ suppressed Th17 cell differentiation and IL-17 production in CIA. IFN-γ maintains IFN-γ-producing Th1 cells by themselves and induces the production of IL-12, another cytokine important for Th1 differentiation from dendritic cells [37,38]. Much evidence suggests that TGF-β and IL-6 induce Th17 differentiation and that IL-23 is required for expansion and maintenance of Th17 cells. In this study, we showed that treatment with α-carba-GalCer attenuated IL-6 and IL-23 expression in the initial phase of CIA. Recently, Chu *et al.* [11] observed that IFN-γ deficiency leads to increased IL-6 production in CIA, indicating that IFN-γ regulates IL-6 production negatively. In addition, Sheikh *et al.* [39] suggested that IFN-γ is a negative regulator of IL-23 in murine macrophages. Thus, we speculate that IFN-γ induced by the treatment with α-carba-GalCer suppressed IL-6 and IL-23 production in the initial phase of CIA and that the reduction of these Th17-related cytokines leads to the amelioration of Th17 cell activation and expansion.

Our results showed an increased population of IFN-γ-producing CD4$^-$ T cells (non-Th cells) in α-carba-GalCer-treated mice, indicating that CD4$^-$ NK T cells, CD8$^+$T cells or other CD4$^-$ T cells are associated with this Th1 polarization. These data support the results of a previous study showing that NK T cells can induce bystander T cell activation [40]. Thus, we believe that α-carba-GalCer treatment polarizes the systemic T cell response, including CII-reactive and CII non-reactive T cells, to the Th1-type response. We confirmed that IL-17-producing CII-reactive T cells were reduced in α-carba-GalCer-treated mice, although IFN-γ production was not significantly different between the α-carba-GalCer-treated mice and the control mice. These results also suggest that α-carba-GalCer can alter the Th1/Th17 balance to Th1 in the CII-reactive T cell response.

It has been reported that anti-CII antibodies are required for the development of CIA [41,42], and that Th1 and Th2 cells are involved in class-switching to IgG2a and IgG1. In fact, IL-4 directs murine IgE and IgG1 production, whereas IFN-γ stimulates selectively the production of IgG2a as well as that of IgG3 under certain circumstances [43,44]. Other studies have reported the association of IL-17 with IgG production in animals with autoimmune disease and the presence of low levels of anti-CII IgG2a antibodies in IL-17-deficient mice [8,45]. In our study, anti-CII IgG2a antibodies but not IgG1 were reduced significantly in α-carba-GalCer-treated mice, implying that the reduction in anti-CII IgG2a antibody in these mice could be due to the decreased number of CII-reactive Th17 cells.

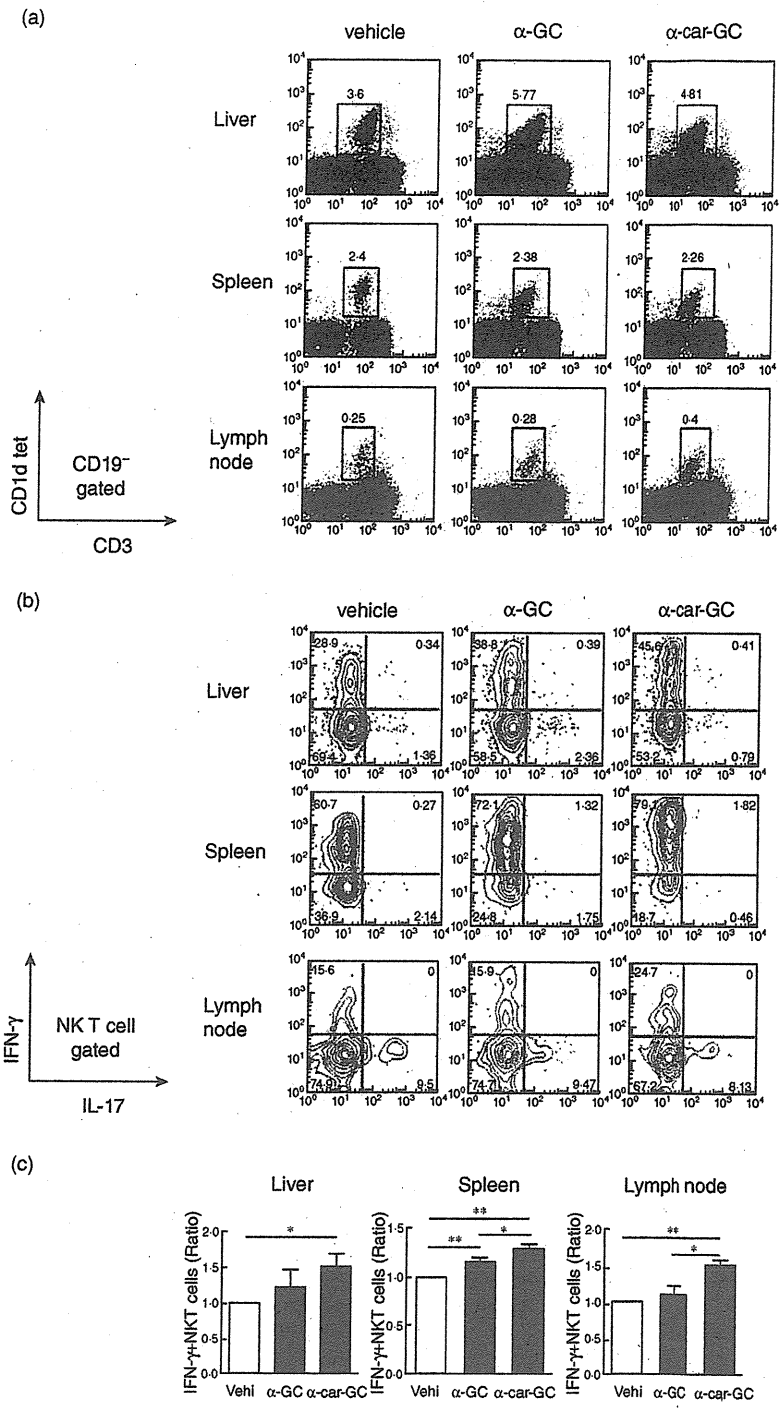

Fig. 8. α-carba-GalCer treatment enhanced natural killer (NK) T cell activation and interferon (IFN)-γ production in the initial phase of collagen-induced arthritis (CIA). Three days after type II collagen (CII) and vehicle, α-galactosylceramide (α-GalCer) or α-carba-GalCer immunization, liver, spleen and draining lymph node (DLN) cells were collected. (a) Numbers in the dot plots indicate the percentage of natural killer (NK) T cells ($CD19^-CD3^+CD1dtetramer^+$ population) from each group. (b) The cells were stimulated with phorbol myristate acetate/ionomycin for 4 h, and intracellular cytokine [IFN-γ, interleukin (IL)-17] concentrations measured by flow cytometry. Flow cytometry results demonstrated IFN-γ and IL-17 expression in NK T cells ($CD19^-CD1dtetramer^+$ population) from each group. (c) The ratio of IFN-γ positive NK T cells in α-GalCer- or α-carba-GalCer-treated mice to that in vehicle-treated mice. Data are representative of two experiments ($n = 3$ per experiment). Values represent mean ± standard deviation.*$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

It has been reported that mice deficient in IFN-γ exhibit severe CIA symptoms, suggesting that IFN- γ plays a role as a suppressor cytokine [11]. In the current study, as expected, IFN-γ neutralization abolished the beneficial effect of α-carba-GalCer in CIA. Thus, we concluded that α-carba-GalCer suppressed CIA in an IFN-γ-dependent manner. However, in the α-carba-GalCer-untreated condition, IFN-γ neutralization seemed to ameliorate the symptoms of CIA compared to isotype Ig treatment (Fig. 2c). Although it is difficult to explain the discrepancy between these results and those of the previous study of IFN-γ deficient mice [11], we speculate an alternative and complementary possibility that IFN-γ has a dual function in CIA. Jacob *et al.* showed that *in vivo* administration of IFN-γ 24 h before CFA immunization

caused an exacerbation of arthritis, whereas administration of IFN-γ 24–48 h after CFA immunization suppressed the disease in an adjuvant arthritis model [46]. These observations indicate that IFN-γ plays a proinflammatory role at steady state or early stage of inflammation (0–24 h after CFA immunization) and subsequently plays an anti-inflammatory role. In fact, several reports have suggested that IFN-γ activates innate immune cells, such as enhancing phagocytosis, proinflammatory cytokine production in response to bacterial component and antigen presentation, although it is also a fact that IFN-γ regulates adaptive Th17 cells negatively [47]. Thus, in our study, anti-IFN-γ treatment at the time of CII/CFA immunization might inhibit the proinflammatory effect of IFN-γ in the early stage of inflammation (0–24 h after immunization) and it might lead to a decreased baseline severity of disease. Meanwhile, although we could not know when the effect of α-carba-GalCer were started after s.c. injection and how long the effect was sustained, it is indisputable that NK T cells in α-carba-GaCer-treated mice were activating and had a higher capacity of IFN-γ-production at 72 h after CII/CFA immunization, which is the time when IFN-γ could play an anti-inflammatory effect in adjuvant arthritis [46]. Thus, we think that α-carba-GalCer-mediated IFN-γ played an anti-inflammatory effect at 72 h after CII/CFA immunization, and anti-IFN-γ mAb could have blocked the IFN-γ and abolished the beneficial effect of α-carba-GalCer. Considering these complex effects of IFN-γ in the development of CIA, IFN-γ should be neutralized for a longer period of time by repeated injections of anti-IFN-γ mAb or by using IFN-γ-deficient mice to evaluate more clearly the IFN-γ dependency of the α-carba-GalCer effect.

