

NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor β 1 production

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Although NKT cells has been known to exert protective roles in the development of autoimmune diseases, the functional roles of NKT cells in the downstream events of antibody-induced joint inflammation remain unknown. Thus, we explored the functional roles of NKT cells in antibody-induced arthritis using the K/BxN serum transfer model. NKT cell-deficient mice were resistant to the development of arthritis, and wild-type mice administrated with α -galactosyl ceramide, a potent NKT cell activator, aggravated arthritis. In CD1d^{-/-} mice, transforming growth factor (TGF)- β 1 was found to be elevated in joint tissues, and the blockade of TGF- β 1 using neutralizing monoclonal antibodies restored arthritis. The administration of recombinant TGF- β 1 into C57BL/6 mice reduced joint inflammation. Moreover, the adoptive transfer of NKT cells into CD1d^{-/-} mice restored arthritis and reduced TGF- β 1 production. In vitro assay demonstrated that interleukin (IL)-4 and interferon (IFN)- γ were involved in suppressing TGF- β 1 production in joint cells. The adoptive transfer of NKT cells from IL-4^{-/-} or IFN- γ ^{-/-} mice did not reverse arthritis and TGF- β 1 production in CD1d^{-/-} mice. In conclusion, NKT cells producing IL-4 and IFN- γ play a role in immune complex-induced joint inflammation by regulating TGF- β 1.

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NKT cells express intermediate levels of a semi-invariant V α 14-J α 281 (V α 14i) TCR in mice or an invariant V α 24-J α 15 TCR in humans (1), which recognizes glycolipid antigens presented by the CD1d (2). Upon activation, NKT cells rapidly produce large amounts of IL-4 and IFN- γ (3), which have been demonstrated to play critical roles in the regulation of innate and adaptive immune responses by NKT cells (4). Thus, it has been proposed that NKT cells exert regulatory functions in autoimmune diseases by establishing an early cytokine environment. In animal models, NKT cells have been reported to affect the development and progression of diabetes mellitus (5), experimental autoimmune encephalitis (6), and systemic lupus erythematosus (7). However, the functional roles of NKT cells in the development of autoimmune arthritis have not been completely explored.

Recently, a spontaneous murine arthritis model was developed by breeding KRN TCR transgenic (Tg) mice in the background of B6

to the nonobese diabetic (NOD) mice (8). KRN TCR has been reported to be specific for a peptide of bovine RNase (42–56) bound to I-A^k presented by APCs (8). The offspring (K/BxN) spontaneously develop a progressive joint-specific autoimmune disease (8). In K/BxN mice, T cells with KRN TCR recognize peptide derived from glucose-6-phosphate isomerase in the context of I-A^{B7} expressed on APCs, and B cells produce arthrogenic Ig against glucose-6-phosphate isomerase (9). The transfer of serum from K/BxN mice into susceptible mice induces a synchronized joint inflammation that mimics K/BxN disease (10). Unlike other arthritis animal models, the K/BxN serum transfer model is confined to the inflammatory responses induced by the deposition of autoantibody in joint spaces (10). Thus, it allows one to explore the downstream events of antibody-induced joint inflammation and the terminal effector mechanism of rheumatoid arthritis (RA). Recently NKT cells were reported to be a subpopulation of T cells that critically exert a functional link between innate and adoptive immunity in immune responses in vivo (11).

The online version of this article contains supplemental material.

Nevertheless, the role of NKT cells at the end-stage of the effector mechanism of joint inflammation where innate immune responses are critically involved remains unclear.

Our results show that NKT cells play an indispensable role in the induction of immune complex-induced joint inflammation by suppressing TGF- β 1 production in joint tissues, which in turn is dependent on IL-4 and IFN- γ secreted by NKT cells.

