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AQ: 1

Natural Killer Cell Degeneration Exacerbates Experimental Arthritis in Mice Via Enhanced Interleukin-17 Production

AQ: 2

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Objective. **An altered phenotype and dysfunction of natural killer (NK) cells have been observed in patients with rheumatoid arthritis. The aim of this study was to determine whether dysregulated NK cells contribute to the pathogenesis of experimental arthritis.**

Methods. **For initiation of collagen-induced arthritis (CIA), DBA/1J mice were immunized with type II collagen in Freund's adjuvant. Control mice were immunized with adjuvant alone. NK cells from the blood, spleens, and bone marrow of immunized mice were analyzed by flow cytometry. Levels of interleukin-17 (IL-17) secretion and autoantibody production were measured by enzyme-linked immunosorbent assays. Immunized mice in which NK cells were depleted by anti–asialo G_{M1} antibody treatment were assessed for the development of CIA. Moreover, sorting-purified NK cells from both mice with CIA and control mice were analyzed for cytokine gene expression.**

Results. **We observed markedly reduced frequencies of NK cells in the blood and spleens of mice with CIA compared with the frequencies in adjuvant-treated control mice. Upon NK cell depletion, immunized mice displayed an early onset of arthritis with more severe clinical symptoms, which correlated with increased plasma cell generation and autoantibody production. Moreover, a substantially increased number of IL-17–secreting cells in synovial tissue and more pronounced joint damage were observed. Freshly isolated NK cells from mice with CIA showed markedly reduced expression of interferon-γ (IFNγ). Furthermore, coculture of normal NK cells and CD4+ T cells revealed that NK cells strongly suppressed production of Th17 cells via their IFNγ production.**

Conclusion. **These results suggest that NK cells play a protective role in the development of experimental arthritis, an effect that is possibly mediated by suppressing Th17 cell generation via IFNγ production.**

Natural killer (NK) cells are lymphocytes of the innate immune system that play an important role in eliminating virus-infected cells and tumor cells. In addition to their involvement in innate immunity, NK cells can shape adaptive immunity by cytokine production and direct killing of other immune cells. Increasing evidence indicates a regulatory role of NK cells in autoimmunity (1). The elimination of NK cells either promotes or suppresses autoimmune diseases, suggesting that NK cells exert divergent effects on the pathogenesis of autoimmunity (2–9). For example, a protective role of NK cells has been reported in a CD4+ T cell transfer mouse model of colitis (6), in Freund's complete adjuvant–mediated protection against diabetes (7), and in experimental autoimmune encephalomyelitis (5,9), in which NK cells were shown to inhibit and kill autoreactive T cells. In contrast, NK cells have been shown to promote autoimmune diseases such as neonatal autoimmune ovarian disease (2) and experimental autoimmune myasthenia gravis (8) by inducing pathogenic T cell responses. Furthermore, recent findings that NK cells can eliminate activated macrophages (10) and

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dendritic cells (11) suggest that NK cells may act as an immune regulator by modulating antigen-presenting cells (APCs).

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint inflammation that leads to destruction of cartilage and bones. Although the etiology of RA remains unclear, numerous studies on the roles of T cells, NK T cells, Treg cells, B cells, macrophages, and mast cells in the onset and progression of disease have been extensively performed (12–21). However, much less is known about the role of NK cells in the pathogenesis of autoimmune arthritis.

Dysfunction of NK cells was observed in patients with systemic-onset juvenile RA (22), and NK cells in synovial fluid from a patient with RA showed a unique phenotype (23). However, whether NK cells play a role in the development of autoimmune arthritis remains largely unclear. Despite RA being recognized as a Th1-mediated autoimmune disease in the past, previous findings that interferon-γ (IFNγ) receptor–deficient mice show increased susceptibility to collagen-induced arthritis (CIA) support the notion that IFNγ suppresses autoimmunity (24). It has become clear that the differentiation of interleukin-17 (IL-17)–secreting Th cells (Th17 cells) requires cytokines, including transforming growth factor β (TGFβ), IL-6, IL-1, and IL-23 (25–27), whereas IL-4 and IFNγ suppress IL-17 production (28,29). CIA is markedly suppressed in IL-17–deficient mice, demonstrating a crucial role of IL-17 in the development of autoimmune arthritis (30,31). Recently, it was shown that IFNγ regulates susceptibility to CIA in mice through the suppression of IL-17 (32).

Although NK cells are among the major IFNγ-producing cells in immune responses, it remains unknown whether or not NK cells can possibly modulate the development of Th17 cells via their production of IFNγ. In the present study, we tested the hypothesis that NK cell dysfunction augments experimental arthritis by modulating Th17 cells via IFNγ production.

MATERIALS AND METHODS

Mice. Male DBA/1J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen–free animal facility at the University of Hong Kong. All animal experiments conducted in this study were approved by the University Committee on the Use of Live Animals in Teaching and Research. Mice ages 8–12 weeks were used for the experiments.

Initiation of CIA. CIA was initiated as previously described (33). Briefly, 100 μg of bovine type II collagen (Chondrex, Redmond, WA) dissolved in 0.1*M* acetic acid was emulsified with an equal volume of Freund's complete adjuvant (2 mg/ml) (Difco, Detroit, MI) and administered intradermally at the base of tail into DBA/1J mice. On day 21, a booster emulsion prepared with type II collagen and Freund's incomplete adjuvant (Difco) was intradermally administered near the primary injection site. Following the same protocol, adjuvant-treated littermates that were given phosphate buffered saline (PBS) in place of type II collagen served as controls. Beginning on day 18, the mice were scored for arthritis severity once every 2 days, as previously described (33).

