autoimmune arthritis, we induced CIA in T-bet–Tg mice and found marked suppression of CIA in T-bet–Tg mice.

To determine the reason for the low incidence of CIA in T-bet–Tg mice, we measured CII-reactive cytokine production and expression in vitro. IL-17 production from CII-reactive CD4+ T cells and *Il17a* expression were reduced in T-bet–Tg mice as compared with B6 mice. Although a predominant Th1 cell response was reported by Ishizuka et al (14), CII-specific IFNγ production was reduced in T-bet–Tg mice, and no significant difference was observed in *Ifng* expression between B6 mice and T-bet–Tg mice. Furthermore, *Il12a* expression was significantly higher in T-bet–Tg mice than in B6 mice, suggesting that overexpression of T-bet on T cells seems to affect innate immune cells, because the main producers of IL-12 are DCs and macrophages, not CD4+ T cells.

In criss-cross coculture experiments with CD4+ T cells and splenic DCs from B6 mice and T-bet–Tg mice, CII-reactive IL-17 production was also reduced even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice, although there was no significant difference in IL-17 production by CD4+ T cells from B6 mice cocultured with DCs from either B6 mice or T-bet–Tg mice. In contrast, no difference in IFNγ production was observed under all coculture conditions examined. Moreover, suppression of RORγt expression and high expression of T-bet on CD4+ T cells were observed even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice. These findings indicate that T-bet overexpression on CD4+ T cells might suppress CII-reactive IL-17 production resulting from suppression of RORγt expression in an IFNγ-independent manner, and that overexpression of T-bet has no direct effect on DC function.

CII-specific IgG levels correlate well with the development of arthritis (15). We observed significant suppression of CII-specific IgG production in the T-bet–Tg mice as compared with the B6 mice. A previous study showed that IL-17 is required for anti-CII antibody production (3). Therefore, the suppression of anti-CII antibody formation might be due to lower CII-reactive IL-17 production in T-bet–Tg mice.

To evaluate the low cytokine response to CII in T-bet–Tg mice, we analyzed lymphocytes obtained after immunization from draining lymph nodes and spleen. The percentage and absolute number of T cells tended to be lower in both the draining lymph nodes and spleen of T-bet–Tg mice compared with B6 mice. Moreover, significantly lower numbers of total thymocytes and an abnormal proportion of T precursor cells were observed in T-bet–Tg mice. The latter phenomenon could be due to T-bet transgene expression on double-negative thymic cells in T-bet–Tg mice. Because previous observations showed that T-bet interferes with GATA-3 function (11) and that GATA-3 was required for the development of early thymic T cells (24), one of the reasons for abnormal T cell development in the thymus might be the dysfunction of GATA-3 by overexpression of T-bet. These results suggest that overexpression of T-bet in thymic T cells affects T cell development, is responsible for the low number of T cells in spleen and lymph nodes, and is related to the low cytokine production against CII in T-bet–Tg mice.

To assess the effect of T-bet on CD4+ T cell differentiation in T-bet–Tg mice, we performed in vitro induction of Th17 cells. Analysis of T-bet–Tg mice showed a reduction in IL-17–producing CD4+ T cells and an increase in IFNγ-producing CD4+ T cells in spite of the condition favoring Th17 differentiation, which indicates suppression of Th17 cell differentiation and predominance of Th1 cell differentiation in vitro in T-bet–Tg mice. These results did not contradict the previous findings that the phenotype of polarized Th1 cells was not affected by Th cell–polarizing conditions (25). It is possible that suppression of CII-reactive IL-17 production in T-bet–Tg mice was not associated with IFNγ. For this reason, we generated T-bet–Tg/IFNγ$^{-/-}$ mice and performed in vitro induction of Th17 cells in these mice. Surprisingly, in T-bet–Tg/IFNγ$^{-/-}$ mice, the levels of IL-17–producing CD4+ T cells were also markedly reduced under Th17 cell differentiation–favoring conditions, indicating an IFNγ-independent suppressive pathway against Th17 cell differentiation. Although previous studies showed that suppression of Th17 cell differentiation was mediated through IFNγ signal transduction (16), our findings allow us to propose a new hypothesis: Th17 cell differentiation is regulated by a pathway that is distinct from the IFNγ signaling pathway. Therefore, we suggest that T-bet expression either directly or indirectly suppresses Th17 cell differentiation via an IFNγ-independent mechanism.

Tbx21 expression was significantly higher in T-bet–Tg mice as compared with B6 mice, and FACS analysis of CII-reactive CD4+ T cells revealed a significantly higher percentage of T-bet+ cells among the CD4+ T cell subset in T-bet–Tg mice. While there was no significant difference in the percentage of RORγt+ cells among the CD4+ T cell subset in T-bet–Tg mice as compared with B6 mice, *Rorc* expression was down-regulated on CII-reactive CD4+ T cells in T-bet–Tg mice. In the case of CD4+ T cells under

conditions favoring Th17 cell differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice. Interestingly, most of the RORγt+ cells also expressed T-bet in T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ T cells in the CD4+ cell subset was lower in T-bet–Tg mice than in B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells, but also inhibits the production of IL-17 from RORγt+ T cells.

Previous studies showed that RORγt expression is positively regulated by several transcription factors, such as runt-related transcription factor 1 (RUNX-1), interferon regulatory factor 4, and STAT-3 (26–28). Lazarevic et al (29) recently reported that T-bet prevented RUNX-1–mediated activation of the gene encoding RORγt, followed by the suppression of Th17 cell differentiation. In addition to direct promotion of RORγt expression, RUNX-1 also acts as a coactivator, together with RORγt, and induces the expression of *Il17a* and *Il17f* (26); therefore, T-bet inhibits IL-17 production by RORγt+ cells induced by RUNX-1 (29). Although further studies will be required to identify the effect of T-bet overexpression on the function of RUNX-1, it might be associated with the suppression of Th17 cell differentiation that was observed in the T-bet–Tg mice.

In conclusion, our results demonstrated that overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of CIA might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways. These findings should enhance our understanding of the pathogenesis of autoimmune arthritis and help in the development of new therapies for RA.

ACKNOWLEDGMENT

We thank Dr. F. G. Issa for critical reading of the manuscript.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sumida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sugihara, Hayashi, Yoh, Takahashi, Matsumoto, Sumida.

Acquisition of data. Kondo, Yao, Tahara.

Analysis and interpretation of data. Kondo, Iizuka, Wakamatsu, Tsuboi, Matsumoto.

REFERENCES

1. Miltenburg AM, van Laar JM, de Kuiper R, Daha MR, Breedveld FC. T cells cloned from human rheumatoid synovial membrane functionally represent the Th 1 subset. Scand J Immunol 1992;35: 603–10.
2. Iwanami K, Matsumoto I, Tanaka-Watanabe Y, Inoue A, Mihara M, Ohsugi Y, et al. Crucial role of the interleukin-6/interleukin-17 cytokine axis in the induction of arthritis by glucose-6-phosphate isomerase. Arthritis Rheum 2008;58:754–63.
3. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171:6173–7.
4. Chu CQ, Swart D, Alcorn D, Tocker J, Elkon KB. Interferon-γ regulates susceptibility to collagen-induced arthritis through suppression of interleukin-17. Arthritis Rheum 2007;56:1145–51.
5. Geboes L, De Klerck B, Van Balen M, Kelchtermans H, Mitera T, Boon L, et al. Freund's complete adjuvant induces arthritis in mice lacking a functional interferon-γ receptor by triggering tumor necrosis factor α–driven osteoclastogenesis. Arthritis Rheum 2007;56:2595–607.
6. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, et al. Human interleukin-17: a T cell–derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 1999;42:963–70.
7. Shen H, Goodall JC, Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis Rheum 2009;60:1647–56.
8. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000;100:655–69.
9. Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive $CD4^+$ T cells. Nat Immunol 2002;57:549–57.
10. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121–33.
11. Hawng ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science 2005;307:430–3.
12. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, et al. TGF-β-induced Foxp3 inhibits T_H17 cell differentiation by antagonizing RORγt function. Nature 2008;453:236–41.
13. Buttgereit F, Zhou H, Kalak R, Gaber T, Spies CM, Huscher D, et al. Transgenic disruption of glucocorticoid signaling in mature osteoblasts and osteocytes attenuates K/BxN mouse serum–induced arthritis in vivo. Arthritis Rheum 2009;60:1998–2007.
14. Ishizaki K, Yamada A, Yoh K, Nakano T, Shimohata H, Maeda A, et al. Th1 and type 1 cytotoxic T cells dominate responses in T-bet overexpression transgenic mice that develop contact dermatitis. J Immunol 2007;178:605–12.
15. Cho YG, Cho ML, Min SY, Kim HY. Type II collagen autoimmunity in a model of human rheumatoid arthritis. Autoimmun Rev 2007;7:65–70.
16. Tanaka K, Ichiyama K, Hashimoto M, Yoshida H, Takimoto T, Takaesu G, et al. Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-γ on STAT3 and Smads. J Immunol 2008;180:3746–56.
17. Kiwamoto T, Ishii Y, Morishima Y, Yoh K, Maeda A, Ishizaki K, et al. Transcription factor T-bet and GATA-3 regulate development of airway remodeling. Am J Respir Crit Care Med 2006;174: 142–51.
18. Shimohata H, Yamada A, Yoh K, Ishizaki K, Morito N, Yamagata K, et al. Overexpression of T-bet in T cells accelerates auto-