RESULTS AND DISCUSSION

NKT cells promote joint inflammation in the antibody-induced arthritis

To determine the specific role of NKT cells in the development of antibody-induced arthritis, we examined NKT cell-

deficient mice, CD1d^{-/-} and J α 281^{-/-} mice, and B6 mice in the K/BxN serum transfer model. Whereas B6 mice showed measurable swelling at 3–4 d after serum transfer, which peaked at 8–9 d, CD1d^{-/-} and J α 281^{-/-} mice were resistant to the development of joint inflammation for 6 d and showed a gradual increase in ankle swelling after 7 d (Fig. 1 A). The maximal thickness of joint swelling in CD1d^{-/-} and J α 281^{-/-} mice was much smaller than that of B6 mice. Histological examination of the ankle joints of B6 mice at 5 d revealed a marked infiltration of neutrophils in synovial fluid and connective tissues (Fig. 1 B). Unlike B6 mice, CD1d^{-/-} and J α 281^{-/-} mice showed mild inflammatory cell infiltration in ankle joints and surrounding connec-

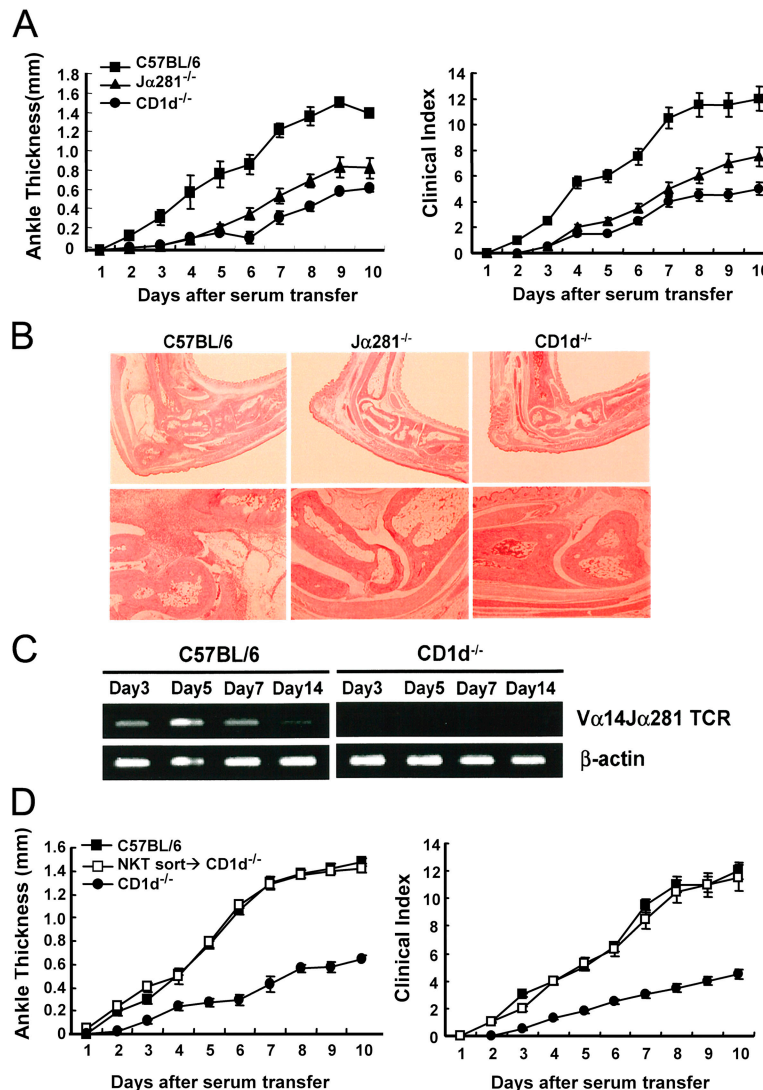


Figure 1. NKT cell-deficient mice attenuate arthritis in the K/BxN serum transfer model. (A) Clinical scores and ankle thickness was monitored in J α 281^{-/-} (black triangle), CD1d^{-/-} (black circle), and B6 mice (black square) after injection of K/BxN serum. (B) Histological examination of the ankle joint of a B6 mouse and of J α 281^{-/-} and CD1d^{-/-} mice 5 d after serum transfer. A magnification of 12.5 in the top panels and 40 in the bottom panels. (C) RT-PCR was used to determine the level of V α 14J α 281 TCR mRNA in the ankle joint tissues of B6 mice and CD1d^{-/-}

mice 3, 5, 7, and 14 d after K/BxN serum transfer. (D) Sorted NKT cells (3×10^5 cells/mouse) from B6 mice (white square) were adoptively transferred into CD1d^{-/-} mice 1 d before K/BxN serum transfer. Clinical scores and ankle thickness were monitored and compared with B6 (black square) and CD1d^{-/-} mice (black circle). Data are the means \pm SEM of three mice in each group. These results are taken from a representative one of six repeated experiments (A and C).