Preparation of cell suspensions from joint tissue. After skin, muscle, and bone were removed under a dissecting microscope, joint samples of the front paw were minced and incubated with collagenase in RPMI 1640 at 37°C for 1 hour. Cell suspensions were filtered through a cell strainer. After lysis of red blood cells, the numbers of viable nucleated cells were determined by trypan blue exclusion.

Flow cytometry and cell sorting. Surface staining was performed using the following anti-mouse monoclonal antibodies from BD Biosciences (San Jose, CA) and BioLegend (San Diego, CA): fluorescein isothiocyanate (FITC)–conjugated antibodies including annexin V, anti-CD49b (clone DX5), anti-IgM (clone R6-60.2), anti-CD23 (clone B3B4), anti-GL7 (clone Ly77), and anti-IFNγ (clone XMG1.2); phycoerythrin-conjugated antibodies including anti-CD49b (clone DX5), anti-CD3ϵ (clone 145-2C11), anti-CD4 (clone GK1.5), anti-CD21 (clone 7G6), anti-CD11c (clone N418), and anti–IL-17 (clone TC11-18H10.1); Cy5-conjugated antibodies including anti-CD3ϵ (clone 145-2C11), anti-IgM (clone R6-60.2), anti-B220 (clone RA3-652), and anti-CD4 (clone GK1.5); and biotinylated anti-B220 (clone RA3-652). A minimum of 20,000 events per sample were collected (34). Splenic DX5+CD3− NK cells were sorted with an Epics Altra flow cytometer (Beckman Coulter, Fullerton, CA), and the purity was routinely >96%.

Cell cycle analysis. CD3+ T cells were removed from the spleen cells of both mice with CIA and control DBA/1J mice, using magnetic microbead separation (Miltenyi Biotec, Auburn, CA) before staining with FITC-conjugated anti-DX5 antibody, followed by fixation with cold 70% ethanol. Cells were treated with 50 μg/ml of RNase (Sigma-Aldrich, St. Louis, MO) and 50 μg/ml of propidium iodide (PI) (Sigma-Aldrich) before flow cytometric analysis (34).

Proliferation assay. Sorted NK cells (2×10^5/well) were seeded into a 96-well round-bottomed plate and pulsed with IL-2 (2,000 IU/ml) for 3 days and 6 days (R&D Systems, Minneapolis, MN). Tritium-labeled thymidine (^{3}H-thymidine; 1 μCi/well) was added for the last 16 hours, and ^{3}H-thymidine incorporation was measured as previously described (35). AQ: 3

Cytotoxicity assay. The killing activity of NK cells was examined by cytotoxicity assay using flow cytometric analysis (36). Briefly, YAC-1 target cells were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Carlsbad, CA) and washed before being cultured with splenic NK cells purified from mice with CIA and control mice, at effector:target ratios of 5:1, 10:1, and 20:1 and incubated in the presence of IL-2 in 5% CO_2 at 37°C for 16 hours. Dead cells were detected by staining with PI (10 μg/ml) for 15 minutes. Flow cytometric analysis was performed to assess NK lytic activity, and cytotoxicity was calculated as follows: % cytotoxi-

city = (specific lysis − spontaneous lysis)/(100% – spontaneous lysis) × 100%.

Real-time quantitative reverse-transcriptase–polymerase chain reaction (RT-PCR). Total RNA was extracted from sorting-purified DX5+CD3− splenic NK cells from mice with CIA and control mice or from the joints of control IgG-treated and asialo G_{M1}–treated mice with CIA, using TRIzol reagent (Invitrogen). Samples of joint tissue were prepared from the front paw after removing skin tissue and bones under a dissecting microscope. The messenger RNA (mRNA) levels of selected genes were determined using the SYBR Green Two-Step qRT-PCR Kit with ROX (Invitrogen) according to the manufacturer's guidelines and as previously described (37).

The sequences of gene-specific primers spanning an intron are as follows: for IFNγ (181 bp), sense 5′-AAGCGTCATTGAATCACACC-3′, antisense 5′-CGAATCAGCAGCGACTCCTT-3′; for tumor necrosis factor α (TNFα) (196 bp), sense 5′-TGGCCTCCCTCTCATCAG-3′, antisense 5′-GGCTGGCACCACTAGTTG-3′; for TGFβ (161 bp), sense 5′-GCGGCAGCTGTACATTGA-3′, antisense 5′-CCGGGTTGTGTTGGTTGT-3′; for IL-10 (166 bp), sense 5′-GGCCCAGAAATCAAGGAG-3′, antisense 5′-CCTTGTAGACACCTTGGT-3′; for IL-13 (211 bp), sense 5′-GCCGGTGCCAAGATCTGT-3′, antisense 5′-GCCATGCAATATCCTCTG-3′; for colony-stimulating factor 1 (CSF-1) (188 bp), sense 5′- ATGGACACCTGAAGGTCCTG-3′, antisense 5′-GTTAGCATTGGGGGTGTTGT-3′; for IL-17A (196 bp), sense 5′-AAAGCTCAGCGTGTCCAAAC-3′, antisense 5′-TGAGCTTCCCAGATCACAGA-3′.