Our results are somewhat different from those of previous studies describing the effects of NK T cells on CIA. For example, Miellot *et al.* [48] demonstrated that treatment with α-GalCer induced IL-10-producing T cells and suppressed CIA. However, in our study, the same treatment had no effect on the development of CIA and we found no IL-10-producing cells in the DLN (data not shown). Coppieters *et al.* [49] suggested that the protective effect of α-C-GalCer on CIA is IFN-γ-independent. In contrast to their results, the beneficial effect of α-carba-GalCer in our study was IFN-γ-dependent. Although the reason for this discrepancy is not known, we speculate that the route and timing of administration of α-carba-GalCer might have influenced the results. Hermans *et al.* [50] reported that enhanced $CD4^+$ and $CD8^+$ T cell responses were observed when α-GalCer was administered at the same time as T cell antigen, and they suggested that the enhancement of T cell responses requires the presentation of T cell antigen and α-GalCer by the same dendritic cell. In our study, CII and glycolipid were administered at the same time and by the same route. Thus, it is possible that CII and glycolipids were captured and presented by the same dendritic cell, which was effective in influencing the T cell response through NK T cell activation. Conversely, Coppieters *et al.* [49] administered glycolipids 5 days after CII immunization, and the route of administration of glycolipids (i.p.) was different from that of CII (intradermally). Further studies are required to examine the effects of several glycolipids on CIA under the same conditions such as dose, route and timing of glycolipid immunization.

In human autoimmune diseases such as RA, Sjögren's syndrome, systemic sclerosis and SLE, the number of NK T cells is decreased and NK T cells function as regulatory cells that inhibit autoimmunity [17–20]. It is anticipated that increasing the population of IFN-γ-producing NK T cells by administration of α-carba-GalCer could be therapeutically useful in autoimmune diseases such as RA, owing to the similarities in the pathogenic processes of RA and CIA.

In conclusion, the present study demonstrated that NK T cells are multi-potent cells and act as regulatory cells by the induction of α-carba-GalCer in CIA. Based on these properties, we believe that further studies are warranted to explore the potential therapeutic benefits of α-carba-GalCer in Th17-mediated autoimmune diseases.

Disclosure

None.

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Involvement of NK 1.1–Positive γδT 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$^+$ γδT cells in ILD. The injection of IL-18 plus IL-2 (IL-18/IL-2) into C57BL6 (B6) mice induced acute ILD that resembled early-stage human ILD. An accumulation of NK1.1$^+$ γδT cells similar to NK cells was evident in the lungs. The T Cell Receptor (TCR) Vγ and Vδ repertoires of NK1.1$^+$ γδT cells indicated polyclonal expansion. The expression of IL-2 receptor β (Rβ) and IL-18Rβ in NK1.1$^+$ γδT cells was higher than in NK1.1$^-$ γδT cells. IL-18/IL-2 stimulated the proliferation of NK1.1$^+$ γδT cells, but not NK1.1$^-$ γδT cells. The IL-18/IL-2–stimulated NK1.1$^+$ γδT cells produced higher concentrations of IFN-γ than did NK1.1$^-$ γδT cells. Moreover, NK1.1$^+$ γδT and NK1.1$^-$ γδT cells constituted completely different cell populations. The IL-18/IL-2–induced ILD was milder in TCRδ$^{-/-}$ and IFN-γ$^{-/-}$ mice, compared with B6 mice. Furthermore, cell-transfer experiments demonstrated that NK1.1$^+$ γδT 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$^+$ γδT cells function as inflammatory mediators in the early phase of IL-18/IL-2–induced ILD.

Keywords: interstitial lung disease; NK1.1; γδT 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 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.

γδT cells exercise nonredundant functions in protecting against infectious agents. Unlike αβT cells and B cells, γδT cells preferentially colonize nonlymphoid tissue (e.g., epithelial or mucosal) (13). γδT cells produce a wide variety of cytokines, chemokines, and growth factors. Furthermore, γδT 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, γδT cells were reported to play a crucial role in lung disease in several murine models. In the airway hyperresponsiveness (AHR) murine model, γδT cells regulated AHR (15–18). By contrast, in the BLM-induced ILD murine model, γδT cells played a protective role against the pathological condition (19). As already described, γδT cells play an effective or suppressive role in lung disease by secreting a variety of cytokines. We recently discovered an increase in γδT 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$^+$ γδT cells could secrete large amounts of IFN-γ compared with NK1.1$^-$ γδT cells in the presence of IL-18/IL-12. On the other hand, NK cells possess the well-known potential to secret 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$^+$ γδT cells can

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produce large amounts of IFN-γ against IL-18/IL-2, the role of NK1.1$^+$ γδT 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$^+$ γδT cell subset. The results provide evidence for NK1.1$^+$ γδT cell subset accumulation in the lungs of mice with IL-18/IL-2–induced ILD. NK1.1$^+$ γδT cells produced IFN-γ and exacerbated ILD. Furthermore, IFN-γ$^{-/-}$ mice showed a resistance to IL-18/IL-2–induced ILD. In T Cell Receptor (TCR) δ$^{-/-}$ 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$^+$ γδT cells could induce the expansion of NK cells in the lungs by IL-18/IL-2. These results suggest that NK1.1$^+$ γδT cells accelerate ILD through the up-regulation of IFN-γ and NK-cell infiltration of the lung. Thus, NK1.1$^+$ γδT 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$^+$ γδT cells in the generation of ILD.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) and IFN-γ–deficient (IFN-γ$^{-/-}$) mice with a C56BL/6 background were purchased from Charles River Japan, Inc. (Tokyo, Japan). TCRδ-deficient (TCRδ$^{-/-}$) mice (22) with the C57BL/6 background were provided by Rikagakukenkyuujo 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 μg). 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$^+$ γδT Cells from the Lung, Spleen, and Liver

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

Cell Purification and Adoptive Transfer of NK1.1$^+$ or NK1.1$^-$ γδT cells

NK1.1$^+$ or NK1.1$^-$ γδT cells from splenocytes were harvested by the method already described. These splenic NK1.1$^+$ or NK1.1$^-$ γδT 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 μl via the tail vein into TCRδ$^{-/-}$ mice. At 24 hours after the adoptive transfer of NK1.1$^+$ or NK1.1$^-$ γδT cells, IL-18 plus IL-2 were injected and induced ILD.

Statistical Analysis

Data are expressed as median or mean ± 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-Vγ and TCR-Vδ profiling, the quantification of gene expression, staining and flow cytometry, intracellular cytokine staining, the measurement of γδT-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$^+$ γδT 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$^+$ γδT cells were increased in the lungs, but NK1.1$^+$ αβT cells were not (Figure 1B). Furthermore, we confirmed that most NK1.1$^-$ and NK1.1$^+$ γδT cells were CD4$^-$ CD8$^-$ dominant (Figure 1C). The number of NK1.1$^+$ γδT 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$^-$ γδT cells was similar in both groups. In the spleens and draining lymph nodes (DLNs), the numbers of NK1.1$^+$ γδT 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$^+$ γδT cells in the liver and intraepithelial lymphocytes (IELs).

