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Involvement of natural killer T cells in C57BL/6 mouse model of collagen-induced arthritis

Chrong-Reen Wang

Section of Allergy, Immunology and Rheumatology, Department of Internal Medicine, Medical College, National Cheng Kung University, Tainan, Taiwan, ROC

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A subset of murine T lymphocytes sharing receptor structures with natural killer cells, named natural killer T cells, has characteristics distinct from conventional T cells and natural killer cells. The DBA/1 strain commonly used for collagen-induced arthritis induction does not express the natural killer 1.1 molecule, a marker for defining murine natural killer T cells. Therefore, collagen-induced arthritis was induced in the C57BL/6 strain carrying natural killer 1.1 marker and the role of natural killer T cells was examined in this model. The collagen-induced arthritis was induced successfully in the C57/BL6 strain with near 70% incidence. Lower percentages of natural killer T cells in lymphoid organs including spleen and lymph node and higher percentages of natural killer T cells in synovium were found in mice with severe inflamed joints as compared with those with mild inflamed joints. The results suggested an infiltration of natural killer $1.1^{+}CD3^{+}$ natural killer T cells into inflamed synovium in the model of collagen-induced arthritis. Although the preliminary results of natural killer 1.1 monoclonal antibody depletion experiments failed to alleviate the gravity of arthritis, such a C57BL/6 mouse model of collagen-induced arthritis may provide a tool in the study of therapeutic manipulation of natural killer T cells in human autoimmune diseases such as rheumatoid arthritis.

Key words: Collagen-induced arthritis, natural killer T cells, rheumatoid arthritis

The regulatory mechanisms in systemic autoimmune diseases remain unclear. There are now compelling evidences that regulatory T cells participate in many immune responses against foreign and self antigens [1]. Recently, a subset in murine T lymphocytes sharing receptor structures with natural killer (NK) cells was found to have characteristics of lymphocyte distinct from conventional T cells, B cells, and NK cells [2,3]. These cells with the name of NK T cells express typical NK cell makers, including members of the NKR-P1 (NK cell activating receptor) and members of the Ly49 (NK cell inhibitory receptor) [4,5]. They also express a heavily biased T-cell receptor (TCR) repertoire with an invariant Vα14-Jα281 chain and a polyclonal Vβ8.2 chain [6,7]. Unlike conventional T cells that recognize peptide antigens in the context of self-MHC class I or class II molecules, NK T cells are specific for glycolipid antigens bound with the MHC class I-like molecule, CD1d [4,8]. As CD1d expression is required for the development of NK T cells, CD1d-deficient mice have a marked reduction in the number of NK T cells [9]. Many NK T cell hybridomas were able to react with splenocytes, thymocytes, or CD1d-transfected cells [4]. Recent studies have shown that CD1d can bind with a variety of glycolipid, including phosphatidylinositol derivatives and glycosylceramides [10,11]. Unlike Th1 and Th2 cells with their restricted ability to produce particular cytokines, murine NK T cells produce both interferon-γ (IFNγ) and interleukin (IL)-4 after stimulation with anti-CD3 or activation by CD1d [12, 13].

Previous studies have suggested a role for NK T cells in the regulation of autoimmunity. Various mouse strains with genetic susceptibility for the development of autoimmune diseases, including non-obese diabetic (NOD) mice with a propensity for development of type I diabetes, SJL mice with a susceptibility for development of experimental allergic encephalomyelitis and lupus-prone lpr strains, were found to have defects in NK T cell number and function [14-16]. Transgenic overexpression of NK T cells in NOD mice was able to inhibit development of diabetes [17]. Moreover, NK T cells isolated from Vα4 transgenic mice either induced or prevented murine lupus in an adoptive transfer model,

Corresponding author: Dr. Chrong-Reen Wang, Section of Allergy, Immunology and Rheumatology, Department of Internal Medicine, Medical College, National Cheng Kung University, 138 Sheng-Li Road, Tainan, Taiwan, 704, ROC. E-mail: wangcr@mail.ncku.edu.tw

depending on the source and cytokine profiles of the NK T cells that were transferred [16].