The quantitative RT-PCR analysis was conducted with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), and the cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed with Sequence Detection System software (Applied Biosystems), and the threshold cycle (C_t) value within the log-linear range of the amplification curve was determined. Relative expression was quantitated by the comparative C_t method, in which fold differences were calculated with normalization to actin and controls.

Coculture of CD4+ cells with NK cells and intracellular staining. DX5+CD3− NK cells were purified from the spleens of DBA/1J mice, using an NK Cell Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. CD4+ T cells were isolated from the spleens of DBA/1J by CD4 (L3T4) microbeads (Miltenyi Biotec). Splenocytes from which CD3+ T cells were depleted by microbeads were used as APCs. CD4+ T cells (1×10^6/ml) were cultured with CD3/CD28 T cell expander (1:5 dilution) (Invitrogen), recombinant murine IL-6 (rMuIL-6) (20 ng/ml), rMuIL-23 (20 ng/ml), and rMuTGFβ (2 ng/ml) (R&D Systems), as previously described (3), in the presence or absence of NK cells (1×10^6/ml), with or without anti-mouse IFNγ antibodies (10 μg/ml) or anti-mouse IL-4 antibodies (10 μg/ml) (Peprotech, Rocky Hill, NJ) for 3 days. For Th17 cell detection by flow cytometry, cells were pulsed with phorbol myristate acetate (100 ng/ml), ionomycin (750 ng/ml) (Sigma-Aldrich), and GolgiPlug (1 μg/ml) (BD Biosciences) for the last 5 hours before surface staining with anti-CD4 antibodies and intracellular staining with anti-IFNγ and anti–IL-17 antibodies, using a Caltag intracellular staining kit (Invitrogen).

NK cell depletion in vivo. To deplete NK cells in vivo, mice were injected intraperitoneally with 50 μg asialo G_{M1} antibody (Wako, Richmond, VA) in 500 μl of PBS, 2 days before the first immunization, followed by 8 injections, once every 5 days (3). Control mice were treated with control normal rabbit IgG (Upstate Biotechnology, Lake Placid, NY).

Histopathologic analysis. Joint tissue specimens were fixed in 10% buffered formalin for 3 days, followed by decalcification in 15% formic acid overnight before being embedded in paraffin. Tissue sections (4 μm thick) were prepared for hematoxylin and eosin staining. Histologic analyses were performed as previously described (33).

ELISPot assay. The numbers of type II collagen–specific antibody-secreting plasma cells and IL-17–producing cells were determined by ELISPot assay. Briefly, 96-well flat-bottomed filtration plates with a cellulose ester membrane (Millipore, Billerica, MA) were coated with bovine type II collagen (5 μg/ml; Chondrex, Redmond, WA) or rat anti-mouse IL-17 (2 μg/ml; R&D systems) in coating buffer (0.05*M* carbonate, pH 9.6) at 4°C overnight. Plates were washed with washing buffer (0.05% Tween 20 in PBS) and then blocked with 5% fetal calf serum at room temperature for 1 hour. Cells were seeded into wells and incubated at 37°C for 4 hours and 24 hours for the detection of antibody-producing plasma cells and IL-17–producing cells, respectively, before being washed. To detect antibody-producing plasma cells, alkaline phosphatase (AP)–conjugated goat anti-mouse IgG (H+L) (1:1,000 dilution; Invitrogen) was added, whereas for detection of IL-17–producing cells, biotinylated goat anti-mouse IL-17 (200 ng/ml; R&D Systems) was added and incubated at room temperature for 2 hours, followed by AP-conjugated streptavidin (1:1,000 dilution; Invitrogen) at room temperature for 1 hour. Plates were washed and developed by adding BCIP/nitroblue tetraziolium (Sigma-Aldrich).

Enzyme linked immunosorbent assay (ELISA). Serum levels of total and type II collagen–specific IgG were measured by a colorimetric sandwich ELISA, as previously described (35). Goat anti-mouse IgG (Invitrogen) and bovine type II collagen (Chondrex) were coated on 96-well MaxiSorp plates (Nunc, Rochester, NY) at 5 μg/ml in coating buffer at 4°C overnight. Plates were washed before blocking with blocking buffer (0.5% gelatin, 0.5% bovine serum albumin, and 0.05% Tween 20 in PBS) at room temperature for 1 hour. Diluted serum samples (1:500) were added and incubated at room temperature for 1 hour. Plates were washed and incubated with AP-conjugated goat anti-mouse IgG (H+L) (1:1,000 dilution; Invitrogen) at room temperature for 1 hour. Plates were washed again, 1 mg/ml of freshly prepared phosphatase substrate (Sigma-Aldrich) in substrate buffer (0.1*M* diethylamine buffer, pH 9.6) was added, and absorbance at 405 nm was measured using a Sunrise microplate reader (Tecan, Durham, NC). Levels of IL-17 in culture supernatant were measured using a DuoSet ELISA Development Kit (R&D Systems), following the manufacturer's protocol.

