NKT-KO mice could not be ascribed to the reduced response to microbial antigen.

Inv-NKT cells have the potential to secrete several cytokines including TNF-_, IFN-γ and IL-4, which are effective or suppressive in the development of arthritis. To investigate whether the cytokine balance changed in NKT-cell-depleted mice during the induction phase of CIA, IFN-γ and IL-4 production was examined after a single immunization *in vivo* and stimulation *in vitro* with CII. The results demonstrate that there is no difference in IFN-γ or IL-4 production between inv-NKT cell positive and negative mice (Fig. 3b, c). Namely, the IFN-_ and IL-4 concentrations in supernatants stimulated with 50 _g/mL of CII were 84.3±50.3 pg/mL and 10.6±1.9 pg/mL in C57BL/6 mice, and 131.2±64.6 pg/mL and 9.1±1.3 pg/mL in NKT-KO mice, respectively. Therefore, the absence of inv-NKT cells did not have an effect on the cytokine balance after primary immunization with antigen and was not considered to have had an influence on the deviation towards a Th1 type response.

T and B cell activity in NKT-KO mice after the booster immunization with CII

To investigate the activation levels of T and B cells *in vivo* during the development phase of CIA (after the booster immunization), the ratio of CD69 expression, an early activation marker, on T and B cells was determined by flow cytometry. The results showed that the percentage of CD69-positive T cells in the spleen was lower in NKT-KO mice than in wild type mice (9.9±1.8 in NKT-KO vs 16.0±3.4 in C57BL/6, $P<0.01$, Figure 4b). The percentage of CD69-positive B cells was also lower in the spleen from NKT-KO mice compared with that of wild type mice (4.1±0.5 vs 5.1±0.7, $P<0.05$, Figure 4c). Therefore, the low incidence of arthritis in NKT-depleted mice was attributable to lower response of T and B cells after the booster immunization.

Cytokine mRNA expression in the spleen from NKT-KO mice after the booster immunization with CII

Further, to examine cytokine production *in vivo* in NKT-KO mice during the development of arthritis, IFN-_ and IL-4 mRNA expression in the spleen was measured by quantitative RT-PCR after the booster

immunization. The results showed that relative IFN-_ mRNA expression in NKT-KO mice was higher than that in wild type mice around 10 or 15 days after the booster injection (1.38±0.58 vs 0.64±0.23 on day 10, $P<0.05$, 1.94±0.06 vs 1.05±0.14 on day 15, $P<0.01$, Figure 5a), while relative IL-4 mRNA expression in NKT-KO mice was lower during the course of arthritis development (mean ±S.D. on days 5, 10, 15, and 30: 1.02±0.02, 1.17±0.56, 1.64±0.17, and 4.02±2.56 in NKT-KO vs 4.08±1.03, 3.04±0.07, 4.19±0.21, and 9.06±2.07 in C57BL/6, $P<0.01$ or 0.05, Figure 5b).

IL-1_ plays a prominent role in the inflammation in CIA [15] and it is controlled by various cytokines. Since IFN-_ and IL-4 secretion was suggested to be changing in NKT-KO mice, the expression level of IL-1_ mRNA in the spleen was also measured. The IL-_ mRNA level was found to be lower in NKT-KO mice than in wild type mice after day 15 of the booster injection (mean ±S.D. on days 15 and 30: 0.84±0.14, 0.73±0.05 in NKT-KO vs 1.27±0.07, 1.10±0.24 in C57BL/6 mice, $P<0.01$, Figure 5c). Therefore, the low incidence of arthritis and alleviation of the symptoms in NKT-depleted mice was probably related to the suppression of IL-1_ secretion.

Discussion

In this study, we revealed that a deficiency of inv-NKT cells induced a lower incidence of arthritis, and the results suggest that inv-NKT cells play a considerable role in arthritis development. Similar results have been shown in a different system by Chiba et al [9]. The repeated administration of antigen for NKT cells, α-GalCer, exacerbated the arthritis of CIA [Ohnishi, Y., et al. submitted], supporting this hypothesis.