Patients with rheumatoid arthritis (RA) are frequently encountered with a high estimated prevalence rate in Taiwan [18]. Rheumatoid arthritis is an autoimmune disease characterized by T lymphocytes accumulation within the synovial compartment and activated $CD4^+$ T cells predominate in the infiltrating mononuclear cells (MNCs) of the rheumatoid joints [19]. In the collagen-induced arthritis (CIA) model, a T-cell-mediated process, the efficacy of anti-TCR antibody (Ab) treatment has been demonstrated [20]. T-helper type 1 (TH1)1 cells play a proinflammatory role, while Th2 cells appear to have an antiinflammatory effect in the CIA model [21]. The DBA/1 strain used for CIA induction does not express the NK1.1 molecule, a marker for defining murine NK T cells. Therefore, the involvement of NK T cells in the murine model of CIA has not been characterized yet. The aim of this study is to induce CIA in the C57BL/6 strain carrying NK 1.1 marker and examined the role of NK T cells in this model.

Materials and Methods

Mice

C57BL/6 mice were purchased from the animal center in Medical College of National Cheng-Kung University and were maintained under specific pathogen-free condition. In these experiments, 5 to 15 mice per group with 10 to 12 weeks of age were used.

Reagents

The monoclonal antibodies (mAbs) against mouse antigens including anti-CD3 (clone 145-2C11), anti-CD16/32 (clone 2.4G2), and anti-NK1.1 (clone PK136) were acquired from PharMingen (San Diego, CA, US). Rat immunoglobulin (Ig) G and bovine type II collagen were purchased from Sigma (St. Louis, MO, US). Complete Freund's adjuvant (CFA) was obtained from DIFCO (Detroit, MI, US). The Lympholyte-M reagents and mouse erythrocyte lysing kit were brought from Cedarlane Laboratories (Hornby, Canada) and R&D System (Minneapolis, MN, US), respectively.

Induction of CIA

We applied a modified protocol for optimal induction of CIA in C57BL/6 mice, where arthritis is consistently developed with around 70% incidence after the secondary immunization [22]. Bovine type II collagen was dissolved in 10 mM acetic acid and combined with an equal volume of CFA. Mice were injected intradermally at several sites into the base of tail with a total of 100 μL emulsion containing 100 μg collagen or CFA only as a control group. The same injection was repeated at Day 21 proximal to the primary injection site. Each mouse was assessed for redness and swelling of limbs every 2 days for up to 10 weeks. The inflammation of each paw was graded by a previously described score system, where 0 = normal, 1 = slight swelling and/or erythema (low score), and 2 = pronounced erythematous swelling with edema (high score) [23].

In vivo depletion of $NK1.1^+$ cells

We also performed *in vivo* experiments to deplete NK1.1 positive cells and assessed this effect on CIA. The purified rat anti-mouse NK 1.1 mAb and purified rat IgG were dialyzed over phosphate buffered saline before use. Mice were injected intraperitoneally with anti-NK1.1 mAb for the experiment group or rat IgG for the control group. Five mice per group were injected with Ab or IgG before priming with bovine type II collagen in CFA. These mice were recorded for their clinical scores every 2 days up to 10 weeks for assessment of treatment effects.

Preparation of MNCs

Spleens and lymph nodes were removed from arthritic mice and were teased apart to make single cell suspensions. Red blood cells were depleted by a mouse erythrocyte lysing kit. The inflamed joint tissues were removed from paws by first stripping the skin and separating the limb below the wrist or ankle joints as described previously [24]. To prepare MNCs from inflamed joints, the minced tissues were further subjected to collagenase (Sigma) 10 mg/mL treatment for 1 h at 37°C. After being filtrated through nylon mesh, these cells were subjected to centrifuge over Lympholyte-M to purify MNCs. Synovial MNCs were stained with fluorescence-conjugated mAbs and further analyzed by flow cytometry.

Flow cytometry analysis

Double fluorescence staining was performed for mouse surface phenotype study. After their Fc receptors were blocked with anti-CD16/CD32, purified MNCs were stained with FITC-conjugated anti-CD3 and PE-conjugated anti-NK1.1 mAbs for 30 min on ice in the dark. The stained cells were analyzed with FACSort (Becton Dickison, Mountain View, CA, US) and CellQuest software programs (Becton Dickison). Ten thousand lymphocytes were gated for analysis of spleen and lymph node (LN), and more than 1500 lymphocytes

were gated for analysis of synovial tissue. Isotype control mAb (PharMingen) staining was included for each sample.

Statistical analysis

The statistical analysis was carried out using Student *t* test to compare mean values between 2 different groups of mice.