tive tissues (Fig. 1 B). To test for the infiltration of NKT cells in joint tissues, we measured the level of V α 14J α 281 TCR mRNA in the joint tissue of B6 mice after K/BxN serum transfer. RT-PCR assays showed V α 14J α 281 TCR mRNA at 3, 5, and 7 d after serum injection and weak intensity band on day 14, which was not demonstrated in CD1d $^{-/-}$ mice (Fig. 1 C). However, no transcripts for V α 14J α 281 TCR were detected in the joint of B6 mice at day 0, indicating that NKT cells infiltrate joint tissues during antibody-induced arthritis (not depicted). To demonstrate that the lack of NKT cells specifically caused the failure of CD1d $^{-/-}$ mice to develop arthritis, we adoptively transferred NKT cells from normal B6 mice (Fig. 1 D) into CD1d $^{-/-}$ mice. Joint swellings in CD1d $^{-/-}$ mice transferred with the NKT cells of normal B6 mice were as thick as those of B6 mice. Furthermore, joint swellings in CD1d $^{-/-}$ mice transferred with splenocytes from RAG $^{-/-}$ V α 14 $^{\text{tg}}$ V β 8.2 $^{\text{tg}}$ (V α 14J α 281 TCR Tg RAG $^{-/-}$) mice were as thick as those of B6 mice, whereas CD1d $^{-/-}$ mice transferred with splenocytes from J α 281 $^{-/-}$ mice did not develop joint inflammation (Fig S1 A, available at <http://www.jem.org/cgi/content/full/jem.20041400/DC1>). Splenocytes from V α 14J α 281 TCR Tg RAG $^{-/-}$ mice contain a large number of V α 14i TCR NKT cells in the absence of conventional $\alpha\beta$ T cells and B cells compared with those from C57BL/6 mice, whereas the splenocytes of J α 281 $^{-/-}$ mice are deficient in V α 14i TCR NKT cells (4). These data indicate that adoptively transferred NKT cells restored arthritis in CD1d $^{-/-}$ mice in the K/BxN serum transfer model. Next, we investigated whether the activation of NKT cells affects arthritis using α -galactosyl ceramide (α -GalCer), a potent NKT cell stimulant. When we injected B6 mice with α -GalCer, joint swelling in these mice was aggravated (Fig. 2). These data indicate that the *in vivo* activation of NKT cells using α -GalCer aggravated joint inflammation during K/BxN serum-transferred arthritis.

It has been reported that patients with RA have abnormalities in the number of TCR α V24 $^+$ β V11 $^+$ double negative NKT cells in peripheral blood lymphocytes compared with healthy persons (12). In the mouse system, the administration of OCH, a sphingosine-truncated analogue of α -GalCer, into B6 mice inhibits collagen-induced arthritis (13). These results provide supporting evidence of the protective roles of NKT cells in autoimmune arthritis. However, our results demonstrate that NKT cells provoke inflammatory responses in the joint tissues of the K/BxN serum transfer model. These contradictory findings for the functional roles of NKT cells in joint inflammation led us to speculate that NKT cells play diverse roles in autoimmune arthritis depending on the disease processes. In the K/BxN serum transfer model, the immune complexes on the cartilage surface were found to engage Fc γ RIII on mast cells and macrophages in the synovium (8, 14). These findings indicate that the K/BxN serum transfer model is one of the typical Arthus reactions, which does not include adoptive immune responses. Thus, the provocative roles of NKT cells in the K/BxN se-