Levels of the cell activation marker CD69 on T and B cells decreased in NKT-cell-deficient mice after secondary (booster) immunization, and serum anti-CII antibody levels were lower in the KO compared with wild type mice. Therefore, the low incidence of arthritis in NKT-deficient mice was due to the low activity of T and B cells during the development phase of arthritis. Some reports have shown that NKT cells activated by _-GalCer induce maturation of DC, and thereby, enhance the antigen-specific T cell response [16, 17]. In addition, inv-NKT cells directly induce B cell proliferation and help antibody production [18]. Therefore, although the level of DC maturation was not investigated in this study, the lower

incidence of arthritis was probably attributable to depletion of inv-NKT cells that effectively influences T and B cell activation. Further experiments, such as adaptive transfer of inv-NKT cells, are required to clarify inv-NKT cell function. Cell proliferative responses and the levels of cytokine secretion in KO mice were comparable to those of wild type mice after primary immunization (Figure 3a, b, c). This suggests that inv-NKT cells are associated with the observed increase in T and B cell activation rather than with the initiation of CIA, including Th1/Th2 polarization, following antigen stimulation. Eberl *et al.* reported that NKT cells contribute to the maintenance and persistent stimulation of memory T cells through cytokine secretion [19]. We speculated that NKT cells are associated with the maintenance of T cells activated by antigen stimulation in CIA.

From the results of the measurement of cytokine mRNA expression levels, lower IL-4 and IL-1_ secretion and temporarily higher IFN-_ secretion were observed in NKT-cell-depleted mice. Examination in IFN-_ KO mice showed that CIA was enhanced by genetic ablation of IFN-_ through up-regulation of IL-1_ production, and therefore, IFN-_ plays a

role in the regulation of IL-1_ in CIA [20]. IL-4 has been reported to be an up-regulator of both type I and type II IL-1 receptors on monocytes. [21, 22, 23] Considering that IL-1_ is a key mediator in the pathology of the CIA model, higher IFN-_ and lower IL-4 levels in NKT-KO mice might contribute to the alleviation of arthritis. In addition, endogeneous IL-4 not only acts directly on B cell activation, but also plays a crucial role in arthritis induced by the injection of anti-CII antibodies. [24]. There is the possibility that IL-4 is a key mediator for the suppression of arthritis in NKT-cell-deficient mice. Since IL-4 was reduced throughout the course of arthritis development, inv-NKT cells might act as the source of IL-4 for arthritis development. Further experiments, such as using anti-cytokine antibodies or cytokine augmentation, are required for confirmation of the involvement of these cytokines on the suppression of CIA.

Although inv-NKT cells have the potential to promote CIA development, arthritis developed at a low incidence with slight symptoms in NKT-deficient mice (40% of incidence and 1.5-2.0 of mean arthritis score, Figure 1a, b, c, d). Therefore, inv-NKT cells are not essential for CIA establishment.

Chiba *et al.* also showed that inv-NKT cells stimulated with OCH, an analog of _-GalCer, could suppress the development of arthritis, and suggested that inv-NKT cells could play a role as suppressor cells [9]. OCH is an artificially synthesized ligand and probably differs from the natural ligands of inv-NKT cells, which are still unknown. Based on the results of this study, inv-NKT cells are considered to have the ability to enhance CIA in a natural state. Recent studies showed that NKT cells could be classified into several subsets based on their capability to secrete cytokines and their phenotype [25, 26, 27]. Subsets activated by OCH might be different from main subsets activated by the as yet unknown natural ligands in CIA.

In conclusion, two KO mouse models clearly show that inv-NKT cells can promote the generation of arthritis, especially during the development phase. Further experiments on the function of inv-NKT cells should shed light on the development and regulation of arthritis.

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Figure legends

Fig. 1 Suppression of arthritis in NKT-KO mice and CD1d-KO mice. Ten NKT-KO (a, b) and ten CD1d-KO mice (c, d) were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. Tuberclosis* H37Ra. Ten C57BL/6 mice were used as the control in each examination. The incidence of CIA (a, c) and the severity of arthritis (b, d) were investigated.

"•" represents each KO mice, "■" represents C57BL/6 mice.

Fig. 2 Reduction of anti-CII Abs in NKT-KO and CD1d-KO mice. Twenty-eight days after the booster injection, the amount of anti-CII IgG antibody in the serum from NKT-KO (a) or CD1d-KO (b) mice and C57BL/6 mice was measured by ELISA.

Fig. 3 Cell proliferation and cytokine production stimulated by CII in NKT-KO mice compared with those in C57BL/6 mice. Five NKT-KO and five C57BL/6 mice were immunized with chicken CII emulsified in IFA plus *M. Tuberclosis* H37Ra. Nine days after

immunization, splenocytes were stimulated with CII. The degree of cell proliferation was evaluated by a BrdU ELISA method (a). The concentrations of IFN-γ (b) and L-4 (c) in the culture supernatants were measured by ELISA.