immune glomerulonephritis in mice with a dominant Th1 background. J Nephrol 2009;22:123–9.
19. Rangachari M, Mauermann N, Marty RR, Dirnhofer S, Kurrer MO, Komnenovic V, et al. T-bet negatively regulates autoimmune myocarditis by suppressing local production of interleukin 17. J Exp Med 2006;203:2009–19.
20. Nath N, Prasad R, Giri S, Singh AK, Singh I. T-bet is essential for the progression of experimental autoimmune encephalomyelitis. Immunology 2006;118:384–91.
21. Yang Y, Weiner J, Liu Y, Smith AJ, Huss DJ, Winger R, et al. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. J Exp Med 2009;206:1549–64.
22. Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. J Exp Med 2002;195:1129–43.
23. Juedes AE, Rodrigo E, Togher L, Glimcher LH, von Herrath MG. T-bet controls autoaggressive CD8 lymphocyte responses in type 1 diabetes. J Exp Med 2004;199:1153–62.
24. Hosoya T, Kuroha T, Moriguchi T, Cummings D, Maillard I, Lim KC, et al. GATA-3 is required for early T lineage progenitor development. J Exp Med 2009;206:2987–3000.
25. Shi G, Wang Z, Jin H, Chen YW, Wang Q, Qian Y. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. J Immunol 2008;181:7205–13.
26. Zhang F, Meng G, Strober W. Interactions among the transcription factors Runx1, RORγt and Foxp3 regulate the differentiation of interleukin 17–producing T cells. Nat Immunol 2008;9: 1297–306.
27. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, et al. The development of inflammatory T_H-17 cells requires interferon-regulatory factor 4. Nat Immunol 2007;8:958–66.
28. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. Immunity 2010;32:605–15.
29. Lazarevic V, Chen X, Shim JH, Hwang ES, Jang E, Bolm AN, et al. T-bet represses T_H17 differentiation by preventing Runx1-mediated activation of the gene encoding RORγt. Nat Immunol 2011;12:96–104.

Clinical & Experimental Immunology
The Journal of Translational Immunology
immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE

Activation of natural killer T cells by α-carba-GalCer (RCAI-56), a novel synthetic glycolipid ligand, suppresses murine collagen-induced arthritis

Y. Yoshiga,* D. Goto,* S. Segawa,* M. Horikoshi,* T. Hayashi,* I. Matsumoto,* S. Ito,* M. Taniguchi† and T. Sumida*

**Division of Clinical Immunology, Doctoral Program in Clinical Sciences, University of Tsukuba, Tsukuba, and †Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan*

Summary

Alpha-carba-GalCer (RCAI-56), a novel synthetic analogue of α-galactosylceramide (α-GalCer), stimulates invariant natural killer T (NK T) cells to produce interferon (IFN)-γ. IFN-γ exhibits immunoregulatory properties in autoimmune diseases by suppressing T helper (Th)-17 cell differentiation and inducing regulatory T cells and apoptosis of autoreactive T cells. Here, we investigated the protective effects of α-carba-GalCer on collagen-induced arthritis (CIA) in mice. First, we confirmed that α-carba-GalCer selectively induced IFN-γ in CIA-susceptible DBA/1 mice *in vivo*. Then, DBA/1 mice were immunized with bovine type II collagen (CII) and α-carba-GalCer. The incidence and clinical score of CIA were significantly lower in α-carba-GalCer-treated mice. Anti-IFN-γ antibodies abolished the beneficial effects of α-carba-GalCer, suggesting that α-carba-GalCer ameliorated CIA in an IFN-γ-dependent manner. Treatment with α-carba-GalCer reduced anti-CII antibody production [immunoglobulin (Ig)G and IgG2a] and CII-reactive interleukin (IL)-17 production by draining lymph node (DLN) cells, did not induce apoptosis or regulatory T cells, and significantly increased the ratio of the percentage of IFN-γ-producing T cells to IL-17-producing T cells (Th1/Th17 ratio). Moreover, the gene expression levels of IL-6 and IL-23p19, Th17-related cytokines, were reduced significantly in mice treated with α-carba-GalCer. In addition, we observed higher IFN-γ production by NK T cells in α-carba-GalCer-treated mice in the initial phase of CIA. These findings indicate that α-carba-GalCer polarizes the T cell response toward Th1 and suppresses Th17 differentiation or activation, suggesting that α-carba-GalCer, a novel NK T cell ligand, can potentially provide protection against Th17-mediated autoimmune arthritis by enhancing the Th1 response.

Keywords: collagen-induced arthritis, glycolipid ligand, natural killer T cells, Th1, Th17

Accepted for publication 25 January 2011
Correspondence: T. Sumida, Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8575, Japan.
E-mail: tsumida@md.tsukuba.ac.jp

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation of the synovial tissues and subsequent destruction of multiple joints [1]. Although the pathogenesis of RA remains unclear, proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, play a central role in this process [2]. There is general agreement that interferon (IFN)-γ-producing T helper type 1 (Th1) cells play a pathogenic role in the development of RA. However, several recent studies on animal models of autoimmune diseases suggested that IL-17-producing Th17 cells, but not IFN-γ-producing Th1 cells, play a crucial role in the development of RA. For example, mice deficient in Th1 cytokines, such as IFN-γ- and IL-12-deficient mice, exhibited severe symptoms in collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) [3–5], whereas those deficient in Th17 cytokines, such as IL-17- and IL-23-deficient mice, were resistant to these diseases [6–9]. Harrington *et al.* [10] suggested that IFN-γ suppresses the differentiation of naive CD4 T cells to Th17 cells. Furthermore, Chu *et al.* [11] showed that IFN-γ also suppressed IL-17 production by differentiated Th17 cells. In addition, IFN-γ plays a suppressor role by inducing myeloid suppressor cells that induce apoptosis of activated T cells in the chronic immune response,

such as the late phase of mycobacterial infection or the autoimmune response [12,13]. It has also been reported that IFN-γ is necessary for the conversion of $CD4^+$ $CD25^-$ T cells to $CD4^+$ regulatory T cells (T_{regs}) during EAE [14]. Thus, IFN-γ is thought to be a suppressive cytokine in several animal models of autoimmune diseases.

Natural killer T (NK T) cells are a subset of T lymphocytes that express NK cell markers, such as NK1·1, in mice. In mice, the majority of NK T cells express an invariant T cell receptor (TCR) encoded by Vα14Jα18, which is associated with highly skewed sets of Vβs, mainly Vβ8·2. The receptor recognizes glycolipid antigen presented by CD1d, a non-classical antigen-presenting molecule [15,16]. Stimulation of TCR induces NK T cells to rapidly secrete large amounts of proinflammatory and anti-inflammatory cytokines, such as IL-4 and IFN-γ [16]. Because of this property, NK T cells are known as immune regulators. Functional defects within NK T cells and reduced numbers of these cells are associated with various human autoimmune diseases [17–20]. In animal models, NK T cells suppress the development and progression of diabetes mellitus [21], EAE [22] and systemic lupus erythematosus (SLE) [23]. However, NK T cells also act as effector cells in some murine models of RA by promoting Th17 responses, producing IL-17 and suppressing the production of transforming growth factor (TGF)-β [24–28]. These evidences suggest a dual function for NK T cells in autoimmunity.

Alpha-galactosylceramide (α-GalCer) is a potent NK T cell ligand. The synthetic ligand induces NK T cell activation and secretion of various cytokines, such as IFN-γ, IL-4, and IL-17 [29]. To control NK T cell activation and cytokine secretion, several analogues of α-GalCer have been synthesized. OCH, which is an α-GalCer analogue with a shorter sphingosine chain, stimulates IL-4 production selectively by NK T cells [30]. The α-GalCer analogue suppressed the EAE-inducing antigen-specific Th2 response. Conversely, α-C-GalCer, a C-glycoside (carbon glycoside) analogue of α-GalCer, activated iNK T cells at very low concentrations and promoted Th1 responses *in vivo* [31]. More recently, Tashiro *et al.* [32] synthesized α-carba-GalCer, which strongly induced NK T cell-mediated Th1 cytokines in a fashion similar to α-C-GalCer [32].