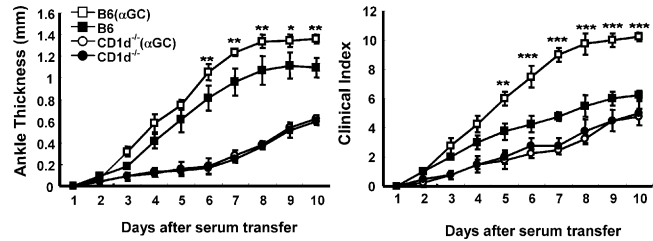


Figure 2. The administration of α -GalCer into B6 mice aggravates arthritis. B6 mice were injected with 1 μ g α -GalCer (white square) three times a week before and after the administration of 70 μ l K/BxN serum and are compared with B6 (black square), CD1d $^{-/-}$ (black circle), and α -GalCer-injected CD1d $^{-/-}$ mice (white circle). Data are the means \pm SEM of four mice in each group. The statistical analysis was performed using the Prism 3.0 program. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

rum transfer model represent a novel function in immune complex-induced inflammatory responses and not the suppressive role of NKT cells described in adoptive autoimmune responses (5, 15). However, NKT cells play different roles in different models for antibody-induced autoimmune diseases. A recent report has provided evidence that α -GalCer suppresses disease in a model for Graves' thyroiditis (16), whereas the functional roles of NKT cells was not found in an experimental autoimmune myasthenia gravis model (17). Taken together, it is conceivable that NKT cells have a dual functionality that depends on the disease processes of RA, a suppressive role in the inductive phase, and a provocative role with respect to antibody-induced joint inflammation.

TGF- β 1 exerts antiinflammatory effects on antibody-induced arthritis

To evaluate the mechanism by which NKT cells contribute to the development of arthritis, we measured the mRNA levels of various proinflammatory cytokines in joint tissues. Upon K/BxN serum transfer, CD1d $^{-/-}$ mice showed higher levels of the TGF- β 1 transcript, whereas B6 mice showed reduced levels of the TGF- β 1 transcript on days 3, 5, 7, and 14. Conversely, IFN- γ and IL-4 were lower in CD1d $^{-/-}$ mice than in B6 mice (Fig. 3 A). In contrast to joint tissues, the transcript levels of TGF- β 1, IL-4, and IFN- γ in the spleen were similar in B6 and CD1d $^{-/-}$ mice in the K/BxN serum transfer model (Fig. 3 B). The reconstitution of CD1d $^{-/-}$ mice with either NKT cells from B6 or V α 14J α 281 TCR Tg RAG $^{-/-}$ mice reduced TGF- β 1 mRNA and increased the levels of IFN- γ and IL-4 transcript in the joint tissues (Fig. 3 B and Fig. S1 B). In contrast, the adoptive transfer of NKT cells did not alter the transcript level of TGF- β 1 or IFN- γ and IL-4 in the spleens of CD1d $^{-/-}$ mice (Fig. 3 B). These results suggest that the TGF- β 1 might be involved in the resistance to joint inflammation shown by CD1d $^{-/-}$ mice, and this is tightly regulated by NKT cells infiltrating joint tissue in the K/BxN serum transfer model. To establish a functional link between TGF- β 1 activity and arthritis, a blocking anti-TGF- β mAb and recombinant TGF- β 1 were injected i.p into CD1d $^{-/-}$ and B6 mice in a K/BxN serum transfer model. TGF- β 1