Immunohistochemical staining. Paraffin sections of joint tissue were rehydrated and treated with 15% hydrogen peroxide. Samples were blocked with 10% rat serum and incubated with biotinylated anti–IL-17 antibodies (BioLegend). Brown precipitates were developed using StreptABComplex/

HRP (Dako, High Wycombe, UK) and 3,3′-diaminobenzidine tetrahydrochloride solution (Sigma-Aldrich). The nucleus was counterstained with Mayer's hematoxylin.

Statistical analysis. Data are expressed as the mean ± SD. Statistical analysis was performed using unpaired 2-tailed *t*-tests. *P* values less than 0.05 and less than 0.01 were considered significant.

RESULTS

Reduced frequencies of NK cells in the spleen and peripheral blood of mice with CIA. To characterize changes in NK cell populations during the pathogenesis of experimental arthritis, both the frequency and absolute number of NK cells were examined in various lymphoid organs and the peripheral blood of mice with CIA and control mice, by flow cytometry. The frequency of DX5+CD3− NK cells was moderately decreased in the peripheral blood before the onset of arthritis symptoms, and this decrease usually occurred 1–3 days after the second type II collagen immunization (data not shown). Subsequently, the frequency of NK cells in the spleen and peripheral blood became markedly reduced in mice with CIA during the early stage of disease development, i.e., 1–4 weeks after the second immunization (Figure 1A).

In contrast, an increased number of CD3+ T cells was detected during the initiation of CIA, which was consistent with our previous findings (33). Although the total number of nucleated cells was significantly increased in the spleens of mice with CIA, the absolute number of splenic NK cells was reduced to levels that were 45% of control values (mean ± SD $2.0 \pm 0.3 \times 10^6$ versus $0.9 \pm 0.2 \times 10^6$; $P < 0.01$) (Figure 1B). Similar patterns of reduced NK frequencies were detected in the peripheral blood and spleens of mice with CIA at the chronic stage of disease, i.e., 12–16 weeks following the second immunization with type II collagen (data not shown). However, no obvious changes in the frequency of NK cells was observed in the bone marrow (Figure 1B).

Increased apoptosis and reduced cytotoxicity of splenic NK cells in mice with CIA. To determine whether increased apoptosis contributed to the reduced population of splenic NK cells in mice with CIA, we evaluated spontaneous apoptosis of splenic NK cells in short-term cultures, in which apoptotic cells accumulate in the absence of phagocytosis (34). Flow cytometric analysis detected a significantly higher incidence of apoptosis of splenic NK cells in mice with CIA after 16 hours of culture (Figure 2A). To examine whether NK cells from mice with CIA were defective in terms of cell proliferation, cell cycle analysis was performed on