In the present study, we found that α-carba-GalCer inhibited the development of CIA. This suppressive effect was dependent on IFN-γ induced by NK T cells. The results also showed that α-carba-GalCer suppressed the production of both anti-type II collagen (CII) antibodies in serum and IL-17 in draining lymph nodes (DLNs) in response to CII. This lower pathogenic Th17 response resulted from enhancement of the Th1 response via α-carba-GalCer-dependent IFN-γ. Thus, α-carba-GalCer could be a potentially useful therapeutic agent for Th17-mediated autoimmune diseases.

Materials and methods

Mice

Male DBA/1J mice were purchased from Charles River Japan (Tokyo, Japan). The animals were kept under specific pathogen-free conditions and studied at 6–9 weeks of age. The Institutional Animal Care and Use Committee of the University of Tsukuba approved all experimental plans.

Reagents

α-GalCer was purchased from Funakoshi (Tokyo, Japan) and α-carba-GalCer (RCAI-56) was kindly provided by Dr Masaru Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan). The structures of these two reagents are shown in Fig. 1a. The stock solutions of these glycolipids were dissolved originally in 100% dimethyl sulphoxide (DMSO) at 1 mg/ml and diluted in phosphate-buffered saline (PBS) just before injection into the mice. The

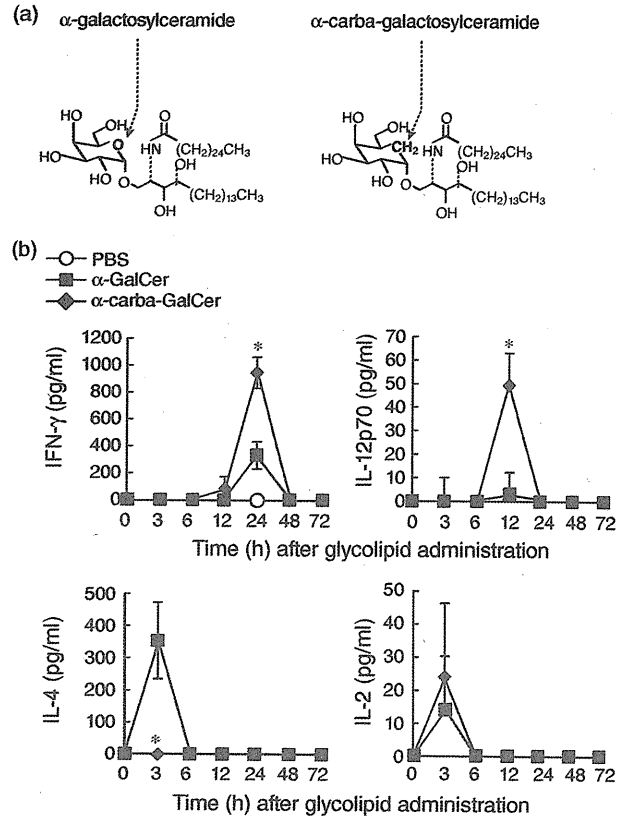

Fig. 1. Natural killer (NK) T cell response to α-carba-GalCer in CIA-susceptible mice. (a) Structure of α-galactosylceramide (α-GalCer) and α-carba-GalCer. (b) DBA/1 mice were injected intravenously (i.v.) with 2 μg of α-GalCer, α-carba-GalCer or vehicle ($n = 3$/group). The levels of interleukin (IL)-2, IL-4, IL-12 and interferon (IFN)-γ were measured by enzyme-linked immunosorbent assay (ELISA). Data are representative of three experiments. Values represent mean ± standard deviation (*$P < 0{\cdot}05$ *versus* α-GalCer).

following monoclonal antibodies (mAbs) were used for flow cytometric analysis: allophycocyanin (APC)-conjugated anti-mouse CD4 (clone: GK1·5; eBioscience, San Diego, CA, USA), peridinin chlorophyll (PerCP)-conjugated anti-CD3, fluorescein isothiocyanate (FITC)- and PerCP-conjugated anti-CD19, FITC- or APC-conjugated anti-IFN-γ (clone XMG1·2; BioLegend, San Diego, CA, USA); and FITC- or phycoerythin (PE)-conjugated anti-IL-17 (clone TC11-18H10·1; BD Pharmingen, Franklin Lakes, NJ, USA), and PE-conjugated CD1d-tetramer (MBL International, Woburn, MA, USA). The following mAbs were used for anti-CII specific IgGs enzyme-linked immunosorbent assay (ELISA): polyclonal rabbit anti-mouse immunoglobulins/HRP (Dako, Glostrup, Denmark), rabbit anti-mouse IgG1-horseradish peroxidase (HRP) (Zymed Laboratories, San Francisco, CA, USA) and rat anti-mouse IgG2a-HRP (Zymed). Bovine type II collagen was purchased from Collagen Research Center (Tokyo, Japan) and dissolved under constant stirring overnight at 4°C in 0·05 M acetic acid in phosphate-buffered saline (PBS) to be used for immunization, or in 0·05 mM Tris-HCl, 0·2 M NaCl, pH 7·4 for ELISA.

Cell preparation

Lymphocytes were isolated from the liver, spleen or DLN, as described previously [33].

Induction of CIA and glycolipid administration

Mice were immunized subcutaneously (s.c.) at the base of their tails with 100 μg of bovine CII emulsified with complete Freund's adjuvant (CFA) (Difco, Detroit, MI, USA). An emulsion was formed by 2 mg/ml of CII with an equal volume of CFA. Two micrograms of either α-GalCer or α-carba-GalCer was added to and emulsified with CII/CFA. The emulsion was injected s.c. into the tail base. A booster dose of 100 μg of CII solution was injected intraperitoneally (i.p.) on day 21. For intracellular cytokine staining, 50 μg was injected into each footpad of the hind paw. Joint swelling was monitored and scored as follows: 0, no swelling or redness; 1, swelling or redness in one joint; 2, involvement of > 2 joints; and 3, severe arthritis affecting all paws and joints. The score for each animal represented the sum of the score for all four paws. The clinical score was calculated using the results of all mice in the group.

Antibody treatment

Systemic IFN-γ neutralization was carried out by treatment with anti-IFN-γ mAb, at 150 μg/mouse injected i.p. on day 0.

Enzyme-linked immunosorbent assay

To determine the CII-specific IgG subtype, bovine CII (10 μg/ml) was coated onto ELISA plates and incubated at 4°C overnight. After two washes with washing buffer (0·05% Tween 20 in PBS), the blocking solution [2% bovine serum albumin (BSA) in PBS] was applied for 1 h at room temperature. After two washes, serially diluted serum samples were added to the CII-coated wells for 1 h. After three washes, HRP-conjugated anti-mouse IgG, IgG1, or IgG2a was added at a final dilution of 1:4000 and incubated for 1 h. After three washes, colour was developed using peroxidase substrate (KPL). The plates were incubated for 15 min at room temperature, and the optical density was read at 450 nm using a microplate reader.

The concentrations of IL-2, IL-4, IL-10, IL-12, IL-17 and IFN-γ in the serum and in the culture supernatants were measured using an ELISA kit (Duoset; R&D Systems, Abingdon, UK), according to the protocols supplied by the manufacturer.

NK T cell response to glycolipid ligand

For the *in vivo* assay, naive DBA/1 mice ($n = 3$) were injected intravenously (i.v.) with 2 μg/mouse of glycolipid ligands and serum was collected at various time points. The concentration of cytokines in serum was determined by ELISA.

CII-reactive T cell response

Twelve days after CII/glycolipid injection, the DLN (inguinal) cells were collected and restimulated with 100 μg/ml of denatured bovine CII (60°C, 10 min) for 72 h. The cells were cultured in complete RPMI-1640 medium containing antibiotics and 5% fetal calf serum (FCS) and incubated at 37°C. The concentration of cytokines in the culture supernatants was determined by ELISA.

Flow cytometry

Cells were stained at 4°C in PBS containing 2% heat-inactivated FCS, incubated for 5 min with anti-CD16/32 to block Fcr receptors, and then incubated for 30 min with various mAbs at appropriate dilutions. A mouse T_{reg} cell staining kit (eBioscience) was used to stain T_{reg} cells following the protocol provided by the manufacturer. Apoptosis was examined by the annexin V/propidium iodide (PI) assay (eBioscience) using the protocol supplied by the manufacturer. Intracellular cytokines were stained using an intracellular staining kit (BD Pharmingen). Lymphocytes from CII-immunized mice were stimulated with phorbol myristate acetate (PMA) (50 μg/ml) and ionomycin (1 g/ml) in the presence of GolgiStop solution (BD Pharmingen) for 4 to 6 h. Flow cytometry was performed on a four-colour fluorescence activated cell sorter (FACS)Calibur. Dead cells were excluded based on the forward- and side-scatter characteristics. The results were analysed using Mac CellQuest software (BD Biosciences, San Jose, CA, USA).