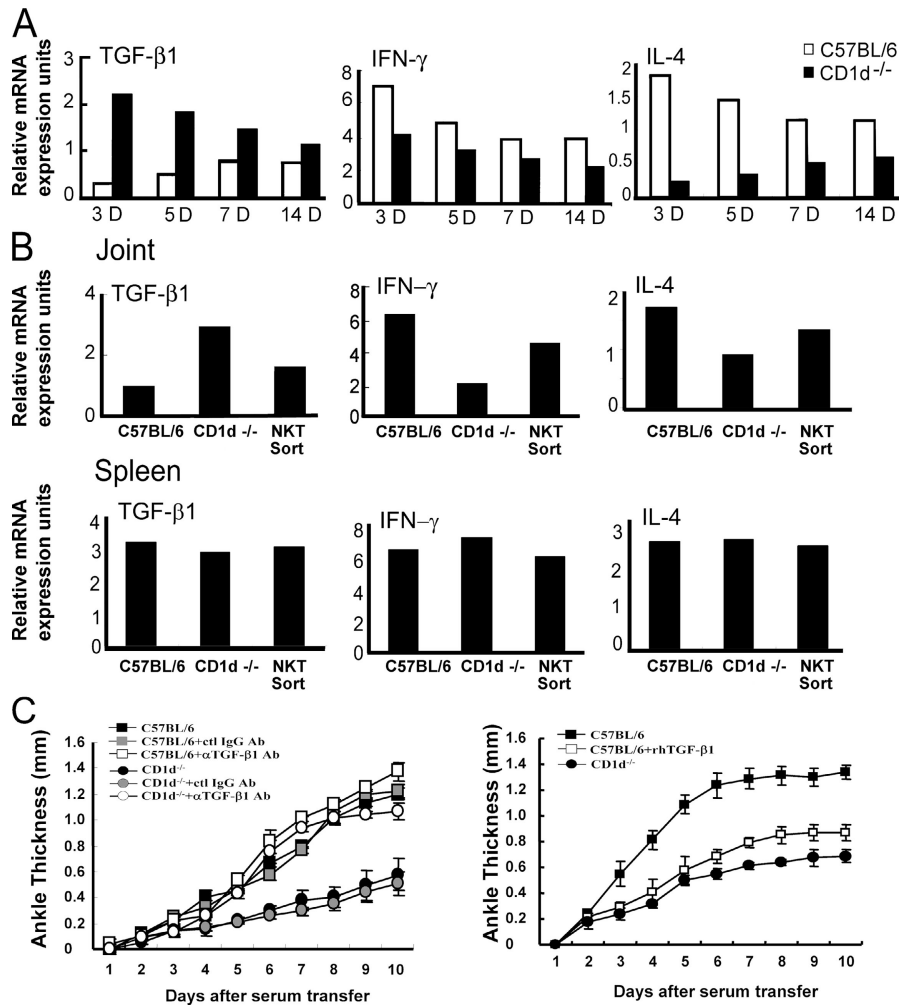


Figure 3. TGF- β 1 exerts antiinflammatory effects on the joint tissues of CD1d^{-/-} mice in the K/BxN serum transfer models. (A) The transcript levels of IL-4, IFN- γ , and TGF- β 1 were measured by real-time PCR in joint. (B) The sorted NKT cells of B6 mice were reconstituted in CD1d^{-/-} mice. The transcript levels of TGF- β 1, IL-4, and IFN- γ were measured by real-time PCR in the joint tissues and the spleens. (C) 500 μ g anti-TGF- β 1 mAb was injected i.p. into CD1d^{-/-} mice three times per week before and after the administration of K/BxN serum. Ankle thickness was monitored

blockade in CD1d^{-/-} mice induced a significant increase in joint swelling and clinical index, whereas TGF- β 1 blockade in B6 mice did not apparently increase joint inflammation (Fig. 3 C). The recombinant TGF- β 1 administered into B6 mice suppressed joint inflammation as compared with B6 mice in the serum transfer model (Fig. 3 D). These findings revealed that the regulatory effects of NKT cells on arthritis depend on the amount of TGF- β 1 produced in joint tissues. However, the roles of NKT cells as effector cells were not completely ruled out in antibody-induced joint inflammation. Therefore, its effector functions in this model should be explored further.

In animal models, TGF- β 1 down-regulates proinflammatory cytokine production and exerts protective effects on collagen-induced arthritis in mice (18). Conversely, it has been demonstrated that TGF- β is also capable of promoting

in B6 (black square), CD1d^{-/-} (black circle), control IgG-injected CD1d^{-/-} (gray circle), and B6 (gray square) mice, and anti-TGF- β 1 mAb-injected CD1d^{-/-} (white circle) and B6 mice (white square). (D) B6 mice were injected i.p. with recombinant TGF- β 1 during the development of arthritis. Joint swelling was measured and compared with B6 (black square), CD1d^{-/-} (black circle), and recombinant TGF- β 1-injected B6 mice (white square). Data are the mean \pm SEM of three mice in each group.

inflammation by enhancing neutrophilic infiltration and the induction of angiogenesis in joint tissues (19). Thus, it has been suggested that the balance between the proinflammatory and antiinflammatory effects of TGF- β in joint inflammation is crucial to RA outcome (20).