Fig 4. Activation level of T and B cells after the booster immunization in NKT-KO mice compared with that in C57BL/6 mice. Eight NKT-KO and eight C57BL/6 mice were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. Tuberclosis* H37Ra. Five days after the booster immunization, splenocytes were collected and stained with FITC-labeled anti-TCR_ or anti-B220, and PE-labeled anti-CD69 antibody. PI-negative cells were gated and FITC-PE double positive cells were counted (a). The proportions of CD69-positive T cells (b) or CD69-positive B cells (c) compared to the total number of T cells ($TCR_^{+}$ cells) or B cells ($B220^{+}$ cells) were calculated.

Fig 5. Cytokine mRNA expression in the spleen after the booster

immunization. Total splenic RNA was collected from three male C57BL/6 mice (•) and three male NKT-KO mice (■) 5, 10, 15, and 30 days after the booster immunization, and the relative expression levels of IFN-_ (a), IL-4 (b), or IL-1_ (c) mRNA were measured by the Taqman quantitative PCR method. * $P<0.05$; ** $P<0.01$

Clinical and Experimental Immunology (in press)

TCRVα14$^+$ NKT cells function as effector T cells in collagen-induced arthritis mice

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Keywords: NKT cells, collagen-induced arthritis, effective, cytokine

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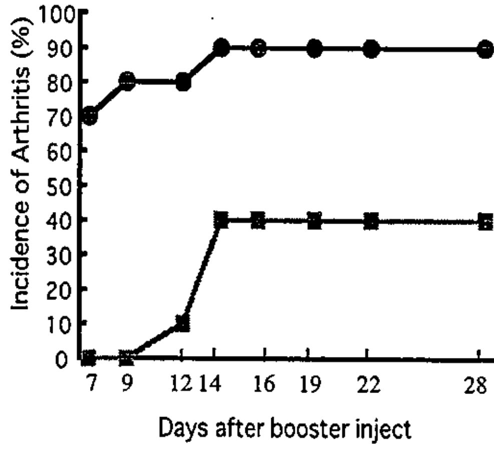
Figure 1

(a)



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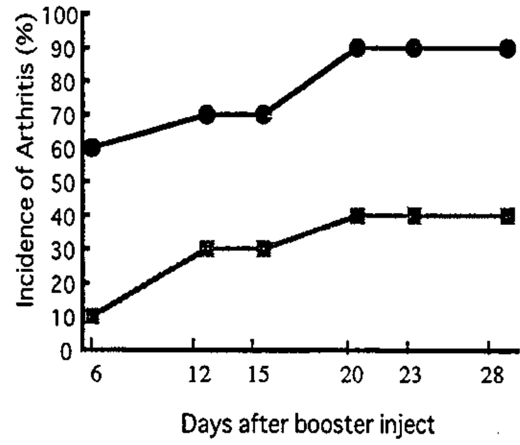

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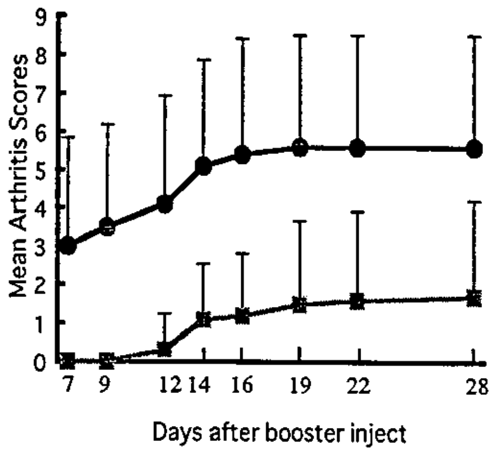

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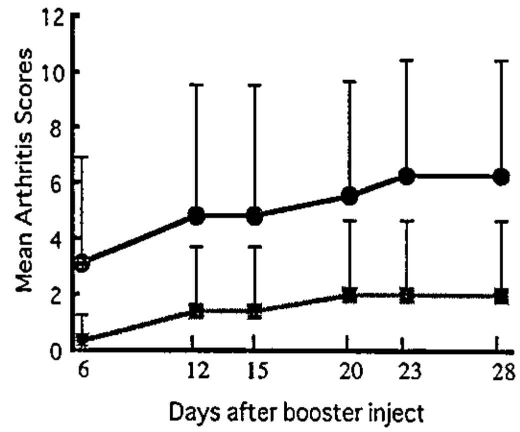