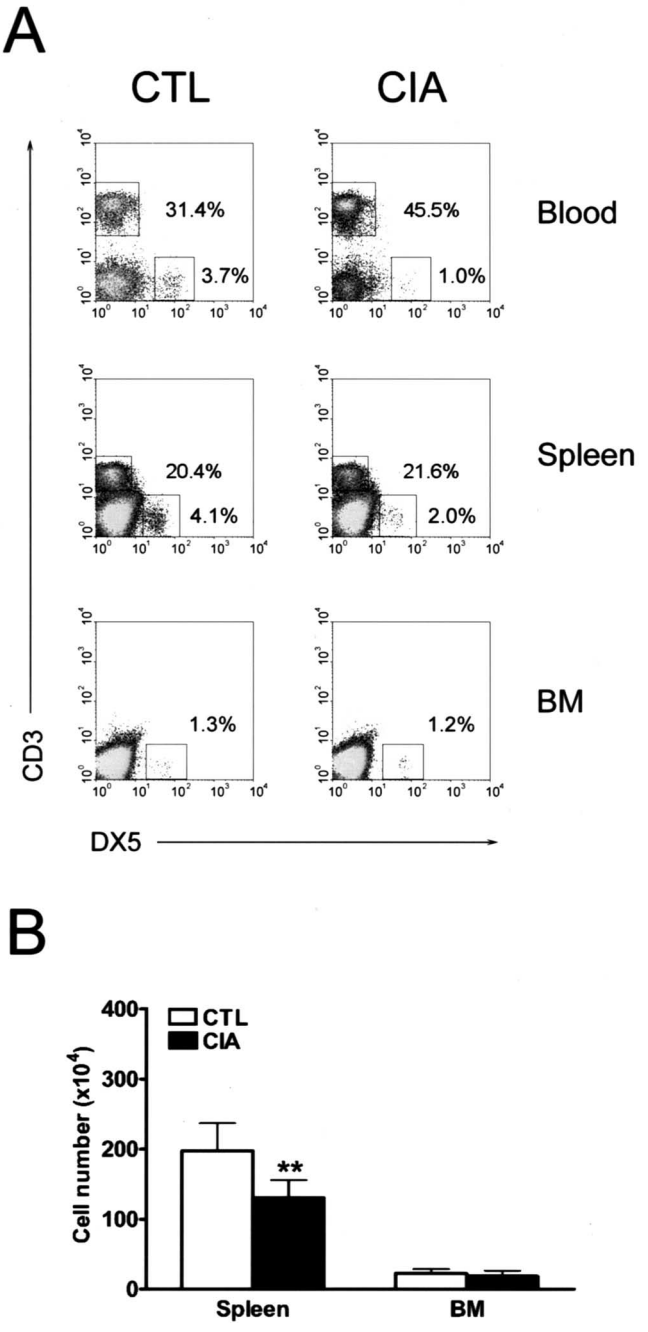

Figure 1. Reduced frequency and number of natural killer (NK) cells in the spleens and peripheral blood of mice with collagen-induced arthritis (CIA). **A,** Single-cell suspensions were prepared from the blood, spleens, and bone marrow (BM) of type II collagen–immunized mice and adjuvant-treated control (CTL) mice on day 30 after the first immunization and stained with anti-DX5 and anti-CD3 antibodies for flow cytometric analysis. Boxed areas show the populations of CD3−DX5+ NK cells and CD3+DX5+ NK cells, with their frequencies indicated. The results are representative of 10 mice per group. **B,** Total number of NK cells from the spleens and bone marrow of mice with CIA and control mice on day 30 after the first immunization. Values are the mean and SD results from 5 independent experiments. ** = $P \leq 0.01$.

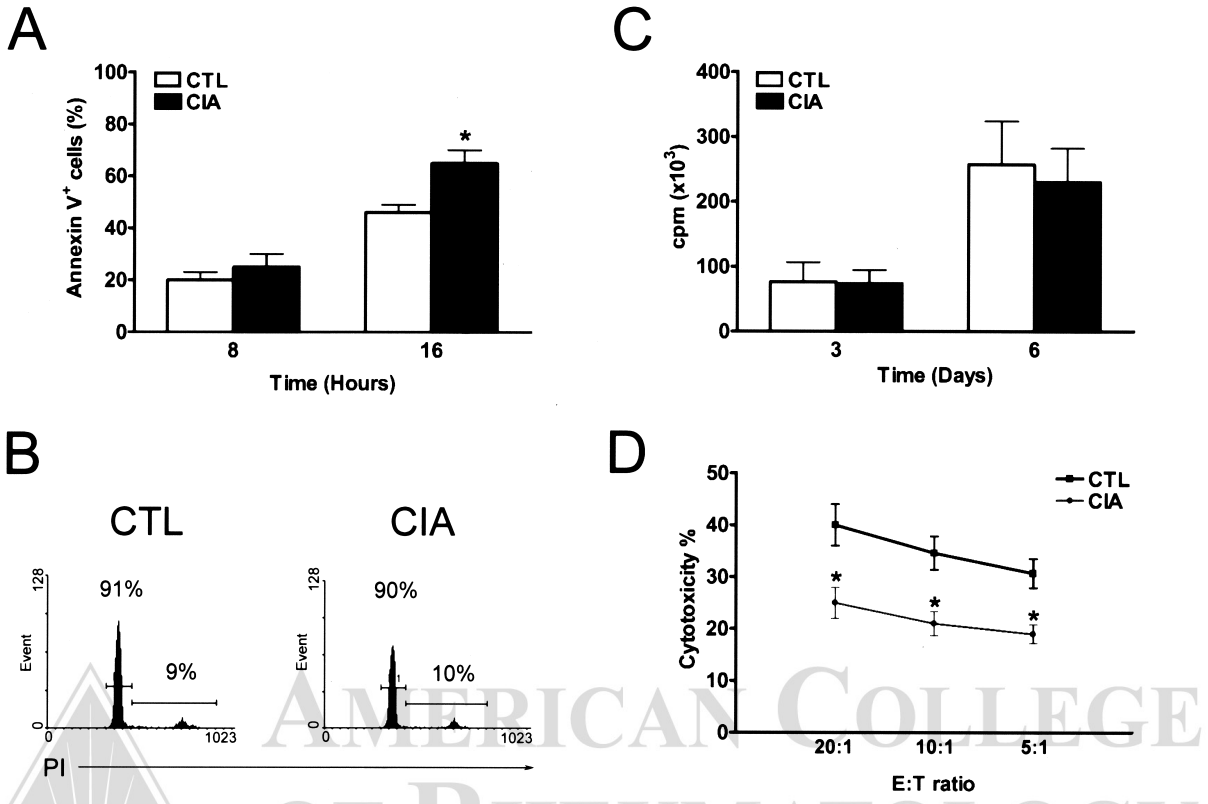

Figure 2. Enhanced apoptosis and defective killing ability of splenic NK cells isolated from mice with CIA. All mice were analyzed on day 30 after the first immunization. **A,** Splenocytes from mice with CIA and control mice were cultured in medium for 16 hours. The percentages of annexin V–positive DX5+CD3− NK cells were measured by flow cytometry. **B,** CD3+ cell–depleted splenocytes were prepared from mice with CIA and control mice and gated on DX5+ NK cells for cell cycle analysis. Histograms are representative of 3 separate experiments, and the percentages of DX5+ NK cells in the G_0/G_1 and $S+G_2/M$ phases are indicated. **C,** NK cells were purified from the spleens of mice with CIA and control mice and cultured with interleukin-2 for 3 days and 6 days before ^{3}H-thymidine was added for the last 16 hours. **D,** Splenic NK cells purified from mice with CIA and control mice were analyzed for their cytotoxicity by incubating with 5,6-carboxyfluorescein succinimidyl ester–labeled YAC-1 cells at the indicated effector:target (E:T) ratios. Flow cytometric analysis was performed to assess NK cell cytotoxicity. Values in **A, C,** and **D** are the mean ± SD results from 3 independent experiments. cpm = counts per minute; PI = ◆◆◆ (see Figure 1 for other definitions). $* = P \leq 0.05$.