TGF- β 1 production in synovium is suppressed by IL-4 and IFN- γ secreted by NKT cells

To explore how NKT cells induce arthritis by regulating the production of TGF- β 1 in our model, synovial cells were taken from mice treated with K/BxN serum and then cultured with Con A to induce the production of TGF- β 1. TGF- β 1 production was suppressed by the activation of NKT cells using α -GalCer and restored by blocking the interaction between CD1d and TCR on NKT cells with anti-CD1d mAb (Fig. 4 A). Furthermore, when we injected B6

mice with α -GalCer, TGF- β 1 production in joint tissues was reduced versus control mice in the K/BxN serum transfer model (Fig. 4 B). These results indicate that activated NKT cells specifically suppress the production of TGF- β 1 by immune cells of synovium. It is well known that NKT cells secrete IL-4, IFN- γ , and IL-13 and enhance the production of IL-10 in vivo upon activation (3, 4, 21). Thus, we attempted to identify cytokines involved in the suppression of TGF- β 1 production by NKT cells. IL-4, IL-10, IL-13, and IFN- γ were neutralized by specific mAbs during synovial and NKT cell activation in culture. Among the blocking mAbs tested, mAbs against IL-4 and IFN- γ partially restored TGF- β 1 production, whereas mAbs against IL-10, IL-13, and control IgG had no effect (Fig. 4 A). These results suggest that IL-4 and IFN- γ secreted by NKT cells might be involved in the suppression of TGF- β 1 production by synovial cells. Interestingly, a recent report provided evidence that CD1d-restricted T cells promote TGF- β production through IL-13 using a tumor model (22). However, IL-13 did not seem to be a major regulatory cytokine for production of TGF- β 1 in the K/BxN serum transfer model (Fig. 4 A). Next, we determined whether these cytokines secreted by NKT cells play critical roles in the development of joint inflammation and in the suppression of TGF- β 1 production in vivo. We transferred sorted NKT cells from either IL-4 $^{-/-}$ or IFN- γ $^{-/-}$ mice into CD1d $^{-/-}$ mice in the K/BxN transfer model and measured joint swelling and the transcriptional level of TGF- β 1 in joint tissues. Joint swelling in CD1d $^{-/-}$ mice adoptively transferred with NKT cells from IL-4 $^{-/-}$ or IFN- γ $^{-/-}$ mice was minimal as in CD1d $^{-/-}$ mice, whereas CD1d $^{-/-}$ mice adoptively transferred with NKT cells from normal B6 showed joint swelling as in B6 (Fig. 4, C and D). TGF- β 1 was increased at the transcript level in CD1d $^{-/-}$ mice adoptively transferred with NKT cells from IL-4 $^{-/-}$ or IFN- γ $^{-/-}$ mice versus CD1d $^{-/-}$ mice transferred with NKT cells from B6 (Fig. 4, C and D). Furthermore, the blockade of either IL-4 or IFN- γ using neutralizing mAb in V α 14J α 281 TCR Tg RAG $^{-/-}$ mice resulted in resistance to the development of joint inflammation and enhanced TGF- β 1 production in joint tissues (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20041400/DC1>). Thus, these results suggest that NKT cells induce arthritis by suppressing TGF- β 1 by producing IL-4 and IFN- γ in vivo. Several experiments demonstrated that IFN- γ is a negative regulator of TGF- β production and of the effects of TGF- β on immune cells in vivo and in vitro (23, 24). These findings explain how IFN- γ regulates joint inflammation by suppressing TGF- β 1 production in joint tissue. On the contrary to previous studies reporting that IL-4 promotes the differentiation of TGF- β -producing CD4 $^{+}$ T cells (23), our study shows that IL-4 secreted by NKT cells suppress the production of TGF- β 1 in joint tissues. It remains unclear how IL-4 suppresses TGF- β 1 production in the joint tissues of the K/BxN serum transfer model. Thus, we speculate that the effects of IL-4-induced cytokine production might be diverse depending on the different maturational stage of the responding cells and/or the murine model system.

