16.0±3.4%, P<0.01, B cells: 4.1±0.5% vs 5.1±0.7%, P<0.05), suggesting that inv-NKT cells contribute to the pathogenicity in the development phase of arthritis. In addition, IL-4 and IL-1_ mRNA expression levels in the spleen during the arthritis development phase were lower in NKT-KO mice, while the IFN-_ mRNA expression level was temporarily higher. These results suggest that inv-NKT cells influence cytokine production in arthritis development.

In conclusion, inv-NKT cells may promote the generation of arthritis, especially during the development rather than initiation phase.

Introduction

Natural killer T cells represent a novel lymphoid lineage distinct from conventional T cells, B cells, and NK cells. As invariant NKT cells (inv-NKT), whose TCR is a single invariant Vα14Jα281 chain, can secrete both Th1 and Th2 cytokines, it was suggested that inv-NKT cells play a role in immunomodulative function. Deficient or defective NKT cells are associated with certain autoimmune diseases [1-3], as well as with several animal models of autoimmune disease [4, 5]. Recent studies showed that inv-NKT cell activation protected against type 1 diabetes in NOD mice [6], EAE [7], and MRL lpr/lpr mice [8]. Chiba *et al.* reported that collagen-induced arthritis (CIA) was suppressed by NKT cell activation with OCH, an analog of _-GalCer [9]. However, the natural function of inv-NKT cells on the development of arthritis remains unclear.

To investigate the role of inv-NKT cells on arthritis in the CIA mouse model, NKT-cell-deficient mice were analyzed. Arthritis was suppressed and anti-CII antibody levels were reduced in these mice. In addition, the ratios of activated T and B cells were lower, and IL-4 and IL-1_ mRNA expression was lower, in the deficient mice during the development phase

rather than induction phase of arthritis, while IFN-_ mRNA expression was temporarily higher. Therefore, we concluded that inv-NKT cells could promote the generation of arthritis and that they affected arthritis development rather than immunological initiation.

Materials and Methods

Mice. Male C57BL/6 mice aged 10-12 weeks old were purchased from Charles River Japan Inc. (Yokohama, Japan). Male NKT cell (TCR Jα281) knock out mice (NKT-KO) [10] and male CD1d knock out mice (CD1d-KO) [11], in which the genetic background was C57BL/6, were used in this study. NKT-KO and CD1d KO mice were established after backcrossing 10 generations or more to B6 mice, respectively, and were kindly provided by Prof. M.Taniguchi (RIKEN Research Center for allergy and Immunology, Yokohama, Japan). The study design was approved by the Ethical Committee of the University of Tsukuba.

Reagents. Chicken type II collagen (CII) was purchased from Sigma-Aldrich Corp. (ST. Louis, USA). CII was dissolved in 0.1M acetic acid

and diluted with 0.01M PBS (pH 7.4). Incomplete Freund's Adjuvant (IFA) and heat-inactivated *M. tuberculosis* (H37Ra) were purchased from Difco Laboratories (Detroit, USA).

Induction of collagen-induced arthritis (CIA) and evaluation of clinical severity. C57BL/6, NKT-KO and CD1d-KO mice were immunized intradermally at the base of the tail with 100 μg chicken CII emulsified in IFA containing 250 μg of inactivated *M. Tuberclosis* (H37Ra). On Day 21 the animals were boosted with an intradermal injection [12]. The animals were observed at 2- or 4-day intervals and evaluated for the severity of arthritis by scoring each paw. The observation period was set for 28 days after the booster immunization, because the clinical scores did not worsen after that in our preliminary experiment using a small number of animals. The scores ranged from 0 to 3 (0, no swelling or redness; 1, swelling or redness in one joint; 2, two joints or more involved; 3, severe arthritis of the entire paw and joints). The score for each animal was the sum of the score for all four paws.

Measurement of anti-CII antibody. C57BL/6, NKT-KO and CD1d-KO mice were sacrificed 28 days after the booster injection and sera were collected. Anti-CII IgG antibodies (anti-CII IgG Abs) were measured by ELISA [13].

Briefly, each mouse serum was diluted 100,000 times with blocking buffer (Block Ace, Dainippon Pharmaceuticals Co., Osaka, Japan) and was incubated in a type II collagen-coated well for 1hr at 37°C. The wells were washed 3 times with washing buffer (0.01M Tris-HCl containing 0.05% Tween 20) and treated with biotinylated goat anti-mouse IgG antibody (Zymed Laboratories, Inc., South San Francisco, USA, diluted 4000 times with the blocking buffer) for 2hr at room temperature. After washing, avidin-alkaline phosphatase (EY laboratories, Inc., San Mateo, USA) diluted 4000 times with the blocking buffer was added to each well and incubated for 1 hr at room temperature. After washing, color development was carried out by an ELISA amplification system (Invitrogen Co., Carlsbad, USA) and the optical density was determined at 490 nm.