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freshly prepared splenic NK cells from mice with CIA and control mice, and no alterations in cell cycle progression were observed (Figure 2B).

To verify the NK cell proliferative capacity, purified splenic DX5+CD3− NK cells from both mice with CIA and control mice were cultured in the presence of IL-2 for up to 6 days and assayed for ^{3}H-thymidine incorporation. NK cells from both mice with CIA and control mice showed a normal proliferative capacity in response to IL-2 stimulation (Figure 2C). We next analyzed the cytotoxicity of purified NK cells as determined by their ability to lyse YAC-1 cells (36). Splenic NK cells from mice with CIA displayed significantly reduced cytotoxic function compared with control mice (Figure 2D), indicating that diminished NK cells with impaired cytotoxicity are associated with the development of CIA. AQ: 11

Early onset of arthritis with significantly increased disease severity and autoantibody production in immunized mice with NK cell depletion. To evaluate the role of NK cells in the development of autoimmune arthritis, we depleted NK cells in type II collagen–immunized mice by administering anti–asialo G_{M1} antibodies once every 5 days, starting 2 days before the first type II collagen immunization. The effectiveness of NK cell depletion was verified by flow cytometry. Two days after treatment with anti–asialo G_{M1} antibodies, the frequency of DX5+CD3− NK cells in peripheral blood was markedly reduced (mean ± SD 0.7 ± 0.3% versus 4.1 ± 0.5% in IgG-treated control mice), whereas the

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Figure 3. Effect of natural killer (NK) cell depletion in exacerbating the development of collagen-induced arthritis (CIA). NK cells were depleted in vivo by intraperitoneal injection of anti–asialo G_{M1} antibodies 2 days before the first immunization, followed by 8 injections, once every 5 days. Mice injected with normal rabbit IgG (control IgG) were used as controls. **A,** Percentage of immunized mice (n = 20 in each group) in which arthritis developed. **B,** Clinical scores for arthritis severity as assessed in anti–asialo G_{M1} antibody– and control IgG-treated mice (n = 20 in each group). **C,** Hematoxylin and eosin–stained sections of inflamed joints from anti–asialo G_{M1} antibody– and control IgG-treated mice on day 40 after the first immunization. **D,** Histopathologic scores for joint tissue from anti–asialo G_{M1} antibody– and control IgG-treated mice on day 40 after the first immunization. Values are the mean and SD results from 10 mice per group. $* = P \leq 0.05$; $** = P \leq 0.01$.

frequencies of DX5+CD3+ NK T cells remained unchanged between these 2 groups (2.5 ± 0.6% and 2.8 ± 0.4%, respectively), indicating that NK cells were specifically depleted by anti–asialo G_{M1} antibody treatment.

Upon depletion of NK cells in vivo, type II collagen–immunized mice showed an earlier onset of arthritis (almost 1 week earlier) as compared with control normal rabbit IgG–treated mice (Figure 3A). Almost 80% of mice in the anti–asialo G_{M1} antibody–treated group developed arthritis 3 days after the booster immunization, while only 5% of mice in the control group showed clinical symptoms. Moreover, the clinical scores for arthritis severity were markedly elevated in NK cell–depleted mice at the acute stage of CIA development (Figure 3B). Histopathologic analysis also showed more pronounced synovial hyperplasia and tissue damage in the joints of anti–asialo G_{M1} antibody–treated mice, as revealed by elevated histopathologic

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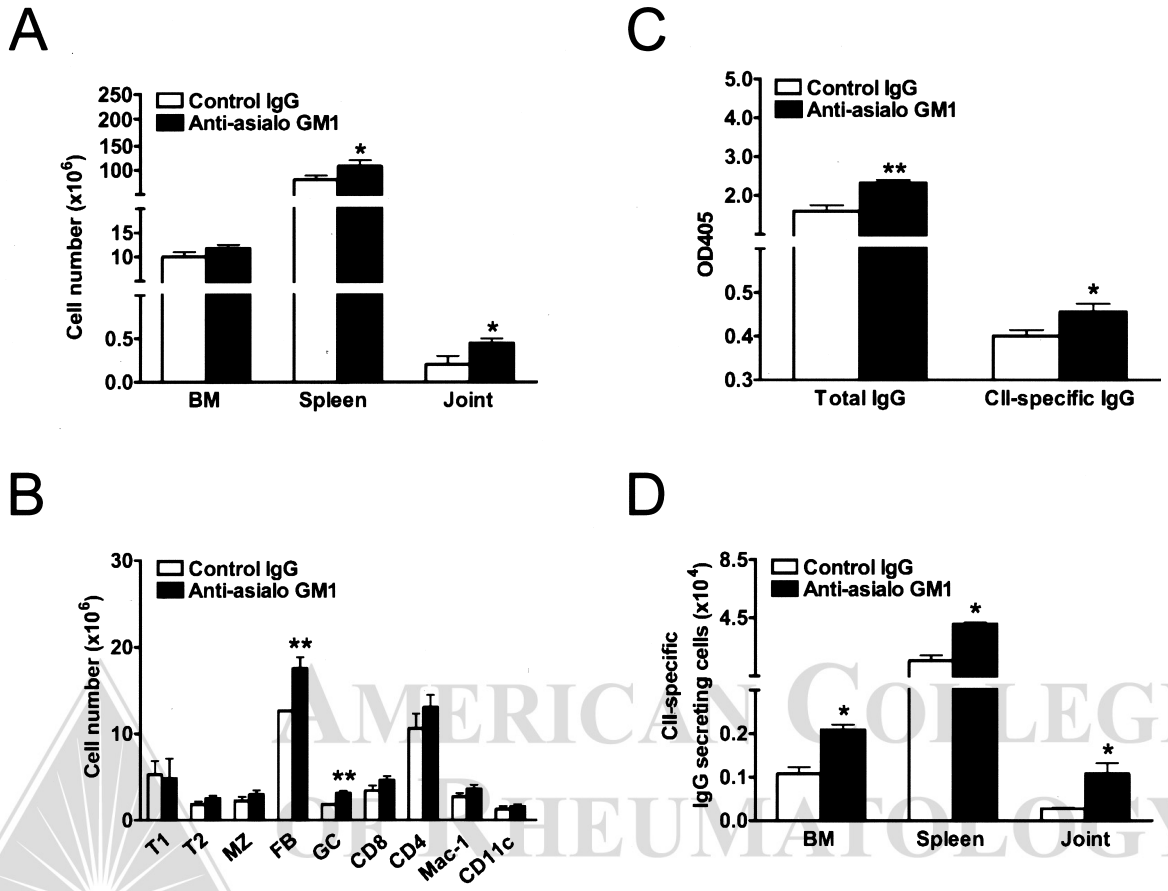