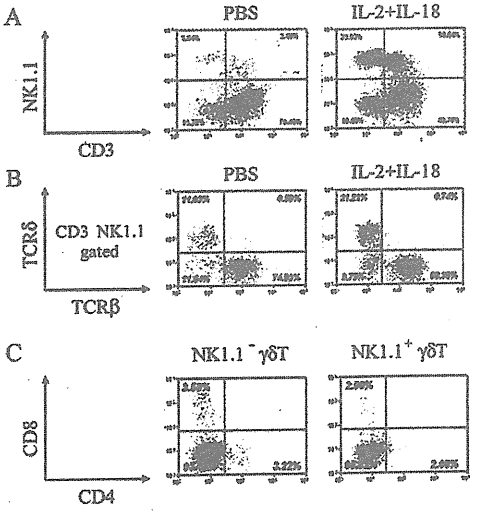

Figure 1. Accumulation of natural killer (NK) 1.1$^+$γδT cells in addition to NK cells in the lungs of mice with IL-18 plus IL-2–induced interstitial lung disease (ILD). (*A*) Pulmonary lymphocytes were harvested from B6 mice treated with PBS or IL-18/IL-2, as described in MATERIALS AND METHODS. Cells were stained with anti-NK1.1 and anti-CD3ε monoclonal antibodies (mAbs). (*B*) Pulmonary lymphocytes from B6 mice treated with IL-18/IL-2 or PBS were stained with anti-NK1.1, anti-CD3ε, anti-T Cell Receptor (TCR)β, and anti-TCRδ mAbs. (C) Pulmonary lymphocytes from B6 mice treated with IL-18/IL-2 were stained with anti-NK1.1, anti-CD3ε, anti-TCRδ, anti-CD4, and anti-CD8 mAbs. Data are representative of more than three independent experiments.

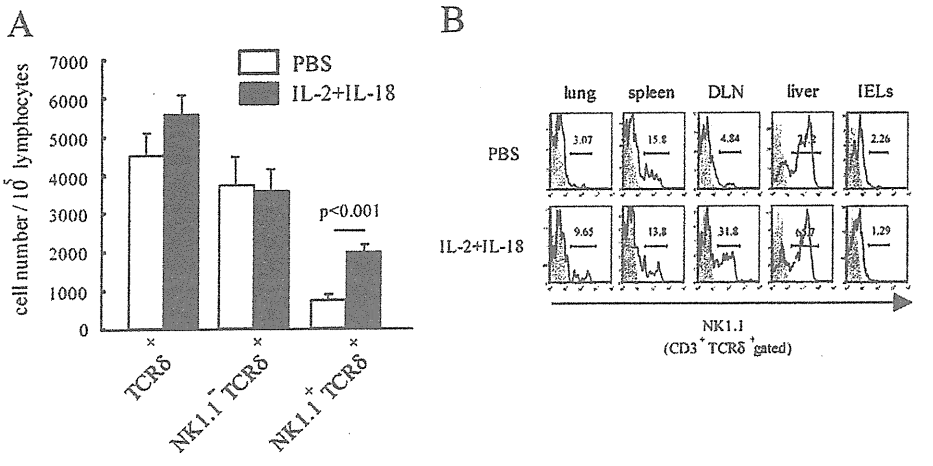

Figure 2. Increased proportion of NK1.1$^+$ γδT cells in lungs, spleens, and draining lymph nodes (DLNs) of mice with IL-18 plus IL-2–induced ILD. (*A*) Pulmonary lymphocytes were harvested from B6 mice treated with IL-18/IL-2 or PBS, as described in MATERIALS AND METHODS. Cells were stained with anti-NK1.1, anti-CD3ε, and anti-TCRδ mAbs. Data represent mean ± 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-CD3ε, and anti-TCRδ mAbs and isotype-matched immunoglobulin, and used for FACS analysis. Data are representative of at least two independent experiments.

TCR Vγ and Vδ Repertoire of NK1.1$^+$ γδT Cells Infiltrating the Lungs of IL-18 plus IL-2–Induced-ILD Mice

To determine the TCR repertoire of NK1.1$^-$ and NK1.1$^+$ γδT cells, we examined the RT-PCR analysis. NK1.1$^-$ and NK1.1$^+$ γδT 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$^+$ γδT cells from each tissue contained a repertoire of Vγ 1, 2, and 4, and of Vδ 1–8 (Figure 3). Moreover, NK1.1$^-$ γδT cells from each tissue contained a repertoire of Vγ 1, 2, 4, 5, and 6, and of Vδ 1–8 (Figure 3). Furthermore, in IL-18/IL-2–treated mice, NK1.1$^+$ γδT cells from each tissue contained a repertoire of Vγ 1, 2, and 4, and of Vδ 1–8 (Figure 3). In addition, NK1.1$^-$ γδT cells from each tissue contained a repertoire of Vγ 1, 2, 4, 5, and 6, and Vδ 1–8 (Figure 3). These results indicate that NK1.1$^+$ and NK1.1$^-$ γδT cells contained a polyclonal TCR repertoire.

NK1.1$^+$ γδT Cells Secrete IFN-γ in the Presence of IL-18 plus IL-2

To determine whether NK1.1$^+$ γδT 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$^+$ γδT cells can produce large amounts of IFN-γ and TNF-α (Figure 4A). Furthermore, the ability of NK1.1$^+$ γδT cells to secrete IFN-γ, TNF-α, and IL-4 was significantly higher than that of NK1.1$^-$ γδT cells (Figure 4B). Conversely, the ability of NK1.1$^+$ γδT cells to secrete IL-17 was significantly lower than that of NK1.1$^-$ γδT cells. Furthermore, we analyzed the production of cytokines from NK1.1$^+$ and NK1.1$^-$ γδT cells when stimulated with IL-18 and IL-2. NK1.1$^+$ γδT 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$^-$ γδT cells secreted IFN-γ in the presence of IL-18/IL-2, and the ability of NK1.1$^+$ γδT cells to secrete IFN-γ was significantly higher than that of NK1.1$^-$ γδT 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-$\gamma^{-/-}$ mice.

Histological examination indicated milder cell infiltration in the lungs of IFN-$\gamma^{-/-}$ mice compared with B6 mice (Figure 4D). Furthermore, pulmonary NK cells in IFN-$\gamma^{-/-}$ mice (10.7% ± 0.52%) were significantly reduced, compared with control mice (29.3% ± 3.79%, $P < 0.05$; Figure 4E).

NK1.1$^+$ γδT 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 γδT cells react to IL-18 and IL-2, we analyzed the expression of IL-2 receptor β (IL-2Rβ) and IL-18 receptor β (IL-18Rβ) in γδT cells of splenocytes from B6 mice. As shown in Figure 5A, NK1.1$^+$ γδT cells expressed more IL-2Rβ and IL-18Rβ than did NK1.1$^-$ γδT cells. To examine the response of NK1.1$^+$ and NK1.1$^-$ γδT 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$^+$ γδT cells against IL-18/IL-2 was significantly higher than that of NK1.1$^-$ γδT cells ($P < 0.05$; Figure 5B).

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

We also examined the expression of NK1.1 in γδT 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$^-$ γδT cells after coculturing with IL-18/IL-2 (Figure 5D). These results indicate that NK1.1$^+$ γδT cells should be completely different for cell populations from NK1.1$^-$ γδT cells.