Quantification of cytokine transcripts

Total RNA was extracted with an RNA extraction kit (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). We used a *Taq*Man assay-on-demand gene expression product (Applied Biosystems, Foster City, CA, USA). The expression levels of IL-6, IL-23p19, TGF-β and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (assay ID IL-6: Mm00446191; IL-23p19: D1160011; TGF- β: 01178819; GAPDH: 99999915, respectively; Applied Biosystems) were normalized relative to the expression of GAPDH. Analysis was performed with an ABI Prism 7500 apparatus (Applied Biosystems).

Statistical analysis

Values were expressed as the mean ± standard error of the mean (s.e.m.). Differences between groups were examined for statistical significance using the *t*-test. Probability values less than 0·05 were considered significant.

Results

NK T cell response to α-carba-GalCer in CIA-susceptible DBA/1 mice

First, we examined whether α-carba-GalCer causes a differential simulation of Th1 cytokine production in DBA/1 mice (known as the CIA-susceptible strain). Mice were injected with 2 μg of either α-carba-GalCer or α-GalCer and their blood cytokine levels were then measured at various time-points by ELISA. α-GalCer, but not α-carba-GalCer, increased IL-4 concentrations at 3 h after injection (Fig. 1b). Conversely, IFN-γ and IL-12 production was induced by both glycolipids, but the levels in α-carba-GalCer-treated mice were higher than those in mice treated with α-GalCer (Fig. 1b). IL-2-production was observed in both α-GalCer- and α-carba-GalCer-treated mice and the concentration of IL-2 in α-carba-GalCer-treated mice was comparable to that in α-GalCer-treated mice (Fig. 1b).

These data support the findings of a previous study [32], and suggest that α-carba-GalCer is a potent ligand for NK T cells and can selectively induce a Th1-type response.

α-carba-GalCer suppresses CIA in an IFN-γ-dependent manner

To examine the effects of α-carba-GalCer on the onset and severity of CIA, male *DBA/1* mice with type II collagen (CII) were injected s.c. with α-carba-GalCer, α-GalCer or vehicle on day 0. As shown in Fig. 2a, α-carba-GalCer treatment tended to reduce the incidence of CIA compared with the vehicle treatment, although the difference was not significant. In contrast, α-GalCer-treatment did not affect the incidence of the disease. The clinical score of arthritis of the α-carba-GalCer-treated group was significantly lower than that of the vehicle-treated groups ($P < 0·05$, Fig. 2b). To

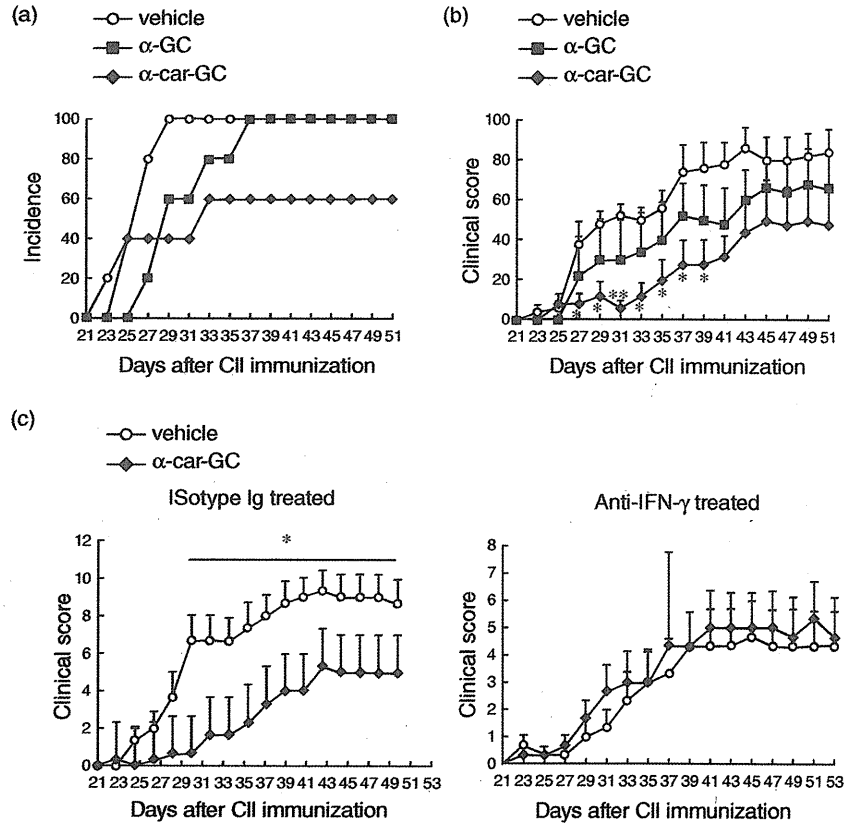

Fig. 2. Effects of α-carba-GalCer on CIA. DBA/1 mice were immunized with CII in CFA and 2 μg of α-galactosylceramide (α-GalCer) ($n = 5$), α-carba-GalCer ($n = 5$) or vehicle ($n = 5$). (a) Incidence and (b) clinical score of arthritis. Mice were immunized with CII/glycolipids as described above and injected intraperitoneally (i.p.) with anti-interferon (IFN)-γ (160 μg/mouse) or isotype on day 0. Subsequently (c) the clinical score of arthritis was monitored serially from day 21. Data are representative of two experiments. Values represent mean ± standard error of the mean of three mice (c) or five mice (a, b) per group (*$P < 0·05$ *versus* vehicle-treated mice).

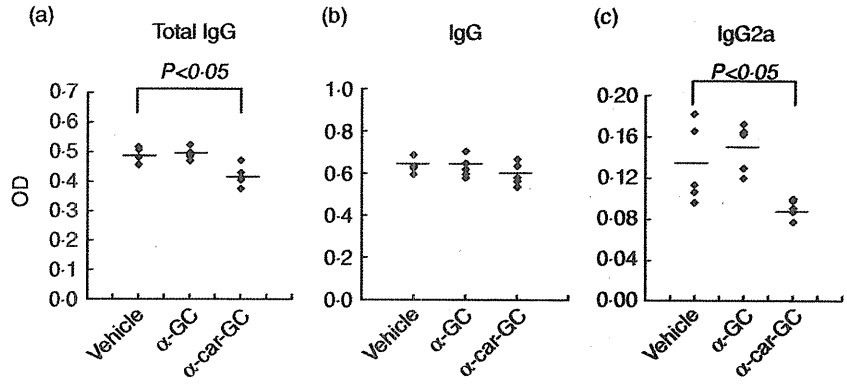

Fig. 3. Production of anti-type II collagen (CII) antibodies in α-carba-GalCer-treated mice. DBA/1 mice were immunized with CII in complete Freund's adjuvant (CFA) and 2 μg of α-galactosylceramide (α-GalCer) ($n = 5$), α-carba-GalCer ($n = 5$) or vehicle ($n = 5$). Sera were obtained on day 35, and (a) the titres of anti-CII-specific immunoglobulins (IgGs) (b) IgG1 and (c) IgG2a were analysed by enzyme-linked immunosorbent assay. Data are representative of three experiments. Values represent mean ± standard error of the mean of five mice per group.

determine whether this therapeutic effect was dependent on IFN-γ, IFN-γ was neutralized in the α-carba-GalCer-treated mice. IFN-γ neutralization at the time of CII immunization abolished the beneficial effect of α-carba-GalCer on CIA, but had no effect in the vehicle-treated mice (Fig. 2c). These data indicate that α-carba-GalCer ameliorates CIA and that this action is mediated through IFN-γ.

α-carba-GalCer suppresses anti-CII antibodies and CII-reactive IL-17 production

In general terms, CIA is thought to be an autoreactive T and B cell-dependent arthritis [34]. Therefore, we determined the anti-CII antibody titre in α-carba-GalCer-treated mice. As shown in Fig. 3a, the anti-CII IgG titre was significantly lower in the α-carba-GalCer-treated mice than in the control mice. Specifically, the anti-CII IgG2a titre was lower in α-carba-GalCer-treated mice, but there were no differences in the anti-CII IgG1 subclass titres among the groups (Fig. 3b,c). Twelve days after injection of CII/α-carba-GalCer, cells were collected from the DLN and restimulated with CII *in vitro*. IL-17 production by DLN cells was lower in the α-carba-GalCer-treated mice than in the control mice (Fig. 4). In contrast, IFN-γ production in α-carba-GalCer-treated mice was comparable to that in α-GalCer- and vehicle-treated mice. None of the cultures showed production of IL-4 and IL-10 (data not shown). These results suggest that α-carba-GalCer treatment suppresses antigen-specific Th17 cell and B cell responses in the development of CIA.