Figure 2

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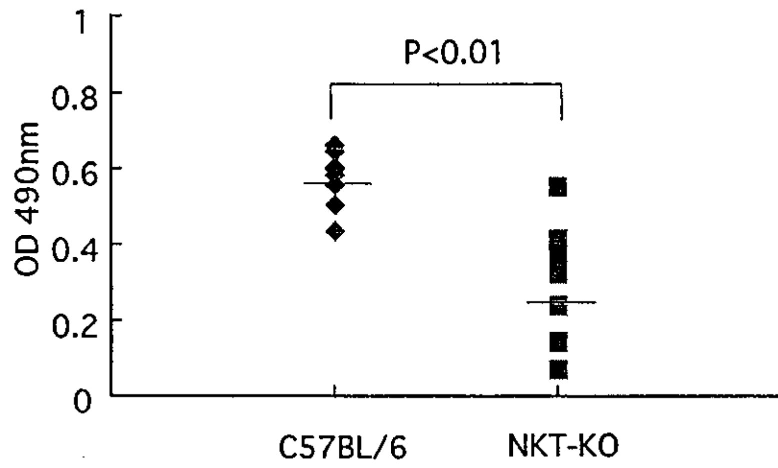

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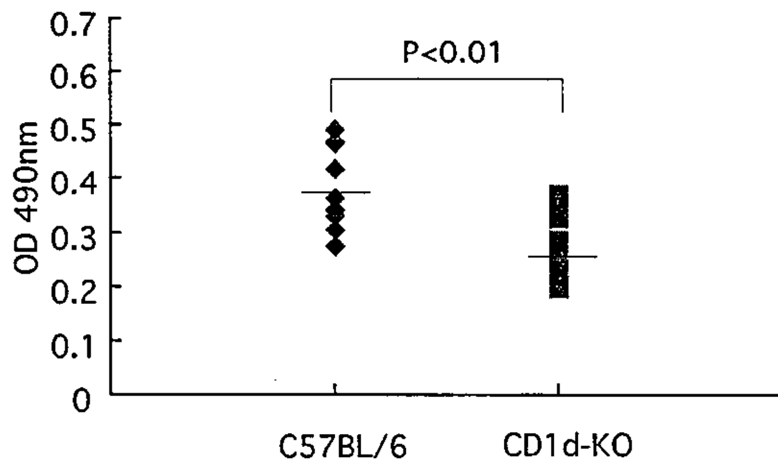
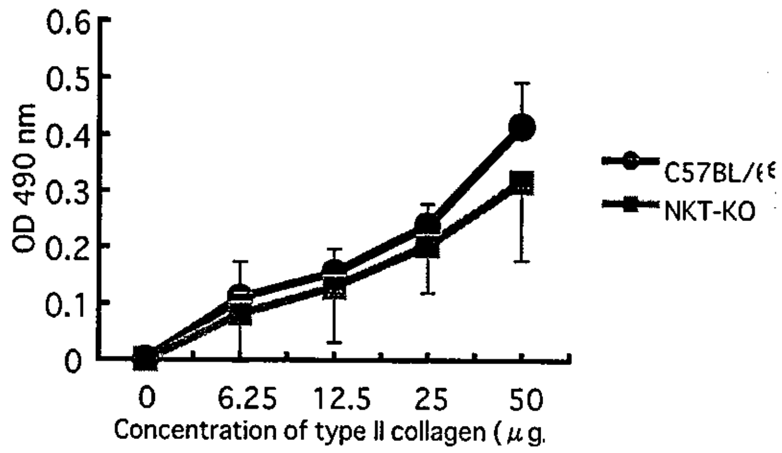


Figure 3

(a)



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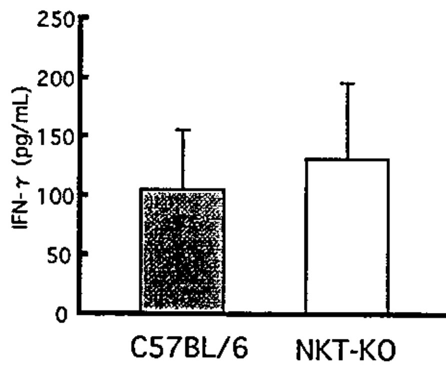