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

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

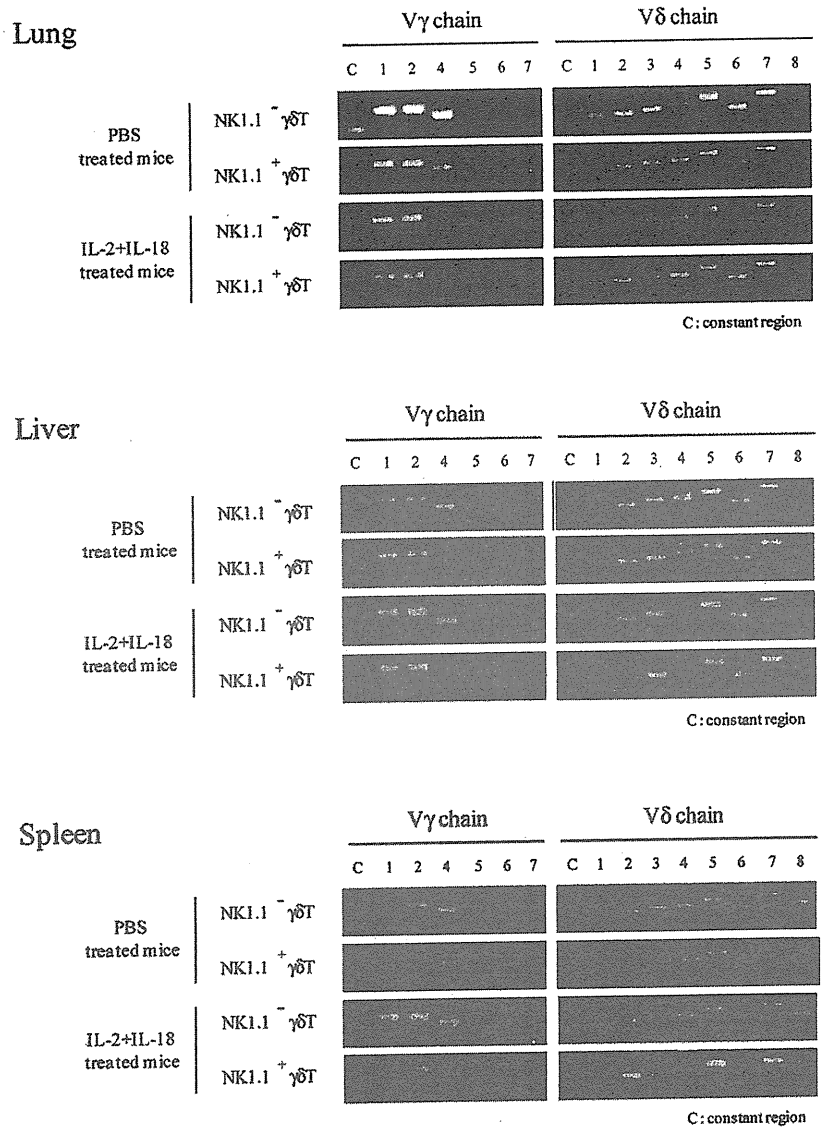

Figure 3. TCR-Vγ and TCR-Vδ repertoire of NK1.1$^+$ γδT cells from mice with IL-18 plus IL-2–induced ILD. TCR-Vγ and TCR-Vδ gene expression in NK1.1$^-$ and NK1.1$^+$ γδT cells were purified from pulmonary lymphocytes, splenocytes, and liver lymphocytes of PBS-treated or IL-18/IL-2–treated mice, and 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 TCRδ$^{-/-}$ 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$^+$ γδT 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$^+$ γδT cells in IL-18/IL-2–induced ILD, we transferred NK1.1$^+$ or NK1.1$^-$ γδT cells to TCRδ$^{-/-}$ mice, and then induced ILD (Figure 7A). The ratio of pulmonary NK cells was increased in TCRδ$^{-/-}$ mice after the transfer of NK1.1$^+$ γδT cells (0.81 ± 0.04), compared with NK1.1$^-$ γδT cells (0.63 ± 0.02, $P < 0.05$; Figure 7B). The expression of IFN-γ mRNA was increased in TCRδ$^{-/-}$ mice with the transfer of NK1.1$^+$ γδT cells (0.34 ± 0.03), compared with control TCRδ$^{-/-}$ mice (0.14 ± 0.03, $P < 0.005$; Figure 7C). These results suggest that NK1.1$^+$ γδT 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$^+$ γδT cells, but not NK1.1$^-$ γδT cells, in the lungs of mice with IL-18/IL-2–induced ILD. These findings suggest that not only NK but also NK1.1$^+$ γδT 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 αβT cells. Most NK1.1$^+$ αβT cells, termed NK T cells, use an invariant T-cell receptor (TCR) such as TCR Vα14 with various TCR Vβ genes. NK1.1$^+$ αβT cells recognize glycolipid antigens presented by the non-polymorphic CD1d molecules (23, 24). Recently, Rosemary and colleagues (25) reported the presence of NK1.1$^+$ γδT cells in naive thymus, liver, spleen, and bone marrow. The proportion of NK1.1$^+$ γδT cells relative to γδT cells varied from 10–15% in the spleen, to greater than 50–70% in the liver. In the present study, NK1.1$^+$ γδT 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

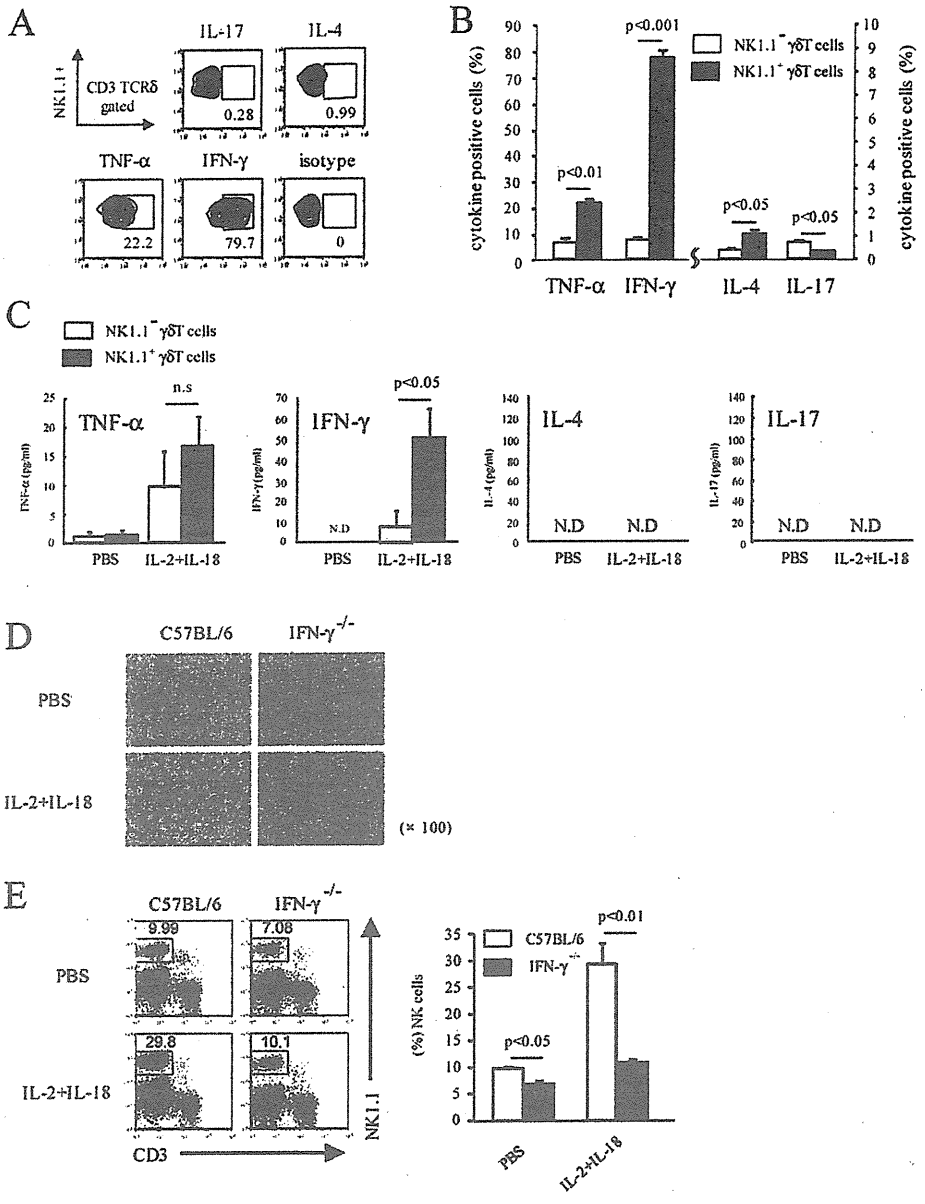

Figure 4. Cytokine profile of NK1.1$^-$ γδT cells and NK1.1$^+$ γδT cells. (*A*) Intracellular staining of TNF-α, IFN-γ, IL-4, and IL-17 was performed after the *in vitro* stimulation of splenocytes from naive B6 mice by phorbol 12-myristate 13-acetate (PMA)/ionomycin for 6 hours, and analyzed among gated CD3$^+$ TCRδ$^+$ 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$^-$ γδT cells. (*C*) Sorted NK1.1$^-$ γδT cells and NK1.1$^+$ γδT 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 ± 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, ×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 ± SEM. $P < 0.05$.

expression of major hisyocompatibility complex (MHC) class II is essential for the development and activation of NK1.1$^+$ γδT 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$^+$ γδT and NK cell accumulation in the lung may involve the high expression of IL-18Rβ and IL-2Rβ in NK1.1$^+$ γδT cells compared with NK1.1$^-$ γδT cells.