α-carba-GalCer does not alter the number of forkhead box P3 (FoxP3$^+$) T_{regs} or apoptotic T cells

IFN-γ is reported to play an important role in the induction of apoptosis and T_{regs} in autoimmune disease [13,14]. Therefore, we examined whether the beneficial effects of α-carba-GalCer were mediated by induction of apoptosis of T cells or T r_{egs}. As shown in Fig. 5a, treatment with α-carba-GalCer did not increase apoptosis, as assessed by the annexin/PI assay. Further analysis indicated that the proportion of FoxP3$^+$ T_{regs} was not significantly different between the α-carba-GalCer-treated mice and the control mice (Fig. 5b). These results suggest that the beneficial effects of α-carba-GalCer on CIA are unlikely to be mediated by induction of apoptosis or T_{regs}.

Alternation of the Th1/Th17 cytokine balance in α-carba-GalCer-treated mice

Because recent studies have shown that IFN-γ suppresses IL-17 production in CIA [10,11], we examined the hypothesis that the beneficial effect of α-carba-GalCer was due to the suppression of IL-17 production by IFN-γ. For this purpose, we determined the proportion of IFN-γ- and IL-17-producing T cells in α-carba-GalCer-treated mice. The proportion of IL-17-producing T cells in α-carba-GalCer-treated mice was significantly smaller than in vehicle-treated mice when analysed immediately *ex vivo* 10 days after α-carba-GalCer immunization with CII (Fig. 6a,b,

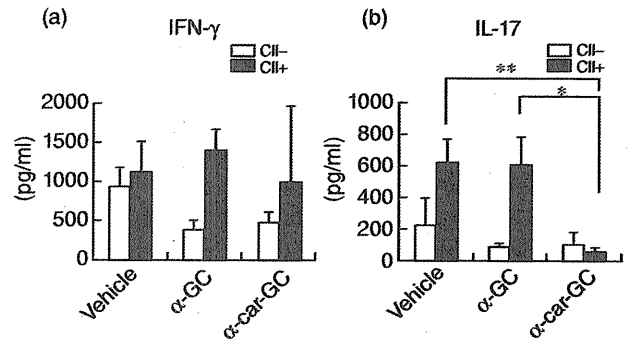

Fig. 4. CII-reactive T cell response in α-carba-GalCer-treated mice. DBA/1 mice were immunized with type II collagen (CII) in complete Freund's adjuvant (CFA) and 2 μg of α-galactosylceramide (α-GalCer) ($n = 3$), α-carba-GalCer ($n = 3$) or vehicle ($n = 3$). Twelve days after CII/glycolipid immunization, draining lymph node (DLN) cells were collected and then stimulated with CII for 72 h. Interferon (IFN)-γ and interleukin (IL)-17 levels in culture supernatant were determined by enzyme-linked immunosorbent assay. Data are representative of three experiments. Values represent mean ± standard error of the mean of three mice per group (*$P < 0.05$; **$P < 0.01$).

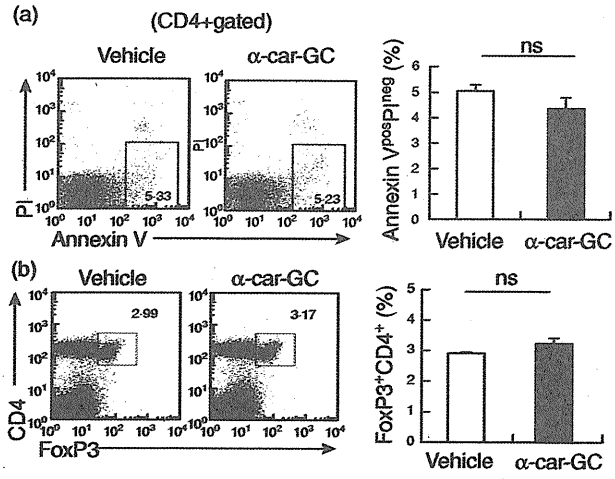

Fig. 5. α-carba-GalCer does not alter the number of forkhead box P3 (FoxP3)$^+$ regulatory T cells (T_{regs}) or apoptotic T cells. DBA/1 mice were immunized with type II collagen (CII) in complete Freund's adjuvant (CFA) and 2 μg of α-carba-GalCer ($n = 3$) or vehicle ($n = 3$). Twelve days after CII/glycolipid 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

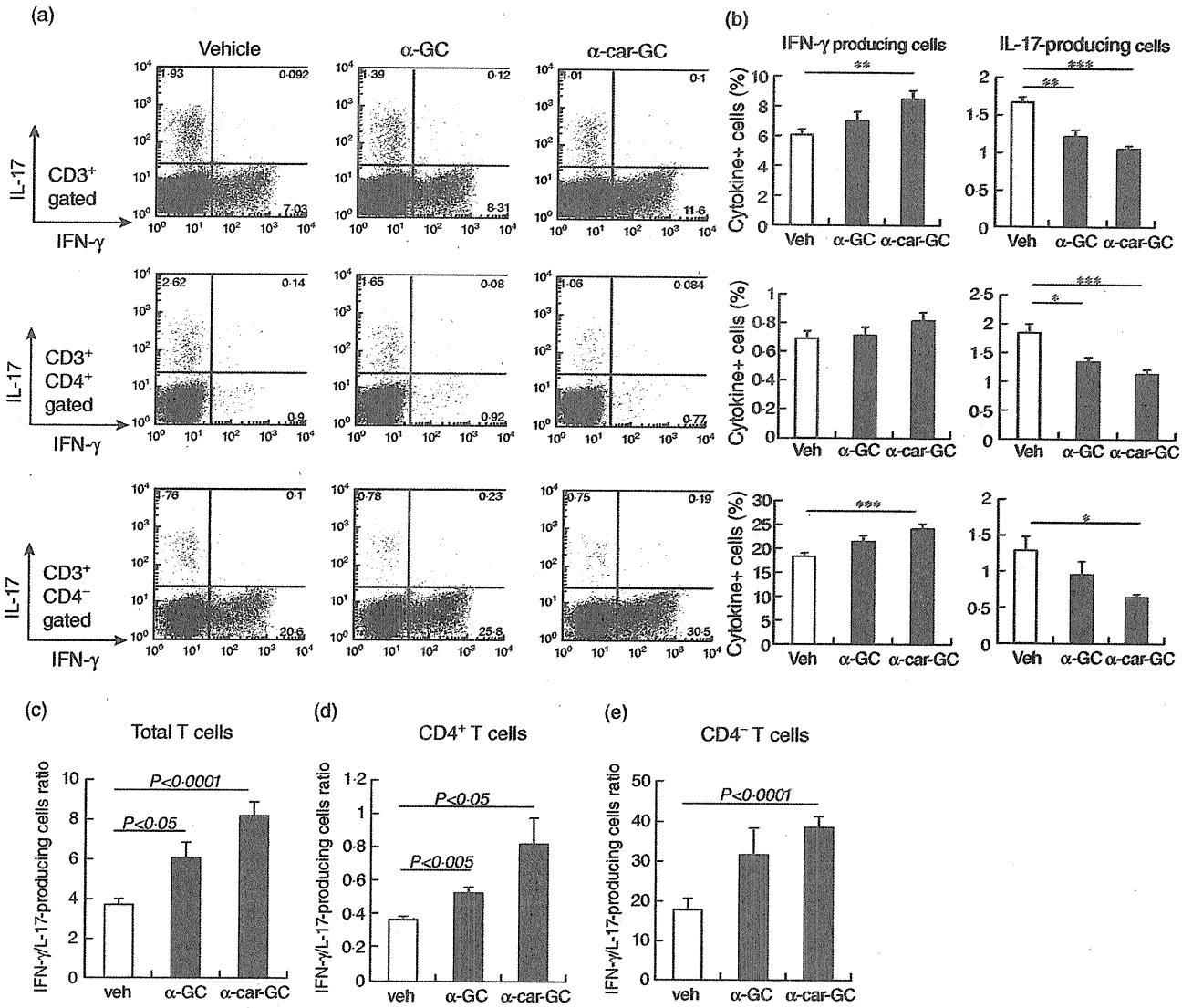

Fig. 6. α-carba-GalCer treatment polarizes T cell response towards T helper type 1 (Th1) and suppresses Th17 cell activation or differentiation. Ten days after type II collagen (CII) and vehicle, α-galactosylceramide (α-GalCer) or α-carba-GalCer immunization, draining lymph node (DLN) cells were stimulated 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

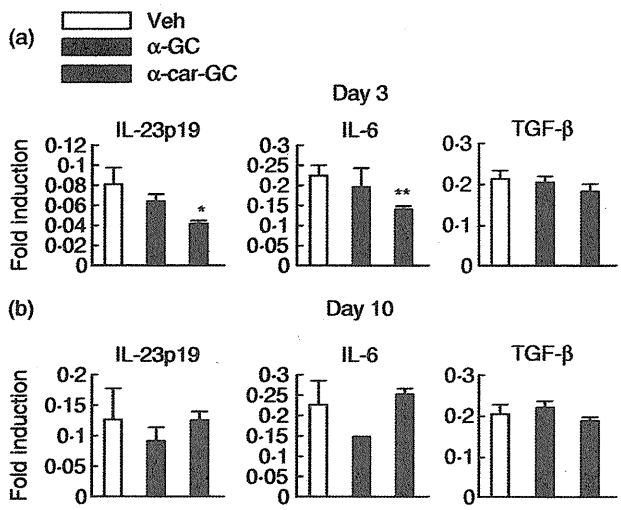

Fig. 7. α-carba-GalCer-treatment attenuates T helper type 17 (Th17)-related gene expression in the initial phase of collagen-induced arthritis (CIA). RNA was purified from draining lymph node (DLN) cells of each mouse (a) 3 days and (b) 10 days after glycolipid and type II collagen (CII) co-immunization. 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.