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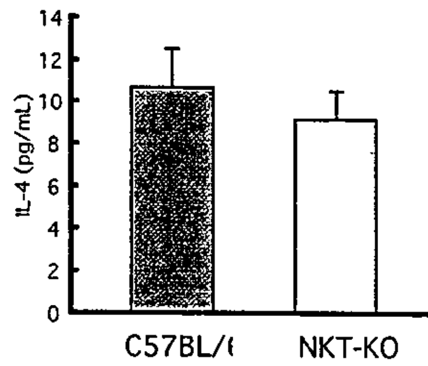
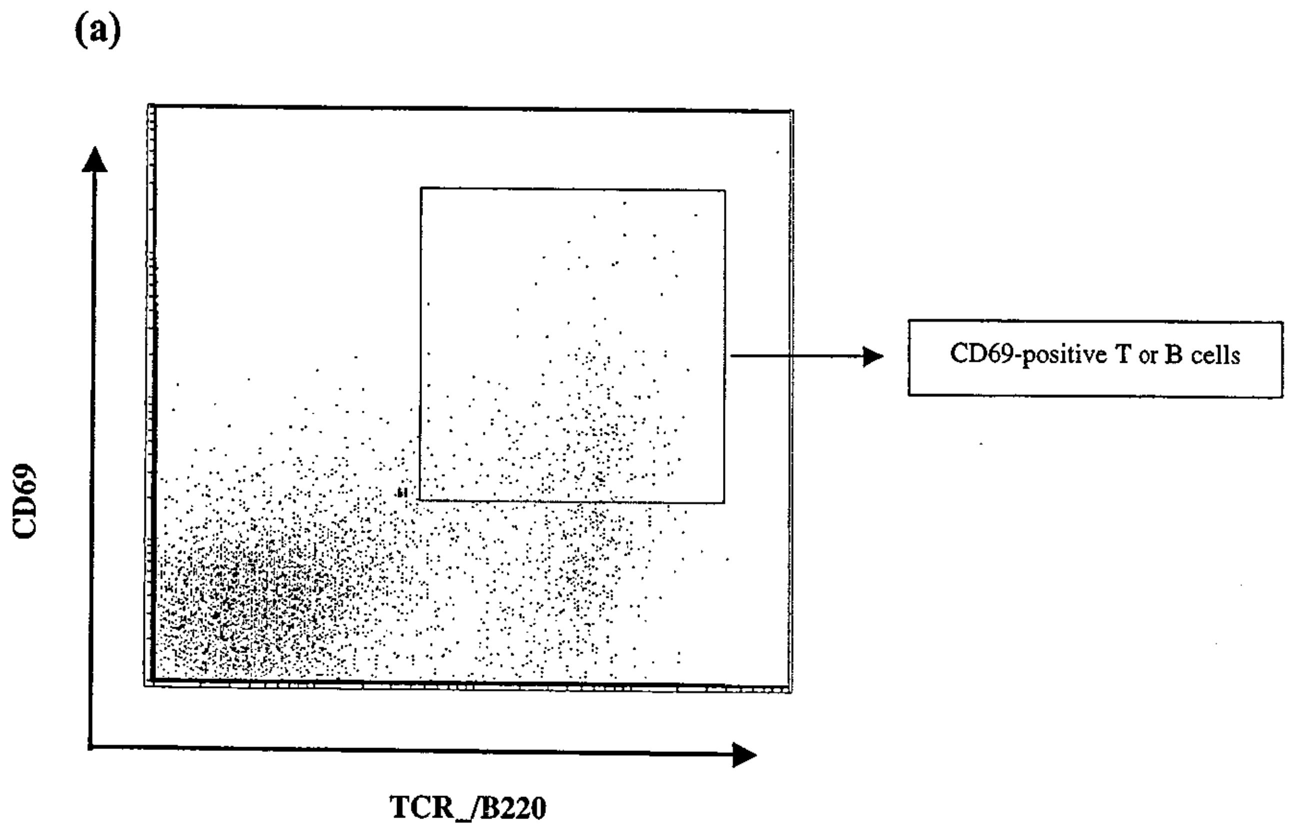


Figure 4

(a)



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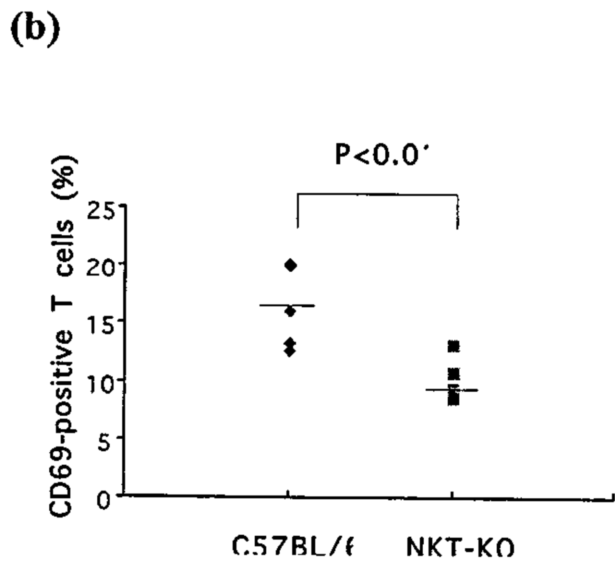

(C)