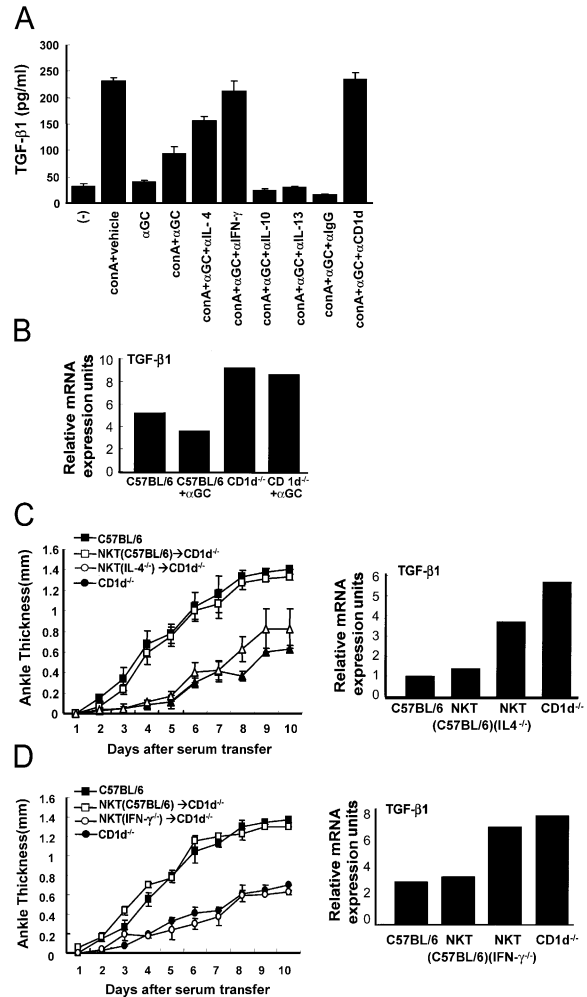


Figure 4. The suppressive effects of NKT cells on TGF- β 1 production in joint tissues are dependent on IL-4 and IFN- γ secreted from NKT cells. (A) Synovial cells were taken from B6 mice treated with K/BxN serum and cultured with Con A and α -GalCer. IL-4, IL-10, IL-13, and IFN- γ were neutralized, and CD1d was blocked using mAbs during synovial cell activation. TGF- β 1 production by synovial cells was determined by ELISA. (B) B6 mice were injected with α -GalCer, and TGF- β 1 transcript levels were measured in the ankle joint tissues by real-time PCR. (C) Sorted NKT cells (5×10^6 cells/mice) from IL-4 $^{-/-}$ (white circle), (D) IFN- γ $^{-/-}$ mice (white circle), and B6 (white square) were adoptively transferred into CD1d $^{-/-}$ mice in the K/BxN serum transfer model. Joint swelling was measured and compared with B6 (black square) and CD1d $^{-/-}$ controls (black circle). The level of TGF- β 1 mRNA was measured in the ankle joint tissues of B6, CD1d $^{-/-}$, and CD1d $^{-/-}$ mice treated with either sorted NKT cells from B6 or IL-4 $^{-/-}$ or IFN- γ $^{-/-}$ mice by real-time PCR 7 d after serum transfer.

Nevertheless, the mechanism for the suppression of TGF- β 1 by IL-4 in this model should be explored further.

In summary, our studies suggest that NKT cells in joint tissue may play an essential role in the end-stage effector phase of RA, and that inflammatory responses in RA might be regulated by modulating NKT cells in joint tissues.

MATERIALS AND METHODS

Mice. KRN TCR Tg mice and NOD mice were provided by D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and from the Institut

de Génétique et de Biologie Moléculaire et Cellulaire, and were maintained in a C57BL/6(B6) background (K/B). Arthritic mice (K/BxN) were obtained by crossing K/B with NOD (N) mice. CD1d^{-/-} mice were provided by Hua Gu (Columbia University, New York, NY). J α 281^{-/-} and RAG^{-/-} V α 14⁸ V β 8.2⁸ (V α 14J α 281 TCR Tg RAG^{-/-}) mice were provided by M. Taniguchi (Chiba University, Chiba, Japan). IL-4^{-/-} and IFN- γ ^{-/-} mice were purchased from The Jackson Laboratory. B6 mice were purchased from DaeHan Biolink. These mice were bred and maintained under specific pathogen-free conditions at the Clinical Research Institute Seoul National University Hospital (CRISNUH). All animal experiments was performed after receiving approval of the Institutional Animal Care and Use Committee of CRISNUH.