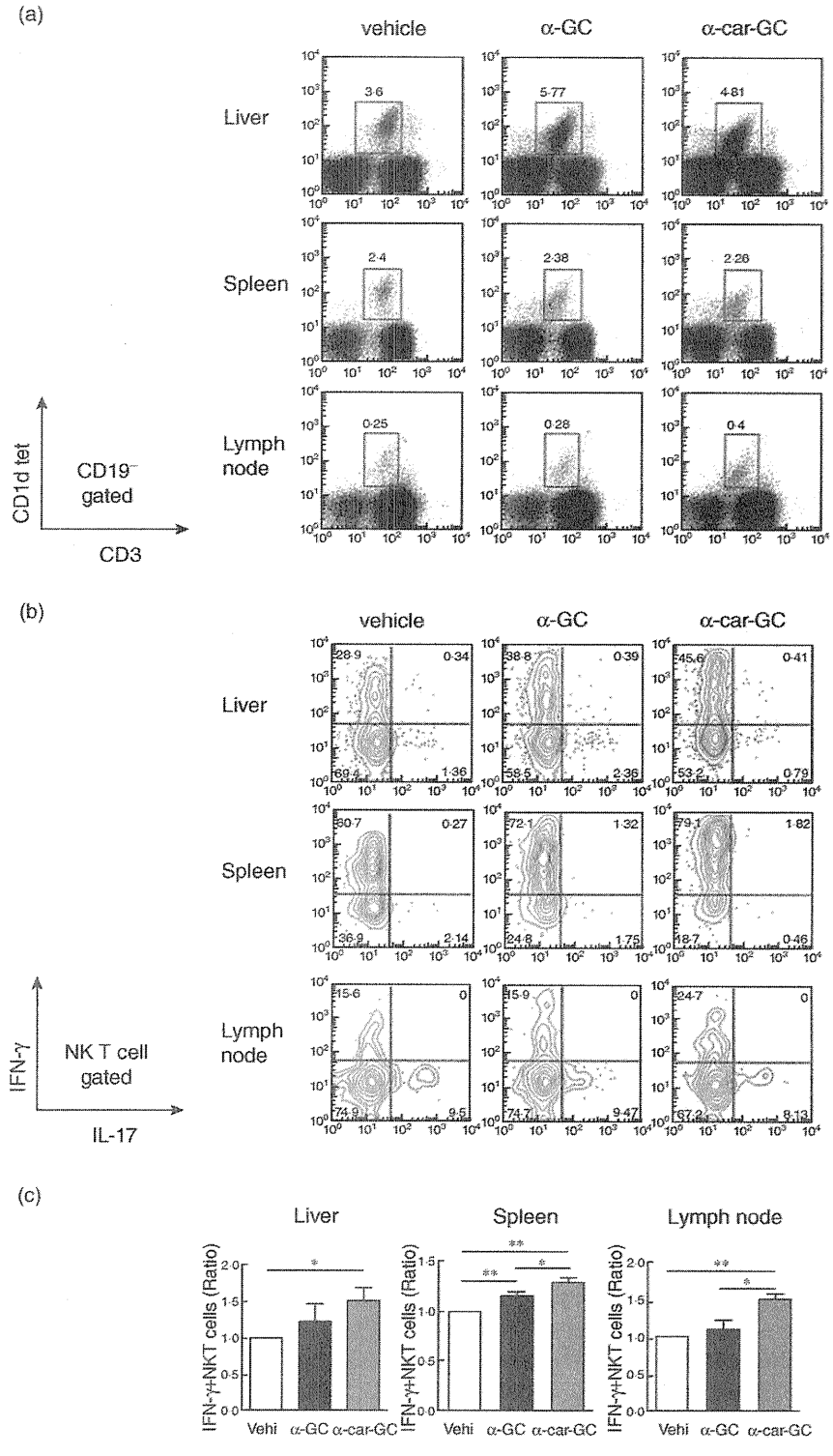

Fig. 8. α-carba-GalCer treatment enhanced natural killer (NK) T cell activation and interferon (IFN)-γ production in the initial phase of collagen-induced arthritis (CIA). Three days after type II collagen (CII) and vehicle, α-galactosylceramide (α-GalCer) or α-carba-GalCer immunization, liver, spleen 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.

References

1 Harris ED Jr. Rheumatoid arthritis. Pathophysiology and implications for therapy. N Engl J Med 1990; **322**:1277–89.

2 Luross JA, Williams NA. The genetic and immunopathological processes underlying collagen-induced arthritis. Immunology 2001; **103**:407–16.

3 Ferber IA, Brocke S, Taylor-Edwards C *et al.* Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J Immunol 1996; **156**:5–7.

4 Willenborg DO, Fordham S, Bernard CC, Cowden WB, Ramshaw IA. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. J Immunol 1996; **157**:3223–7.

5 Vermeire K, Heremans H, Vandeputte M, Huang S, Billiau A, Matthys P. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. J Immunol 1997; **158**:5507–13.

6 Cua DJ, Sherlock J, Chen Y *et al.* Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003; **421**:744–8.

7 Murphy CA, Langrish CL, Chen Y *et al.* Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 2003; **198**:1951–7.

8 Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003; **171**:6173–7.