NK1.1$^+$ γδT cells were reported to secrete several cytokines, including IL-4, IL-10, IL-13, and TNF-α (20, 27). We showed that NK1.1$^+$ γδT cells were able to secrete large amounts of TNF-α, IFN-γ, and IL-4 after stimulation with PMA/ionomycin, compared with NK1.1$^-$ γδT cells. However, under conditions where IL-18/IL-2 was present, NK1.1$^+$ γδT cells were able to secrete large amount of IFN-γ compared with NK1.1$^-$ γδT cells, but not TNF-α and IL-4. Haas and colleagues (20) showed that NK1.1$^-$ γδT cells could produce IL-17. In the present study, we also showed that NK1.1$^-$ γδT cells could produce more IL-17 compared with NK1.1$^+$ γδT cells via stimulation with PMA/ionomycin. However, when the stimulus involved IL-18/IL-2, we could not detect IL-17–producing γδT (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 γδT cells (18). Indeed, IFN-γ$^{-/-}$ mice showed a significant amelioration of IL-18/IL-2–induced ILD. Thus, NK and NK1.1$^+$ γδT 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 TCRδ$^{-/-}$ mice clearly showed a significant amelioration of IL-18/IL-2–induced ILD in comparison with B6 mice. These results indicate that NK1.1$^+$ γδT 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 γδT cells used TCRγ 1, 4, 5, 6, and 7 genes. TCR Vγ1$^+$ and TCR

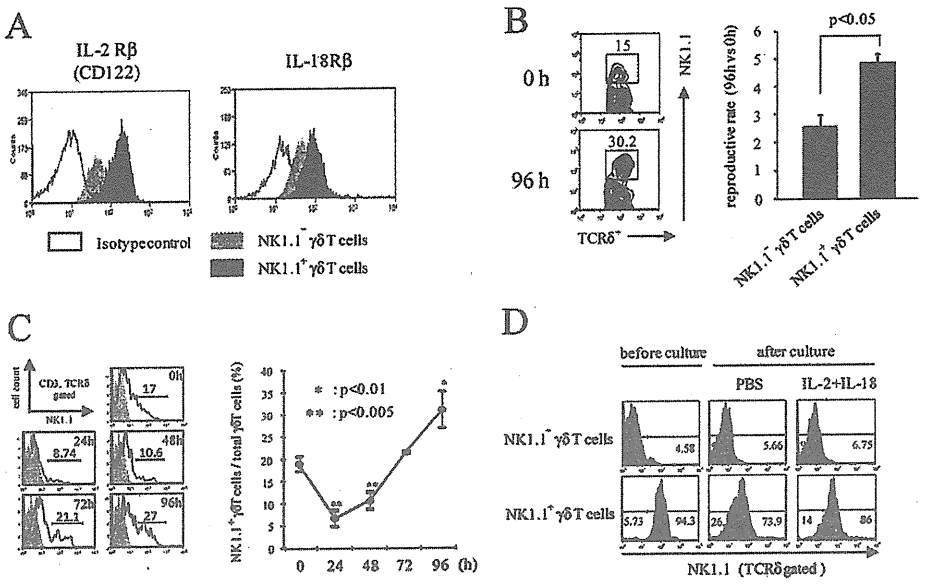

Figure 5. High expression levels of IL-2Rβ and IL-18Rβ and increased proliferation of NK1.1$^+$ γδT cells, after stimulation with IL-18 plus IL-2. (*A*) The expression levels of IL-2 receptor β (IL-2Rβ) and IL-18Rβ on NK1.1$^+$ and NK1.1$^-$ γδT cells were analyzed by flow cytometry. Splenocytes from B6 mice were enriched by a TCRγ/δ$^+$ isolation kit, as described in MATERIALS AND METHODS. Cells were stained with anti-CD3, anti-TCRδ, anti-NK1.1, anti-IL-2Rβ, and anti-IL-18Rβ mAbs, and isotype-matched immunoglobulin. (*B*) Splenocytes from B6 mice were cocultured with IL-18/IL-2 for 96 hours. (*Left*) Representative flow cytometry demonstrates NK1.1$^+$ γδT cells of splenocytes after culturing with IL-18/IL-2 for 96 hours. After culturing, cells were stained with anti-CD3ε, anti-TCRδ, and anti-NK1.1 mAbs for flow cytometry. (*Right*) Reproduction rate of NK1.1$^+$ and NK1.1$^-$ γδT cells under 0-hour and 96-hour culture conditions. Data represent mean ± 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 ± 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 predominantly 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

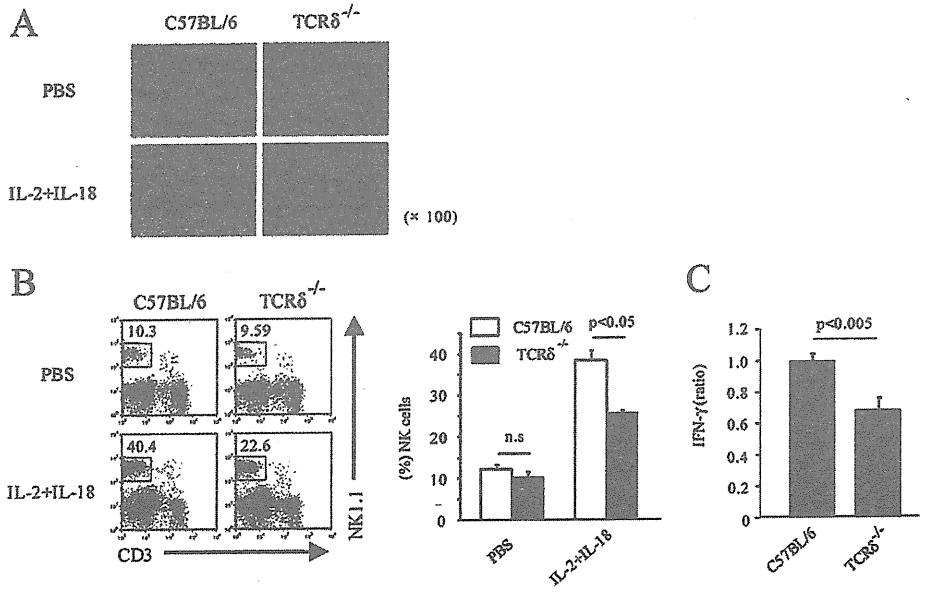

Figure 6. Amelioration of IL-18 plus IL-2–induced ILD, and reduced number of pulmonary NK cells and production of IFN-γ, in TCRδ$^{-/-}$ mice. (*A*) Lung tissues were harvested from B6 and TCRδ$^{-/-}$ mice at 6 hours after treatment with IL-18/IL-2 for 4 days. Lung tissues were stained with hematoxylin and eosin. Original magnification, ×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 ± 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 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 ± SEM. $P < 0.05$.

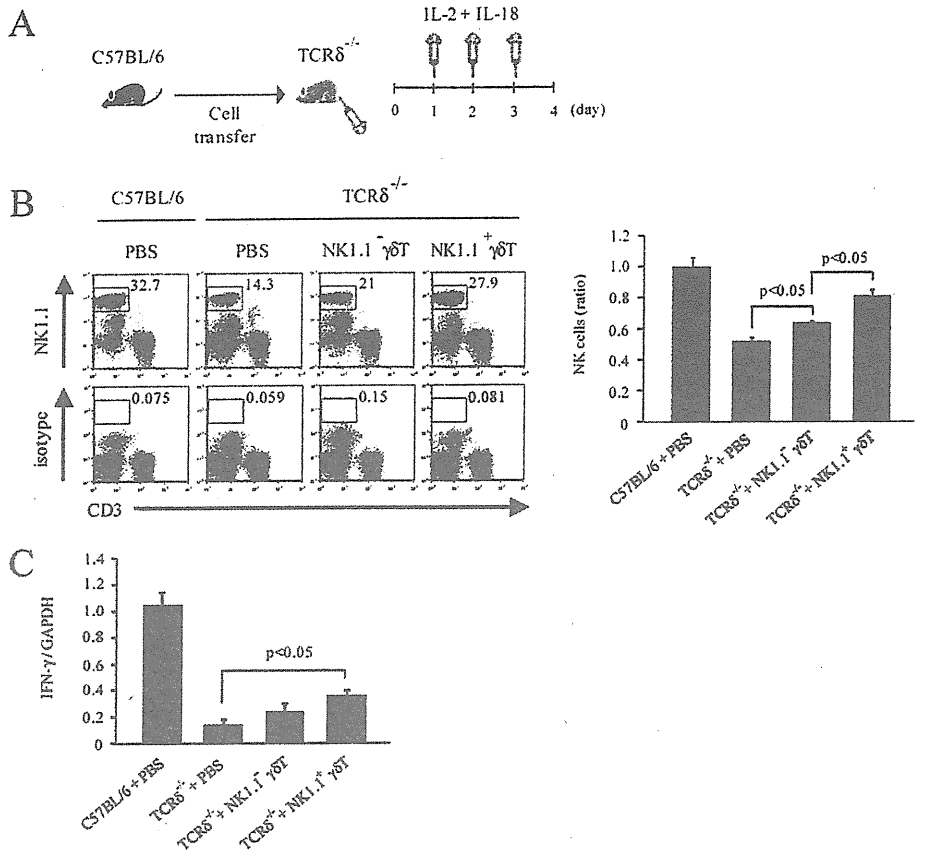

Figure 7. NK1.1$^+$ γδT cells accelerate the severity of IL-18 plus IL-2–induced ILD. (*A*) Naive B6 mice were harvested, and NK1.1$^-$ γδT and NK1.1$^+$ γδT cells were purified from splenocytes, as described in MATERIALS AND METHODS. These cells were transferred into TCRδ$^{-/-}$ mice (2×10^5/mouse). 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$^+$, or NK1.1$^-$ γδT 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 ± SEM of three independent mice, using the average value of the control mouse. The values as mean ± SEM for B6 mice + PBS were 1 ± 0.061; for TCRδ$^{-/-}$ mice + PBS, 0.514 ± 0.030; for TCRδ$^{-/-}$ mice + NK1.1$^-$ γδT cells, 0.636 ± 0.015; and for TCRδ$^{-/-}$ mice + NK1.1$^+$ γδT 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$^-$ γδT 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 ± SEM of three independent mice, using the average of the control mouse. Values as mean ± SEM for B6 mice + PBS were 1 ± 0.094; for TCRδ$^{-/-}$ mice + PBS, 0.132 ± 0.041; for TCRδ$^{-/-}$ mice + NK1.1$^-$ γδT cells, 0.219 ± 0.059; and for TCRδ$^{-/-}$ mice + NK1.1$^-$ γδT cells, 0.327 ± 0.048; $P < 0.05$.