Serum transfer and arthritis scoring. Arthritic adult K/BxN mice were bled and sera were pooled. Recipient mice were injected i.p. with 150 μ l (except for the experiment for α -GalCer stimulation in vivo) of pooled K/BxN sera on days 0 and 2. Ankle thickness was measured with a caliper (Manostat). Joint swellings were monitored and scored as follows. 0, no joint swelling; 1, swelling of one finger joint; 2, mild swelling of wrist or ankle; 3, severe swelling of wrist or ankle.

Histological examination. Whole knee joints and hind paws were fixed in 10% formalin, decalcified, and paraffin embedded. Sections were prepared from the joint tissue blocks and stained with hematoxylin and eosin.

Adoptive transfer experiment. After mice were killed, livers were homogenized and resuspended in loading buffer (PBS plus 10% FBS and 1 mM EDTA) and overlaid onto lympholyte-M (Cedarlane). After centrifugation for 20 min at 900 *g* at 25°C, liver mononuclear cells were isolated from the interface. Obtained cells were stained with PE-conjugated anti-NK1.1 (BD Biosciences) and CyChrome-conjugated anti-TCR β (BD Biosciences). Stained cells were then sorted by FACStarPlus using CELLQuest software, and the purity of sorted cells was >98%.

Real-time PCR and RT-PCR analysis. The joint tissues were homogenized using a Polytron and TRIzol reagent, and total RNA was isolated. 5 μ g RNA was reverse transcribed with Molony Munne Leukemia virus RT (Koschem) and then PCR was performed. The following primers were synthesized by Applied Biosystems: GAPDH: TGCACCACCAACTGCTTA forward, GGATGCAGGATGATGTT reverse, and CAGAAGACTGTGGATGGCCCTC-VIC; IFN- γ : AGCAACAGCAAGGCGAAAA forward, CTGGACCTGTGGGTTGTTGA reverse, and CTCAAACCTGGCAATACTCATGAATGCATC-TAMRA; TGF- β 1: GCAACATGTGGAACCTACCAGAA forward, GACGTCAAAAAGACAGCCACTCA reverse, and ACCTTGTAACCGGCTGCTGACCC-TAMRA. The real-time PCR primer for IL-4 was purchased from Biosource International. The results for each cytokine are normalized with respect to GAPDH expression. The primers for V α 14J α 281 TCR are CTAAGCAGCAGCTGCACA forward and AGGTATGACAATCAGCTGAGTCCC reverse.

Synthesis of α -GalCer. α -GalCer was synthesized using the method developed by Kim et al. (25).

Cytokine neutralization and ELISA. Synovial cells were taken from B6 mice, administrated K/BxN serum, and cultured with Con A (Sigma-Aldrich) and α -GalCer. Anti-IL-4, anti-IL-10, and anti-IL-13 mAbs (R&D Systems) and anti-IFN- γ mAb (American Type Culture Collection [ATCC]) were used to neutralize the respective cytokines in the concentration of 50 μ g/ml. CD1d was blocked using 5 μ g of blocking mAbs (BD Biosciences) during synovial cell activation. TGF- β 1 production was measured using ELISA (BD Biosciences). The hybridomas for anti-TGF- β (clone: ID11.16.8), anti-IFN- γ (clone: R4-6A2), and IL-4 mAb (clone HB188) were purchased from ATCC. 500 μ g of each mAb was injected i.p. three times per week. 1 μ g rhTGF- β 1 (R&D Systems) was injected i.p. into B6 mice twice before and after the administration of K/BxN serum.

Online Supplemental Materials. Fig. S1 shows that the adoptive transfer of V α 14i cells restores joint inflammation and suppresses TGF- β 1 production in CD1d^{-/-} mice. Fig. S2 shows that the blockade of IL-4 or IFN- γ in V α 14J α 281 TCR Tg RAG^{-/-} mice reduces joint inflammation and does not suppress TGF- β 1 production in synovium. Figs. S1 and S2 are available at <http://www.jem.org/cgi/content/full/jem.20041400/DC1>.

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