Results

Induction of CIA in the C57BL/6 strain mice

We successfully induced CIA in the common laboratory C57/BL6 strain. The time kinetics of average clinical scores in 12 CFA-treated control mice and 15 CFA plus type II collagen-treated mice are shown in Fig. 1. After the secondary immunization, 10 of 15 CFA plus type II collagen-treated mice developed arthritis with a 66.7% incidence.

Decrease in NK T cells of spleen and LN from mice with severe arthritis

Representative figures of the percentages of $NK1.1^+$ $CD3^+$ NK T cells in spleen and LN were shown in Fig. 2A (high score) and 2B (low score). Table 1 shows the average percentages of $NK1.1^+CD3^+$ NK T cells in spleen and LN from 3 mice in each group. Mice with high clinical score have lower percentages of $NK1.1^+$ $CD3^+$ NK T cells than those with low clinical score ($p<0.05$).

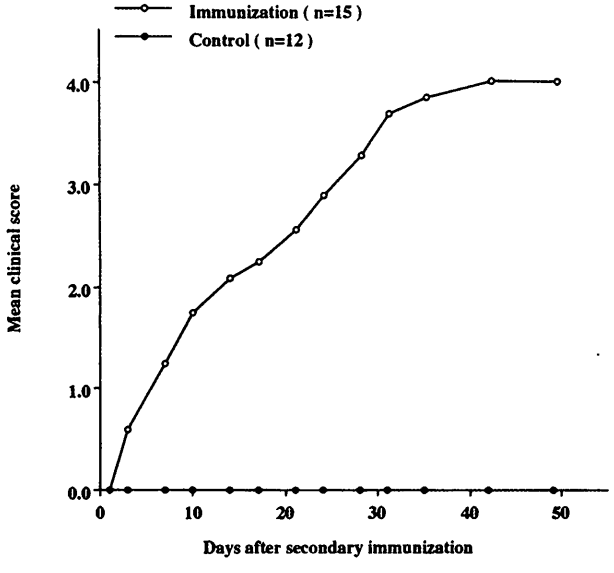

Fig. 1. Evaluation of clinical scores of collagen-induced arthritis in C57BL/6 mice. Mice were injected intradermally into the base of tail with a total of 100 μL emulsion containing CFA plus 100 μg bovine type II collagen or CFA only as a control group. The same injection was repeated at Day 21. Each mouse was assessed for redness and swelling of limbs every 2 days for up to 10 weeks.

Increase in NK T cells of synovium from mice with severe arthritis

The presence of NK T cells was demonstrated in MNCs purified from synovium of arthritic mice with high and low clinical score as shown in Fig. 2A and 2B, respectively. As shown in Table 1, inflamed joints of high score showed higher percentages of $NK1.1^+CD3^+$ NK T cells as compared with those of low score ($p<0.05$).

No alleviation of arthritis by depletion of $NK1.1^+$ cells

For anti-NK1.1 mAb depletion experiments, there was no statistical significance in clinical scores between the experiment group and the control group ($p>0.1$).

Discussion

The use of animal models to study RA is essential and CIA is the most widely studied form induced in susceptible strains of mice by immunization with type II collagen emulsified in CFA [25]. Early works reported a hierarchy of responsiveness to CIA linked to special H-2 haplotype with DBA/1 ($H\text{-}2^q$) being the most responsive strain; nevertheless, with a novel immunization protocol, C57BL/6 ($H\text{-}2^b$) mice, one of the commonest laboratory strains, can develop CIA with high incidence and severity [22]. The C57BL/6 strain model of CIA can also be induced in the author's laboratory with similar incidence and severity as shown in the results.

Lower percentages of NK T cells in lymphoid organs including spleen and LN and higher percentages of NK T cells in synovium were found in mice with severe inflamed joints (high clinical score) as compared with those with mild inflamed joints (low clinical score). The results of this study suggest an infiltration of NK 1.1^+CD3^+ NK T cells into inflamed synovium of CIA. Although the preliminary results of NK 1.1 mAb depletion experiments failed to alleviate the gravity of arthritis, such a C57BL/6 mouse strain model of CIA may provide a tool in the study of therapeutic manipulation of NK T cells in human RA.