γδT cell expansion in the lung. These results indicate that NK1.1$^+$ γδT cells may recognize a wide variety of antigens, and thus are unlikely invariant NK T cells. However, whether NK1.1$^+$ γδT 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 αβ 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$^+$ γδT cells after stimulation with IL-18/IL-2. The expression of NK1.1 in γδT 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 αβ NK T cells. Moreover, NK1.1$^-$ γδT cells did not express the NK1.1 molecule, even after culturing with IL-18/IL-2 for 96 hours, indicating that NK1.1$^+$ γδT cells may have different cell populations than NK1.1$^+$ γδT cells. Further examination will be necessary to clarify whether NK1.1$^+$ γδT cells develop from immature NK1.1$^-$ γδT cells or NK1.1$^+$ γδT 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 γδT 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 γδT cells. The results demonstrated that γδT 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$^+$ γδT cells into TCRδ$^{-/-}$ mice compared with NK1.1$^-$ γδT cells. Therefore, these findings in TCRδ$^{-/-}$ mice support the notion that NK1.1$^+$ γδT 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$^+$ γδT cells in peripheral blood mononuclear cells in patients with HIV (32) and multiple sclerosis (33). However, no reports on CD161$^+$ γδT cells in patients with interstitial pneumonitis are available. Thus, further experiments on human γδT cells in lungs from patients with ILD are necessary for a better understanding of the pathogenesis of ILD.

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ORIGINAL ARTICLE

Comparative suppressive effects of tyrosine kinase inhibitors imatinib and nilotinib in models of autoimmune arthritis

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Abstract Imatinib and nilotinib are inhibitors that selectively target a set of protein tyrosine kinases, including abelson kinase (Abl), together with the chimeric oncoprotein, breakpoint cluster region-abelson kinase (Bcr-Abl), as well as stem cell factor receptor (KIT), platelet-derived growth factor receptor (PDGFR), discoidin domain receptor (DDR), and colony stimulating factor-1 receptor (CSF-1R). The aim of the present study was to investigate whether imatinib or nilotinib was effective against arthritis in the glucose-6-phosphate isomerase (GPI)-induced arthritis mouse model. Imatinib or nilotinib was administered orally to the arthritic mice at different time points. Efficacy was evaluated by visual scoring and by determining the production of anti-GPI antibody. Splenocytes from the arthritic mice were cultured with GPI in the presence of imatinib or nilotinib in vitro, and cytokine levels in the culture supernatants were analyzed. To investigate the effects of imatinib and nilotinib on T-cell proliferation, lymph node cells from the arthritic mice were cultured with GPI in the presence of imatinib or nilotinib in vitro. Interleukin (IL)-17 mRNA expression in the arthritic ankle joints from the onset of arthritis was analyzed by real-time polymerase chain reaction (PCR). The administration of imatinib from day 0 showed suppression of arthritis ($P < 0.05$), the administration of nilotinib from day 0 resulted in pronounced suppression of arthritis ($P < 0.01$), and that from day 7 showed significant inhibition of the progression of arthritis ($P < 0.05$). A reduction in anti-GPI antibodies was correlated with the therapeutic efficacy of imatinib, but not with that of nilotinib. Imatinib dose-dependently inhibited tumor necrosis factor (TNF)-α, IL-6, interferon (IFN)-γ, and IL-17 production by splenocytes in vitro, while nilotinib inhibited only IL-17 and IFN-γ production in a dose-dependent fashion. Imatinib at 3 μM exerted a mild antiproliferative effect on CD4+ T cells ($P < 0.05$), whereas imatinib at 10 μM and nilotinib at 3 and 10 μM demonstrated a marked antiproliferative effect ($P < 0.01$). The IL17 gene expression level on day 7 tended to be higher than that on day 14. These findings suggest that imatinib and nilotinib could prevent autoimmune arthritis, essentially via distinct mechanisms, in that imatinib inhibits both inflammatory and T-cell-derived cytokine production, whereas nilotinib suppresses T-cell-derived cytokine production. Imatinib and nilotinib could have therapeutic potential for rheumatoid arthritis (RA) and other inflammatory diseases.

Keywords Imatinib · Nilotinib · GPI and arthritis

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Introduction

Rheumatoid arthritis (RA) is characterized by symmetrical polyarthritis and joint destruction. Although the etiology is considered to be autoimmune reactivity to some antigens, the exact mechanisms are not fully understood. Thus far, several models of arthritis have been described and analyzed to understand the etiological mechanisms of RA. Glucose-6-phosphate isomerase (GPI) was first identified as an

arthritogenic target of T and B cells in KRN T cell receptor transgenic mice on the C57Bl/6xNOD genetic background (K/BxN mice) developing severe inflammatory arthritis [1]. GPI-induced arthritis, caused by immunization with recombinant human GPI (rhGPI) to DBA/1 mice, has recently emerged as a new disease mechanism [2]. We have previously demonstrated that the T-helper (Th) 17 subset of CD4+ T cells plays a central role in the pathogenesis of GPI-induced arthritis; GPI-specific CD4+ T cells were skewed to Th17 at the time of onset, and blockade of interleukin (IL) 17 resulted in a significant amelioration of arthritis [3]. Furthermore, the finding that the administration of cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4 Ig) ameliorated the progress of arthritis implies the importance of Th17 cells [4]. We recently demonstrated a clear therapeutic effect of anti-tumor necrosis factor (TNF) monoclonal antibody (mAb) in mice with GPI-induced arthritis, and the therapeutic response correlated with the in vitro regulation of TNF production [4]. These results indicate that the GPI-induced arthritis model is suitable for studying the mechanisms of action of IL-17 antagonists as well as TNF-α antagonists in RA patients.

Imatinib is a small-molecule tyrosine kinase inhibitor that targets breakpoint cluster region-abelson kinase (Bcr-Abl) and is used to treat chronic myelogenous leukemia (CML). Nilotinib has been developed as a new more potent and selective inhibitor of Bcr-Abl [5]. These drugs also inhibit a narrow spectrum of additional protein tyrosine kinases, including Abl, lymphocyte-specific protein tyrosine kinase (Lck), and stem cell factor receptor (KIT), platelet-derived growth factor receptor (PDGFR), discoidin domain receptor (DDR), and colony stimulating factor-1 receptor (CSF-1R) kinases [6]. The synovium in RA patients contains activated macrophages and T cells which secrete several cytokines, and both imatinib and nilotinib inhibit CSF-1R signaling in macrophages and Lck during T-cell activation [7–10]. Dose-dependent inhibition of the proliferative response of purified CD4+ T cells by imatinib was observed, which associated with a reduction in interferon (IFN)-γ production [11]. We hypothesized that imatinib and its successor, nilotinib, may affect the Th17 subset, as well as the Th1 subset, to inhibit cytokine production. Therefore, in this study we investigated whether imatinib or nilotinib was effective against GPI-induced arthritis in mice and whether these agents suppressed the production of inflammatory cytokines and T-cell-derived cytokines in splenocytes in vitro.

Materials and methods

Reagents and mice

Imatinib mesylate and nilotinib were kindly provided by Novartis (Basel, Switzerland). Male DBA/1 mice were purchased from Charles River Laboratories (Yokohama, Japan). All mice were maintained under specific pathogen-free conditions, and all experiments were conducted in accordance with the institutional ethics guidelines.

GPI-induced arthritis and administration of imatinib or nilotinib

The rhGPI was produced as described previously [12]. Mice were immunized intradermally with 300 μg of rhGPI in Freund's complete adjuvant (Difco, Detroit, MI, USA) and the immunization day was defined as day 0. The rhGPI and Freund's complete adjuvant were emulsified at a 1:1 ratio (volume/volume). For the induction of arthritis, 150 μl of the emulsion was injected intradermally into the base of the tail. Arthritis was evaluated visually, and changes in each paw were scored on a scale of 0–3 according to our previous report [4]. A twice-daily oral dose of 100 mg/kg imatinib in distilled water was administered to mice, in which the pharmacokinetic profile provided a plasma peak level similar to that in humans on a mid-range dose of 400 mg once daily [13]. Nilotinib, which was well absorbed and displayed good bioavailability in mice, with a longer half-life than imatinib, was diluted in 0.5% methylcellulose containing 0.05% Tween 80, and administered once daily at a dose of 100 mg/kg [14]. The administrations of these agents started on days 0 and 7 after immunization for experiments on the prevention of arthritis, and on day 14 after immunization for treatment experiments.