Figure 4. Enhanced type II collagen–specific autoantibody production and increased number of plasma cells in NK cell–depleted mice with CIA. All mice were analyzed on day 30 after the first immunization. **A,** Total number of cells in bone marrow (BM), spleen, and joint tissue from anti–asialo G_{M1} antibody– and control IgG-treated mice with CIA. **B,** Analysis of immune cell populations in the spleens of anti–asialo G_{M1} antibody– and IgG-treated mice with CIA. The total numbers of T1 ($B220^+CD23^-IgM^{high}CD21^{low}$), T2 ($B220^+CD23^+IgM^{high}CD21^{high}$), marginal zone (MZ; $B220^+CD23^-IgM^{high}CD21^{high}$), follicular (FB; $B220^+CD23^+IgM^{low}CD21^{low}$), and germinal center (GC; $GL7^+B220^+$) B cells, as well as Th cells ($CD4^+$), cytotoxic T cells ($CD8^+$), macrophages ($Mac\text{-}1^+$), and dendritic cells ($CD11c^+$) were enumerated. **C,** Serum levels of total and type II collagen (CII)–specific autoantibodies in NK cell–depleted mice with CIA and control mice as measured by enzyme-linked immunosorbent assay. **D,** Type II collagen–specific IgG-producing plasma cells in bone marrow, spleen, and joint tissue from NK cell–depleted mice with CIA and control mice as measured by ELISPot assays. Values are the mean and SD results from 3 independent experiments. OD_{405} = optical density at 405 nm (see Figure 3 for other definitions). $* = P \leq 0.05$; $** = P \leq 0.01$.

scores for synovial inflammation, cartilage damage, and bone erosion (Figures 3C and D). These data strongly indicate the involvement of NK cells in the development of arthritis.

Consistent with augmented inflammatory responses in NK cell–depleted mice with CIA, the total numbers of nucleated cells in spleen and joint tissue were substantially increased in anti–asialo G_{M1} antibody–treated mice (Figure 4A). In addition, the numbers of follicular B cells and germinal center B cells were significantly increased after depletion of NK cells (Figure 4B). Serum levels of anti–type II collagen–specific IgG were significantly elevated, together with a marked increase in the number of type II collagen–specific IgG-producing plasma cells in bone marrow, spleen, and joint tissue from NK cell–depleted mice with CIA (Figures 4C and D). These results indicate that NK cell depletion enhances plasma cell generation and autoantibody production during the development of CIA.

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Figure 5. Elevated numbers of interleukin-17 (IL-17)–secreting cells in draining lymph nodes (LNs) and joints from natural killer (NK) cell–depleted mice with collagen-induced arthritis (CIA). All mice were analyzed on day 30 after the first immunization. **A,** ELISPot analysis for IL-17–secreting cells in spleens, draining lymph nodes, and inflamed joints of anti–asialo G_{M1} antibody– and control IgG-treated mice with CIA. Results are representative of 3 independent experiments. **B,** IL-17 levels in the supernatant of cultured spleen and lymph node cells isolated from anti–asialo G_{M1} antibody– and control IgG-treated CIA mice as analyzed by enzyme-linked immunosorbent assay. Results are representative of 3 independent experiments. **C,** IL-17 mRNA expression in inflamed joint tissue from anti–asialo G_{M1} antibody– and control IgG-treated mice with CIA as determined by real-time polymerase chain reaction analysis. Relative mRNA expression levels were normalized to actin. Results are representative of triplicate samples in 2 independent experiments. **D,** Immunohistochemical staining of IL-17–producing cells in paraffin sections of inflamed joint tissue from anti–asialo G_{M1} antibody– and control IgG-treated mice with CIA. Photomicrographs are representative of 4 independent experiments. Values in **A–C** are the mean and SD. ** = $P \leq 0.01$.