Studies in humans have described populations of peripheral blood lymphocytes that express TCR and NK cell makers including CD16, CD56, CD57, and CD94 [26,27]. Recent reports indicate that an invariant Vα24 α chain is preferentially expressed on double-negative

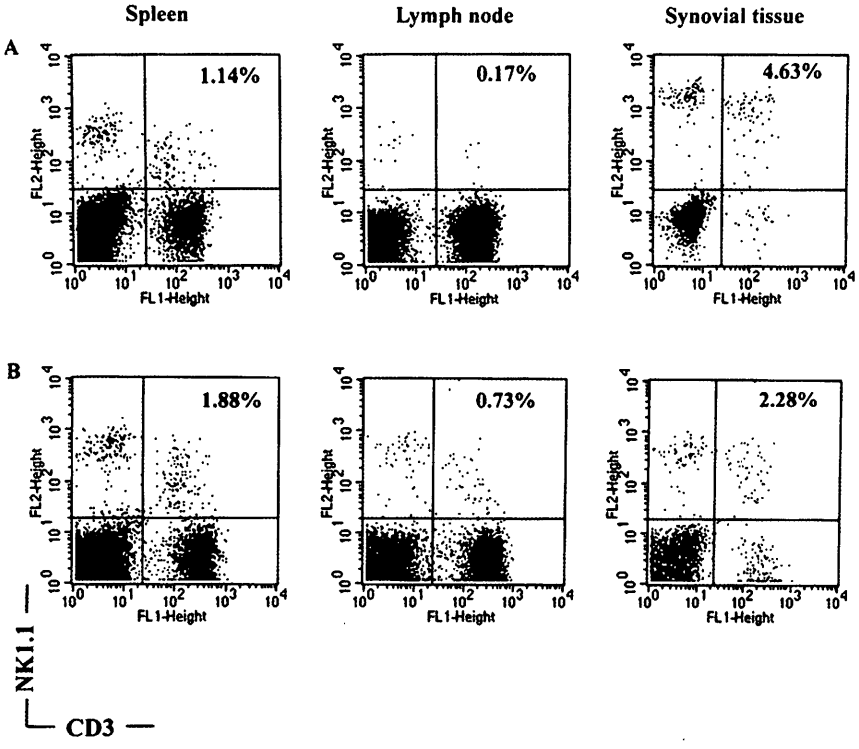

Fig. 2. Percentages of NK1.1$^+$CD3$^+$ NK T cells in lymphocytes from spleen, lymph node, and synovial tissue. Percentages of NK T cells in a mouse with severe joint inflammation (high score), a representative figure from mice with high score, are shown in **(A)** and those in a mouse with mild inflammation (low score), a representative figure from mice with low score, are shown in **(B)**.

T cells from healthy individuals [28,29]. The homology of amino acid sequences is found to be 90% in the complementarity-determining region 3 between murine Vα14 and human Vα24 NK T cells antigen receptor [29]. Another similarity to murine Vα14 NK T cells is that human Vα24 cells also express a limited TCR β repertoire including Vβ11 [28]. All invariant Vα24 T cell clones recognize the CD1d molecule and discriminate between CD1d and other CD1 proteins such as CD1a, CD1b, and CD1c [29]. A glycolipid ligand, α-galactosylceramide, can selectively activate freshly isolated human Vα24 T cells [30]. Therefore, humans may have an equivalent of murine Vα14 NK T cells, that is, Vα24 NK T cells. These cells have been demonstrated to play a role in the rheumatoid synovium [31]. Therefore, Vα24 NK T cells could influence the disease process of Th1-dominated autoimmunity.

The realization that NK T cells play a critical role in immune regulation and maintenance of self-tolerance raises the possibility of manipulating these cells to modulate immune responses during prophylaxis and therapy. A recent study has shown that administration of α-galactosylceramide to mice can polarize adaptive immune responses towards the Th2-dominated immunity [32]. Immunotherapy in human diseases such as RA may be possible with the glycolipid reagents such as α-galactosylceramide that selectively activates NK T cells.

Table 1. Percentages of NK1.1$^+$CD3$^+$ NK T cells in lymphocytes from spleen, lymph node, and synovial tissue

Score	% of NK1.1$^+$CD3$^+$ NK T cells		
	Spleen	Lymph node	Synovial tissue
High (severe)	1.25 ± 0.10	0.33 ± 0.14	4.86 ± 0.51
Low (mild)	1.79 ± 0.24	0.71 ± 0.08	2.64 ± 0.755
p	<0.05	<0.05	<0.05

Note: each group includes 3 mice.

In conclusion, the results of this study demonstrate a potential role of NK1.1$^+$CD3$^+$ NK T cells in the CIA model of C57BL/6 strain. Such a model may provide a tool in the study of therapeutic manipulation of NK T cells in RA.

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