Histological assessment of arthritis

Mice were killed on day 26 and the hind-paw joints were removed, fixed, decalcified, and paraffin-embedded. The sections were stained with hematoxylin and eosin, and evaluated for histological changes indicating synovitis and pannus formation.

Measurement of anti-GPI antibody in serum

To detect the levels of anti-GPI antibodies, we prepared microtiter plates coated with 5 μg/ml rhGPI for 12 h at 4°C. After a washing with 0.05% Tween 20 solution in phosphate-buffered saline (PBS), Block Ace (diluted 1/4 in PBS; Dainippon Pharmaceuticals, Osaka, Japan) was used for 2 h at room temperature. After two washes, sera (diluted 1/500, whose validity was confirmed in the previous study) were added and incubated for 2 h at room temperature [3]. After three washes, alkaline phosphatase-conjugated anti-mouse IgG (diluted 1/5,000) was added for 1 h at room temperature. After three washes, color was developed with alkaline phosphatase (AP) reaction solution

containing 9.6% diethanolamine and 0.25 mmol/l $MgCl_2$ (pH 9.8) with AP substrate tablets (Sigma Chemical, St. Louis, MO, USA; one AP tablet per 5 ml AP reaction solution). Plates were incubated for 20 min at room temperature and the optical density was measured by plate spectrophotometry at 405 nm.

Analysis of cytokine profiles

Spleens were dissected from immunized DBA/1 mice on days 3, 7, and 14 after immunization. A single-cell suspension was prepared and hemolysed with a solution of 0.83% NH_4Cl, 0.12% $NaHCO_3$, and 0.004% disodium ethylenediamine tetraacetic acid (EDTA). The number of splenocytes was counted, and the preparation was centrifuged again, and resuspended in RPMI medium (Gibco, Rockville, MD, USA), supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 10% fetal bovine serum, and 50 μM 2-mercaptoethanol. Splenocytes (2×10^6 cells/ml) were cultured with 5 μg/ml of rhGPI and 1, 3, and 10 μM imatinib or nilotinib for 72 h at 37°C in an atmosphere containing 5% CO_2 [4]. The supernatants were assayed for IL-6, TNF-α, monocyte chemotactic protein-1 (MCP-1), and IFN-γ by cytometric bead array (BD Bioscience, San Jose, CA, USA) and for IL-17 by enzyme-linked immunosorbent assay (BD Biosciences, Bedford, MA, USA).

Cell proliferation assay

GPI-immunized DBA/1 mice were killed on day 10. Inguinal lymph nodes were harvested to prepare single-cell suspensions, as these suspensions were suitable tissue for investigating the proliferation of CD4+ T cells in GPI-induced arthritis [3]. Cells (2×10^7 cells/ml) in PBS were stained with 1.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE-DA; Molecular Probes, Eugene, OR, USA) for 10 min. Stained cells (2×10^6 cells/ml) were cultured in 96-well round-bottomed plates with 5 μg/ml of rhGPI and 1, 3, and 10 μM imatinib or nilotinib for 4 days. Collected cells were stained with allophycocyanin (APC)-conjugated anti-CD4 (Biolegend, San Diego, CA, USA) and then analyzed by flow cytometry. Samples were measured on a FACScalibur instrument (BD Pharmingen, San Diego, CA, USA) and data were analyzed with FlowJo software (Treestar, Ashland, OR, USA).

Analysis of IL-17 gene expression

GPI-immunized mice were killed on days 0, 7, and 14. Synovial tissues from the ankle joints were isolated and minced with scissors. Total RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) in accordance with the instructions provided by the manufacturer. cDNA was obtained by reverse transcription with a commercially available kit (Fermentas, Glen Burnie, MD, USA). For real-time PCR, we used a TaqMan Assay-on-Demand gene expression product (Applied Biosystems, Foster City, CA, USA). The expression levels of IL-17 (assay ID: Mm00439619_m1; Applied Biosystems) were normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID: Mm99999915_g1; Applied Biosystems). Analysis was performed with an ABI Prism 7500 apparatus (Applied Biosystems) by the standard procedure.

Statistical analysis

Data are expressed as means ± SD or SEM. Comparisons of arthritis scores, antibody values, and gene expression were examined by the Mann–Whitney *U*-test. Differences in cytokine profiles and cell proliferation were examined using repeated analysis of variance (ANOVA) followed by the Dunnett's test (GraphPad Prism v. 3.0, GraphPad Software, San Diego, CA, USA).

Results

Inhibition of GPI-induced arthritis by imatinib

To determine the preventive and therapeutic effects of imatinib, 100 mg/kg of the agent was orally administered twice daily from days 0, 7, and 14 after immunization, and findings were compared with those of the control vehicle from day 0. The administration of imatinib from day 0 showed a suppressive effect on GPI-induced arthritis, which was correlated with the arthritis score (Fig. 1a) ($P < 0.05$ or $P < 0.01$). However, imatinib administered from days 7 and 14 only tended to ameliorate the arthritis. Histological analysis of joints from imatinib-treated mice indicated the suppression of synovial layer thickness and immune cell invasion (Fig. 1c).

Inhibition of GPI-induced arthritis by nilotinib

To investigate whether nilotinib prevented or treated arthritis, we administered 100 mg/kg nilotinib orally once daily from days 0, 7, and 14 after immunization and administered control vehicle from day 0. The administration of nilotinib from day 0 drastically blocked the development of arthritis ($P < 0.01$) and that from day 7 showed significant inhibition of the progression of arthritis (Fig. 1b) ($P < 0.05$). Histological analysis of joints from nilotinib-treated mice indicated the suppression of synovial layer thickness and immune cell invasion (Fig. 1d). These results suggest that nilotinib prevented GPI-induced arthritis more intensively than imatinib.