9 Komiyama Y, Nakae S, Matsuki T *et al.* IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol 2006; **177**:566–73.

10 Harrington LE, Hatton RD, Mangan PR *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct

from the T helper type 1 and 2 lineages. Nat Immunol 2005; 6:1123–32.
11 Chu CQ, Swart D, Alcorn D, Tocker J, Elkon KB. Interferon-gamma regulates susceptibility to collagen-induced arthritis through suppression of interleukin-17. Arthritis Rheum 2007; 56:1145–51.
12 Dalton DK, Haynes L, Chu CQ, Swain SL, Wittmer S. Interferon gamma eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. J Exp Med 2000; 192:117–22.
13 Chu CQ, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. J Exp Med 2000; 192:123–8.
14 Wang Z, Hong J, Sun W *et al.* Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25− T cells to CD4+ Tregs. J Clin Invest 2006; 116:2434–41.
15 Kronenberg M, Gapin L. The unconventional lifestyle of NK T cells. Nat Rev Immunol 2002; 2:557–68.
16 Van Kaer L. alpha-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. Nat Rev Immunol 2005; 5:31–42.
17 Sumida T, Sakamoto A, Murata H *et al.* Selective reduction of T cells bearing invariant Vα24JαQ antigen receptor in patients with systemic sclerosis. J Exp Med 1995; 182:1163–8.
18 Sumida T, Maeda T, Taniguchi M, Nishioka K, Stohl W. TCRAV24 gene expression in double negative T cells in systemic lupus erythematosus. Lupus 1998; 7:565–8.
19 Maeda T, Keino H, Asahara H, Taniguchi M, Nishioka K, Sumida T. Decreased TCR AV24AJ18+ double negative T cells in rheumatoid synovium. Rheumatology 1999; 38:186–8.
20 Kojo S, Adachi Y, Keino H, Taniguchi M, Sumida T. Dysfunction of T cell receptor AV24AJ18+, BV11+ double-negative regulatory natural killer T cells in autoimmune diseases. Arthritis Rheum 2001; 44:1127–38.
21 Hong S, Wilson MT, Serizawa I *et al.* The natural killer T-cell ligand a-galactosylceramide prevents autoimmune diabetes in NOD mice. Nat Med 2001; 7:1052–6.
22 Singh AK, Wilson MT, Hong S *et al.* Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. J Exp Med 2001; 194:1801–11.
23 Yang JQ, Singh AK, Wilson MT *et al.* Immunoregulatory role of CD1d in the hydrocarbon oil-induced model of lupus nephritis. J Immunol 2003; 171:2142–53.
24 Chiba A, Kaieda S, Oki S, Yamamura T, Miyake S. The involvement of Vα14 natural killer T cells in the pathogenesis of arthritis in murine models. Arthritis Rheum 2005; 52:1941–8.
25 Ohnishi Y, Tsutsumi A, Goto D *et al.* TCR Valpha14 natural killer T cells function as effector T cells in mice with collagen-induced arthritis. Clin Exp Immunol 2005; 141:47–53.
26 Yoshiga Y, Goto D, Segawa S *et al.* Invariant NK T cells produce IL-17 through IL-23-dependent and -independent pathways with potential modulation of Th17 response in collagen-induced arthritis. Int J Mol Med 2008; 22:369–74.
27 Kim HY, Kim HJ, Min HS *et al.* NK T cells promote antibody-induced joint inflammation by suppressing transforming growth factor β1 production. J Exp Med 2005; 201:41–7.
28 Kim HY, Kim S, Chung DH. FcgammaRIII engagement provides activating signals to NK T cells in antibody-induced joint inflammation. J Clin Invest 2006; 116:2484–92.
29 Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNK T cells, the 'Swiss-Army knife' of the immune system [Review]. Curr Opin Immunol 2008; 20:358–68.
30 Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature 2001; 413:531–4.
31 Schmieg J, Yang G, Franck RW, Tsuji M. Superior protection against malaria and melanoma metastases by a C-glycoside analog of the natural killer T cell ligand alpha-galactosylceramide. J Exp Med 2003; 198:1631–41.
32 Tashiro T, Sekine-Kondo E, Shigeura T *et al.* Induction of Th1-biased cytokine production by alpha-carba-GalCer, a neoglycolipid ligand for NK T cells. Int Immunol 2010; 22:319–28.
33 Biburger M, Tiegs G. Activation-induced NK T cell hyporesponsiveness protects from alpha-galactosylceramide hepatitis and is independent of active transregulatory factors. J Leukoc Biol 2008; 84:264–79.
34 Cho YG, Cho ML, Min SY, Kim HY. Type II collagen autoimmunity in a mouse model of human rheumatoid arthritis. Autoimmun Rev 2007; 7:65–70.
35 Harada M, Seino K, Wakao H *et al.* Down-regulation of the invariant Valpha14 antigen receptor in NK T cells upon activation. Int Immunol 2004; 16:241–7.
36 Doisne JM, Becourt C, Amniai L *et al.* Skin and peripheral lymph node invariant NK T cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. J Immunol 2009; 183:2142–9.
37 Bradley LM, Dalton DK, Croft M. A direct role for IFN- in regulation of Th1 cell development. J Immunol 1996; 157:1350–8.
38 Yang YF, Tomura M, Ono S, Hamaoka T, Fujiwara H. Requirement for IFN-gamma in IL-12 production induced by collaboration between v(alpha)14(+) NK T cells and antigen-presenting cells. Int Immunol 2000; 12:1669–75.
39 Sheikh SZ, Matsuoka K, Kobayashi T, Li F, Rubinas T, Plevy SE. Cutting edge: IFN-gamma is a negative regulator of IL-23 in murine macrophages and experimental colitis. J Immunol 2010; 184:4069–73.
40 Eberl G, Brawand P, MacDonald HR. Selective bystander proliferation of memory CD4+ and CD8+ T cells upon NK T or T cell activation. J Immunol 2000; 165:4305–1.
41 Seki N, Sudo Y, Yoshioka T *et al.* Type II collagen-induced murine arthritis: induction and perpetuation of arthritis require synergy between humoral and cell-mediated immunity. J Immunol 1998; 140:1477–84.
42 Watson WC, Townes AS. Genetic susceptibility to murine collagen II autoimmune arthritis: proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC), and non-MHC loci. J Exp Med 1985; 162:1878–91.
43 Snapper CM, Peçanha LM, Levine AD, Mond JJ. IgE class switching is critically dependent upon the nature of the B cell activator, in addition to the presence of IL-4. J Immunol 1991; 147:1163–70.
44 Bossie A, Vitetta ES. IFN- enhances secretion of IgG2a from IgG2a-committed LPS-stimulated murine B cells: implications for the role of IFN- in class switching. Cell Immunol 1991; 135:95–104.
45 Doreau A, Belot A, Bastid J *et al.* Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus. Nat Immunol 2009; 10:778–85.

46 Jacob CO, Holoshitz J, Van der Meide P, Strober S, McDevitt HO. Heterogeneous effects of IFN-gamma in adjuvant arthritis. J Immunol 1989; 142:1500–5.
47 Young HA, Hardy KJ. Role of interferon-gamma in immune cell regulation. J Leukoc Biol 1995; 58:373.
48 Miellot A, Zhu R, Diem S, Boissier MC, Herbelin A, Bessis N. Activation of invariant NK T cells protects against experimental rheumatoid arthritis by an IL-10-dependent pathway. Eur J Immunol 2005; 35:3704–13.
49 Coppieters K, Van Beneden K, Jacques P *et al.* A single early activation of invariant NK T cells confers long-term protection against collagen-induced arthritis in a ligand-specific manner. J Immunol 2007; 179:2300–9.
50 Hermans IF, Silk JD, Gileadi U *et al.* NK T cells enhance CD4+ and CD8+ T-cell responses to soluble antigen *in vivo* through direct interaction with dendritic cells. J Immunol 2003; 171:5140–7.

Involvement of NK 1.1–Positive γδT Cells in Interleukin-18 Plus Interleukin-2–Induced Interstitial Lung Disease

Seiji Segawa[1], Daisuke Goto[1], Yohei Yoshiga[1], Masanobu Horikoshi[1], Makoto Sugihara[1], Taichi Hayashi[1], Yusuke Chino[1], Isao Matsumoto[1], Satoshi Ito[1], and Takayuki Sumida[1]

[1]Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

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.

CLINICAL RELEVANCE

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

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

(Received in original form July 14, 2010 and in final form December 22, 2010)

This study was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science, and by the Japanese Ministry of Health, Labor, and Welfare.

Correspondence and requests for reprints should be addressed to Takayuki Sumida, M.D., Ph.D., Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: tsumida@md.tsukuba.ac.jp

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 45. pp 659–666, 2011
Originally Published in Press as DOI: 10.1165/rcmb.2010-0298OC on January 21, 2011
Internet address: www.atsjournals.org

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-$\gamma^{-/-}$ 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$^+$ 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-$\gamma^{-/-}$) mice with a C56BL/6 background were purchased from Charles River Japan, Inc. (Tokyo, Japan). TCRδ-deficient (TCR$\delta^{-/-}$) 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 TCR$\delta^{-/-}$ 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).

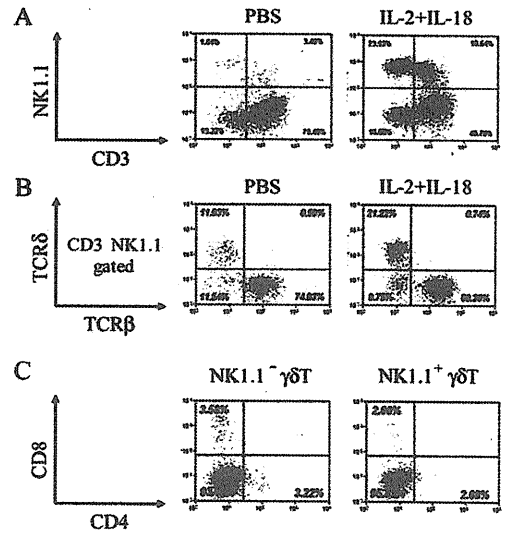

Figure 1. Accumulation of natural killer (NK) 1.1$^+$γδT cells in addition to NK cells in the lungs of mice with IL-18 plus IL-2–induced interstitial lung disease (ILD). (*A*) Pulmonary lymphocytes were harvested from B6 mice treated with PBS or IL-18/IL-2, as described in MATERIALS AND METHODS. Cells 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.