Measurement of the primary CII-specific response *ex vivo*. C57BL/6 and NKT-KO mice were immunized intradermally with 100 μg chicken CII

emulsified in IFA containing 250 μg of inactivated *M. Tuberclosis* (H37Ra). Nine days after immunization, spleens were removed. The red blood cells were removed from the splenocytes by treatment with 0.16M Tris-NH_4Cl solution, and $2x10^5$ cells were restimulated in triplicates with several concentrations of chicken CII (6.25, 12.5, 25, and 50 _g/mL) for 72 hr and the proliferative response was estimated using the BrdU ELISA system (Cell Proliferation ELISA kit, Roche Diagnostics GmbH, Mannheim, Germany). IFN-γ and IL-4 concentrations in the culture supernatants were measured by ELISA using an immunoassay kit (Biosource International Inc., Camarillo, USA).

Flow cytometric analysis. Fluorescein isothiocyanate (FITC)-labeled anti-TCR_ mAb (clone H57-597), anti-CD45R(B220) mAb (clone RA3-6B2), and PE-labeled anti-CD69 mAb (clone H1.2F3) were purchased from eBioscience, Inc. (San Diego, USA). Rat anti-mouse Fc_R II/III mAb (clone 2.4G2, BD Biosciences, San Diego, USA) was used as the Fc block. Splenocytes were collected from eight C57BL/6 male mice and eight NKT-KO male mice 5 days after the booster immunization and were treated with 0.16M Tris-NH_4Cl solution. The cells were stained with mAbs and propidium iodide (PI, BD Biosciences, San Diego, USA), and were

analyzed by flow cytometry using EPICS XL-MCL (Beckman Coulter, Inc., Fullerton, USA).

Quantitative RT-PCR. The spleen was removed from three of the C57BL/6 male mice and three of the NKT-KO male mice 5, 10, 15, and 30 days after the booster immunization and they were preserved in RNA*later* (QIAGEN GmbH, Hilden, Germany). Total RNA was extracted by TriZol reagent (Invitrogen Co., Carlsbad, USA) and no genomic DNA contamination was confirmed using the GAPDH primer. First strand cDNA was synthesized using SuperScript III First Strand System (Invitrogen Co., Carlsbad, USA). The relative expression levels of IFN-_, IL-1_, and IL-4 mRNA were determined by Taqman RT-PCR technology (ABI PRISM 7700, Applied Biosystems, Foster city, USA). The target gene copy number of each sample was standardized by GAPDH gene expression. The primer-probe set for each cytokine and GAPDH was purchased from Applied Biosystems (Assay-on demand system).

Statistical analysis. Statistical analysis was carried out using the Fisher's exact test for the incidence of arthritis, and the Student's or Welch t-test for arthritis score, anti-CII antibody titer, percentage of CD69-positive cells,

and relative cytokine mRNA expression.

Results

CIA in NKT-KO and CD1d-KO mice

To evaluate the association of Vα14-Jα281 NKT cells with the development of CIA, two different KO mice, Jα281-KO (NKT-KO) and CD1d-KO mice, were used and the incidence and severity of arthritis in each were compared to genetically matched C57BL/6 mice. In NKT-KO mice, the incidence (40%) and arthritis score (1.5 ± 2.2) were significantly reduced compared with the control mice (90%, 5.4 ± 3.2, $p<0.01$)(Fig. 1a, b). In CD1d-KO mice, the incidence (40%) and arthritis score (2.0 ± 2.7) were also significantly reduced compared with control mice (90%, 6.4 ± 4.2, $p<0.01$)(Fig. 1c, d). Judging from these findings, we hypothesize that inv-NKT cells function as effector T cells.

Anti-CII antibody in sera from NKT-KO and CD1d-KO mice

Serum anti- CII IgG Abs were also significantly decreased in NKT-KO and CD1d-KO mice (OD value: 0.32 ± 0.16 and 0.29 ± 0.06 respectively) compared with their controls (OD value: 0.58 ± 0.08 and 0.38 ± 0.08) ($P<0.01$) (Fig. 2). The decrease in pathogenic anti-CII Ab levels is one

reason why arthritis is suppressed in the NKT-cell-deficient mice.

Primary anti-CII response in NKT-KO mice

The immune system of mice was stimulated with adjuvant including microbial antigens as well as CII for the induction of CIA. NKT cells were stimulated with microbial antigens in the context of CD1d, an MHC class-I like molecule on APC [14]. Therefore, it is possible that the suppression of the incidence and severity of arthritis in NKT-KO mice is attributable to the reduction of the immune response to microbial antigens, leading to a reduced response to CII. To address this possibility, the degree of cell proliferation in NKT-KO mice was compared with those in C57BL/6 mice when stimulated with chicken CII after *in vivo* immunization with CII and inactivated *M. Tuberculosis*. The results of the cell proliferation assay, mean OD values and S.D. at 6.25, 12.5, 25, and 50 _g/mL of CII, were 0.11±0.06, 0.16±0.04, 0.24±0.04, and 0.42±0.08 in C57BL/6 mice, and 0.08±0.09, 0.13±0.10, 0.20±0.08, and 0.32±0.14 in NKT-KO mice, respectively (Fig 3a). This indicates that the cell response to CII is not significantly different between NKT-KO and C57BL/6 mice at any antigen concentration tested, suggesting that the suppression of CIA in

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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