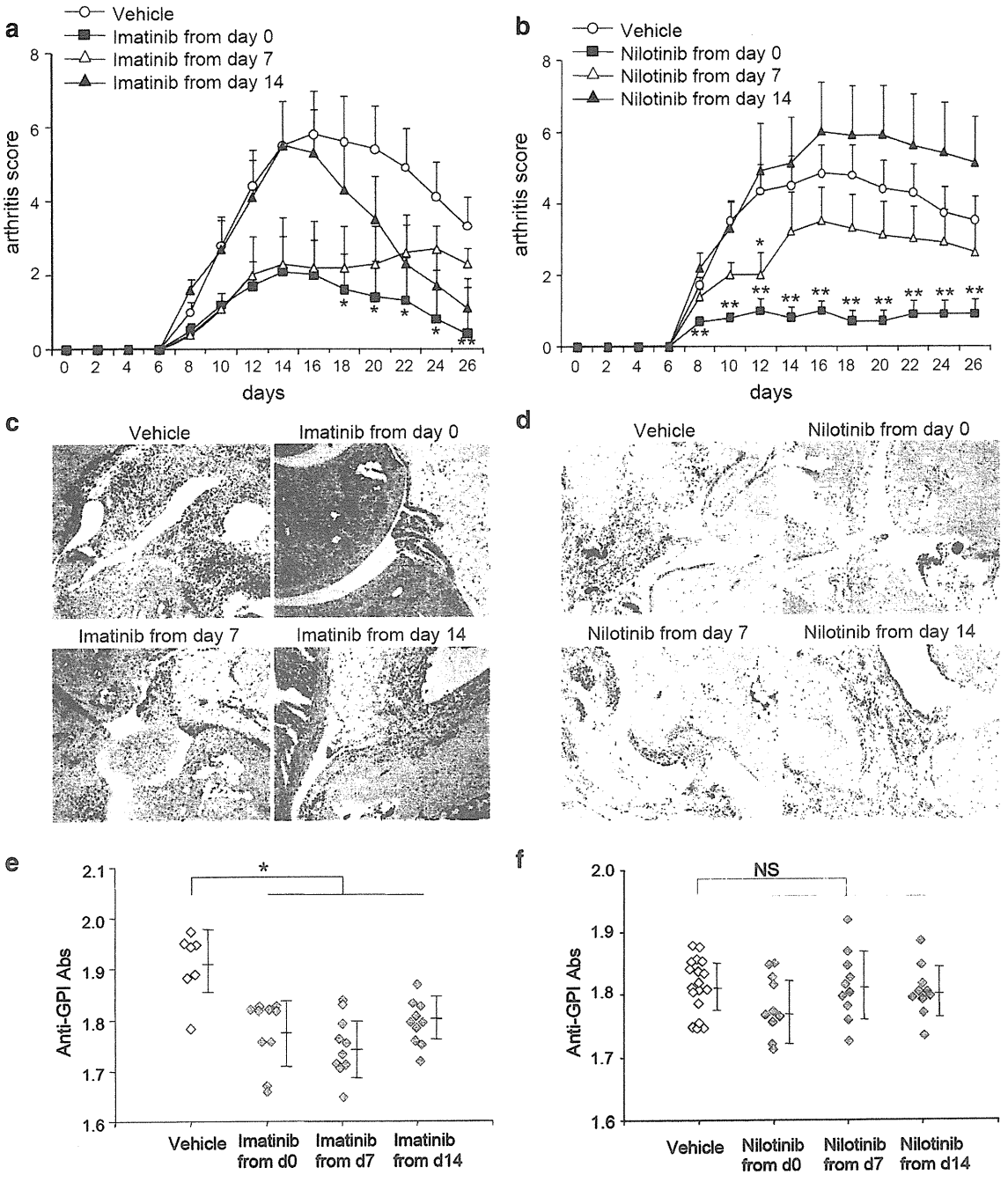

Fig. 1 Treatments with imatinib and nilotinib suppress glucose-6-phosphate isomerase (*GPI*)-induced arthritis in mice. Mice with GPI-induced arthritis were orally administered **a** 100 mg/kg imatinib twice daily, **b** 100 mg/kg nilotinib once daily from days 0, 7, and 14 after GPI immunization, compared with vehicle administration from day 0. The development of arthritis was monitored visually, and scores based on a scale of values of 0–3 are the means ± SEM of 10–18 mice per group. Ankle joints on day 26 in mice treated with vehicle from day 0 and those treated with **c** imatinib and **d** nilotinib from days 0, 7, and 14 were sampled and stained with H&E. Representative images are shown at a magnification of ×40. Titers of anti-GPI antibody in sera obtained on day 26 following oral administration of **e** 100 mg/kg imatinib twice daily or **f** 100 mg/kg nilotinib once daily from day 0, day 7, and day 14 were determined by enzyme-linked immunosorbent assay. *Each symbol* represents a single mouse. *Bars* show the mean ± SD of optical density at 405 nm. $*P < 0.05$, $**P < 0.01$ versus vehicle by Mann–Whitney *U*-test, *Abs* Antibodies, *d* day, *NS* not significant

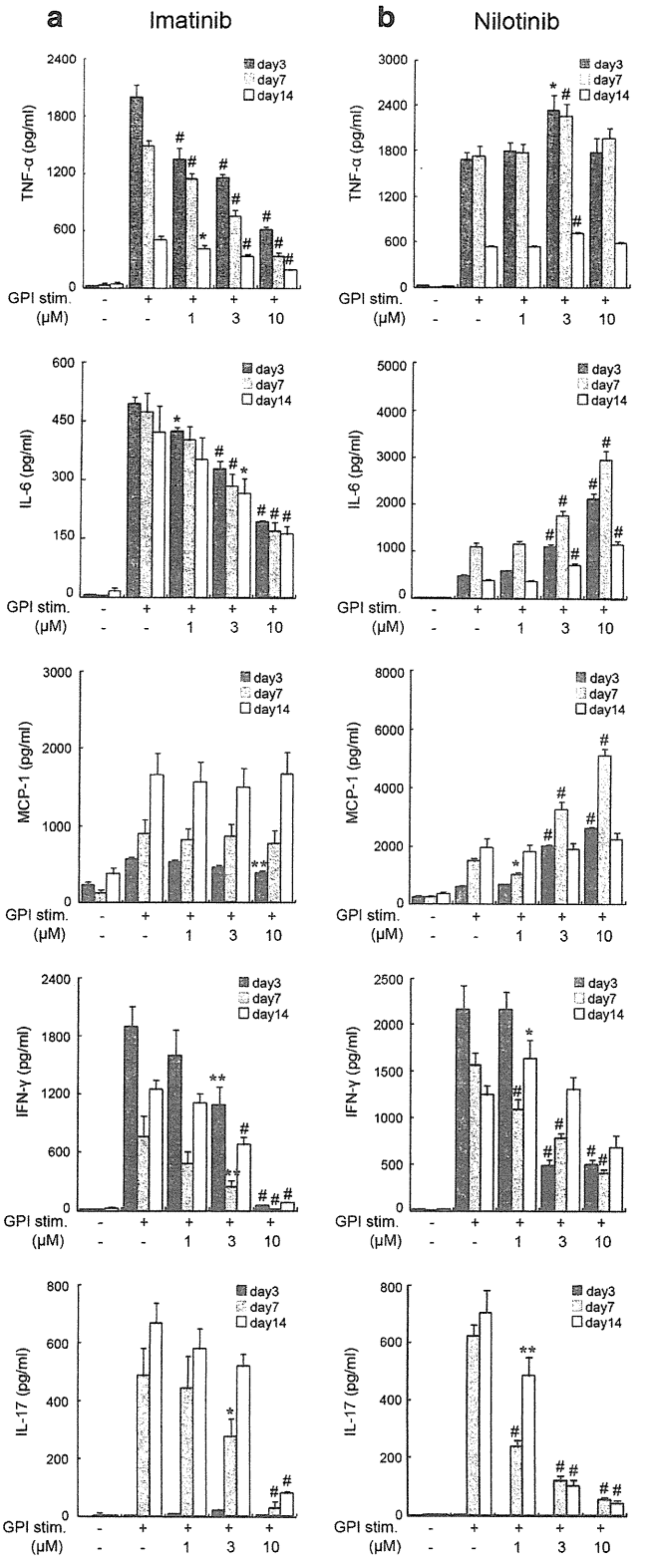

Fig. 2 Imatinib and nilotinib suppress cytokine production by splenocytes from arthritic mice in vitro. Spleens were removed from GPI-immunized mice on days 3, 7, and 14 after immunization, and then single-cell suspensions were prepared. Splenocytes (2×10^6 cells/ml) were stimulated (*stim.*) with 5 μg/ml GPI in the presence of 1, 3, and 10 μM **a** imatinib or **b** nilotinib for 72 h; culture supernatants were analyzed for tumor necrosis factor-α (*TNF-α*), interleukin 6 (*IL-6*), MCP-1, and interferon-γ (*IFN-γ*) by cytometric bead array, and the supernatants were analyzed for IL-17 by enzyme-linked immunosorbent assay. Values are means ± SEM of at least two mouse experiments in triplicate. $*P < 0.05$; $**P < 0.01$; $^{\#}P < 0.001$ compared with GPI-stimulated values on the corresponding sampling day

Suppression of antigen-specific antibody production by imatinib

We examined whether imatinib and nilotinib affected the production of anti-GPI antibodies. GPI-immunized mice were administered with imatinib or nilotinib from day 0, day 7, and day 14 to day 26. In all groups with each dosing period, sera were obtained on day 26 and the titers of anti-GPI antibodies were analyzed by enzyme-linked immunosorbent assay [3]. Treatment of mice with imatinib resulted in significant reductions of anti-GPI antibody titers from day 0, day 7, and day 14, as compared with the control (Fig. 1e) ($P < 0.05$). These results emphasized that the inhibitory effects of imatinib on the production of anti-GPI antibodies occurred irrespective of the phase of arthritis when the treatment was administered. On the other hand, treatment of mice with nilotinib from day 0, day 7, and day 14 did not appreciably affect the titers of anti-GPI antibody (Fig. 1f).

Imatinib inhibits the production of proinflammatory cytokines in splenocytes

GPI-immunized DBA/1 mice were killed on days 3, 7, and 14 after immunization. Spleens were taken and prepared as single-cell suspensions. Splenocytes were cultured with stimulation by rhGPI for 72 h in the presence of 1, 3, and 10 μM imatinib, and analysis of cytokines in supernatants was performed. Imatinib inhibited TNF-α and IL-6 production in a dose-dependent fashion, significantly at concentrations of 1 μM and higher (Fig. 2a). Imatinib did not affect the GPI-induced production of MCP-1, except for 10 μM treatment on day 3. IFN-γ production was inhibited by imatinib concentrations as low as 3 μM. IL-17 production was not detected on day 3; however, imatinib at 3 and 10 μM suppressed the production of IL-17 on day 7, and at 10 μM suppressed the production of IL-17 on day 14.

Nilotinib inhibits the production of T-cell-derived cytokines in splenocytes

GPI-immunized DBA/1 mice were killed on days 3, 7, and 14 after immunization. Spleens were taken and prepared as single-cell suspensions. Splenocytes were cultured with stimulation by rhGPI for 72 h in the presence of 1, 3, and 10 μM nilotinib, and the concentrations of cytokines were analyzed as described above. Nilotinib did not influence the production of TNF-α except at a concentration of 3 μM,