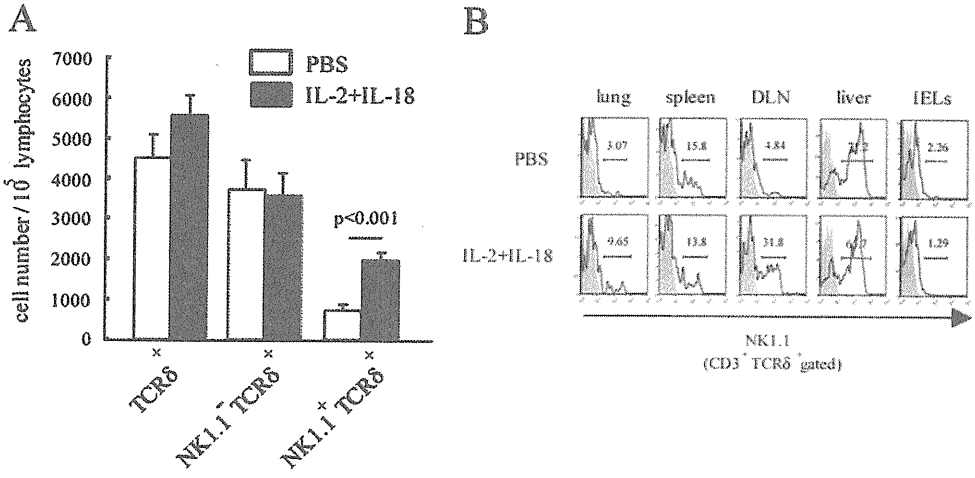

Figure 2. Increased proportion of NK1.1$^+$ γδT cells in lungs, spleens, and draining lymph nodes (DLNs) of mice with IL-18 plus IL-2–induced ILD. (*A*) Pulmonary lymphocytes were harvested from B6 mice treated with IL-18/IL-2 or PBS, as described in MATERIALS AND METHODS. Cells were stained with anti-NK1.1, 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-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% ± 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% ±

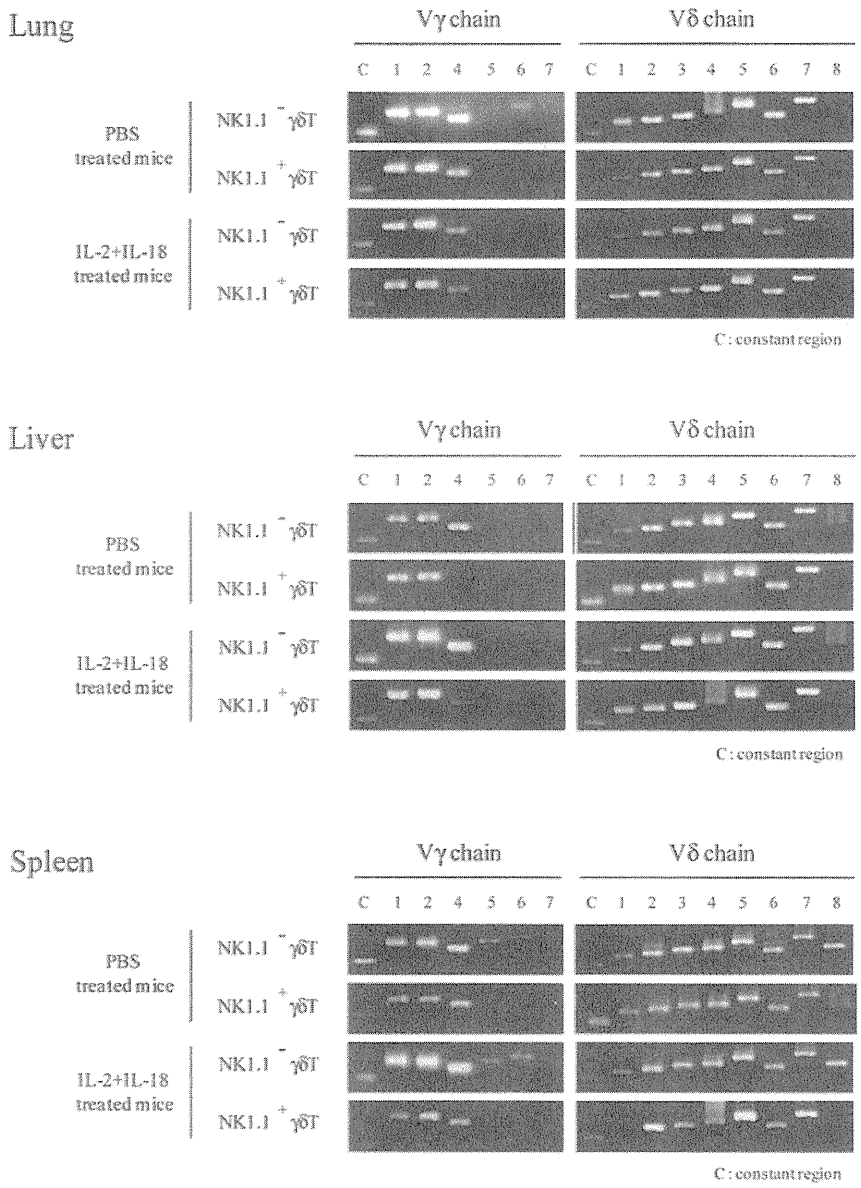

Figure 3. TCR-Vγ and TCR-Vδ repertoire of NK1.1$^+$ γδT cells from mice with IL-18 plus IL-2–induced ILD. TCR-Vγ and TCR-Vδ gene expression in NK1.1$^-$ and NK1.1$^+$ γδT cells were purified from pulmonary lymphocytes, splenocytes, and liver lymphocytes of PBS-treated or IL-18/IL-2–treated mice, and 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

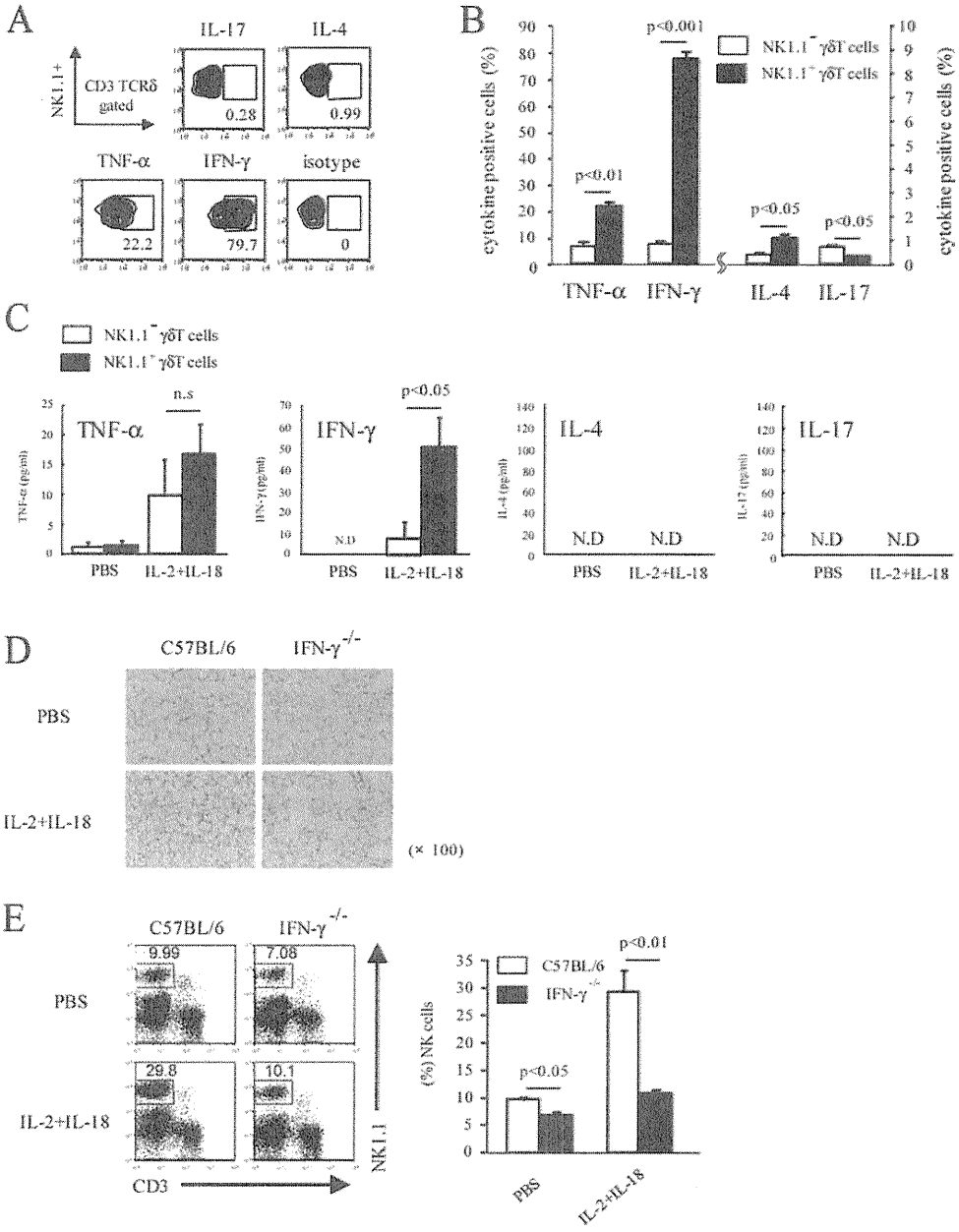

Figure 4. Cytokine profile of NK1.1$^-$ γδT cells and NK1.1$^+$ γδT cells. (*A*) Intracellular staining of TNF-α, IFN-γ, IL-4, and IL-17 was performed after the *in vitro* stimulation of splenocytes from naive B6 mice by phorbol 12-myristate 13-acetate (PMA)/ionomycin for 6 hours, and analyzed among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