Increased IL-17–secreting cells in draining lymph nodes and joints of NK cell–depleted mice with CIA. Recent studies have shown that Th17 cells are indispensable for the development of autoimmune arthritis. To determine the effect of NK cell depletion on IL-17 production in vivo, we found that the number of IL-17–secreting cells was significantly increased in the draining lymph nodes and joint tissue of anti–asialo G_{M1} antibody–treated mice with CIA, as detected by ELISPot analysis (Figure 5A), indicating a role of NK cells in IL-17 induction within local joint tissue and lymph nodes. In addition, levels of secreted IL-17 in culture supernatants of lymph node cells increased ~2.5-fold in the NK cell–depleted group (Figure 5B). Real-time PCR analysis revealed dramatically increased levels of IL-17 mRNA expression in joint tissue from NK cell–depleted mice with CIA; this finding was further supported by immunohistochemical detection of abundant IL-17–secreting cells in the joint tissue (Figures 5C and D). Taken together, these findings provide strong

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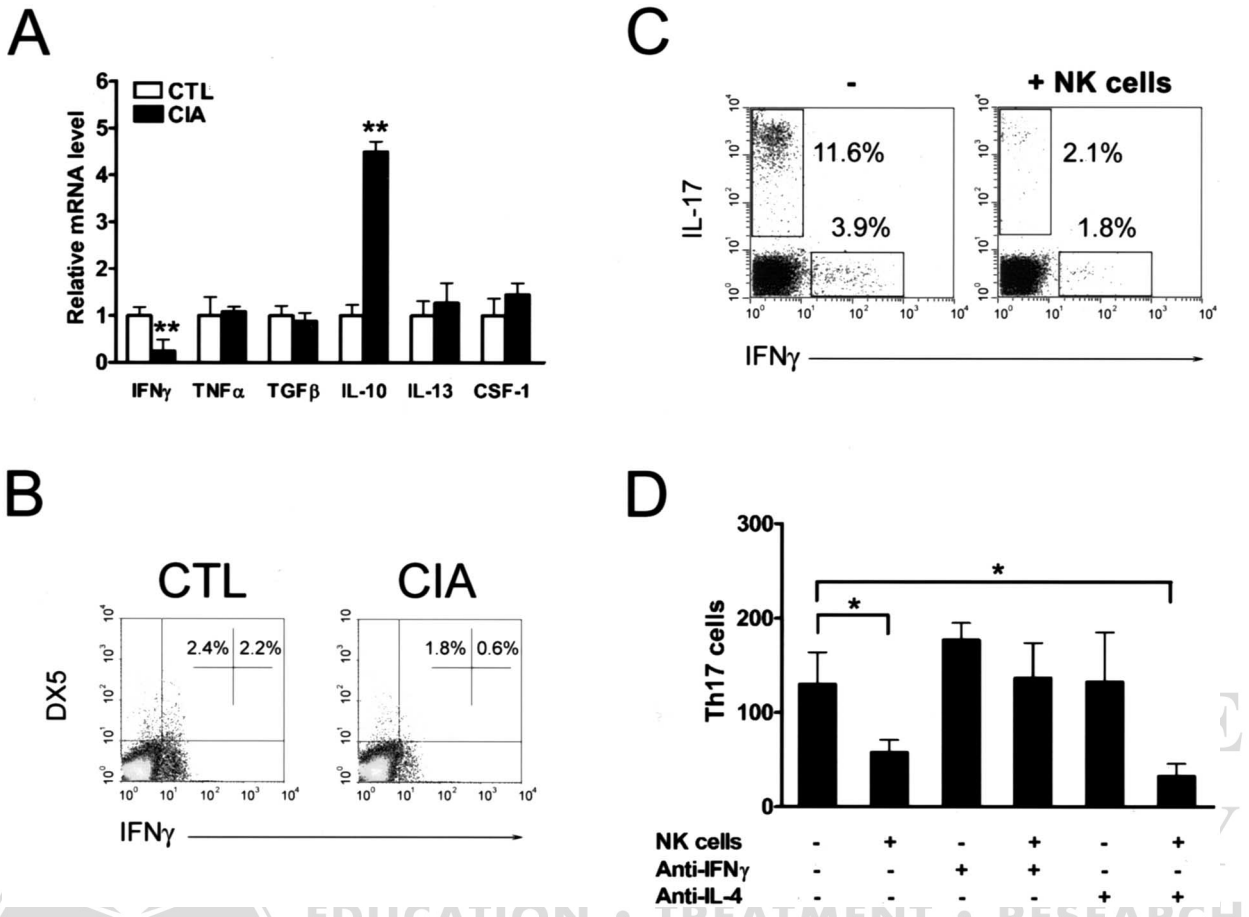

Figure 6. Natural killer (NK) cell–mediated suppression of the generation of Th17 cells via interferon-γ (IFNγ) production. **A,** Messenger RNA levels of IFNγ, tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), interleukin-10 (IL-10), IL-13, and colony-stimulating factor 1 (CSF-1) normalized to actin as determined in DX5+CD3+ NK cells isolated from the spleens of mice with CIA and control mice by real-time polymerase chain reaction. Results are representative of triplicate samples in 3 independent experiments. **B,** Percentages of CD3−DX5+IFNγ− and CD3−DX5+IFNγ+ cells. Splenocytes from mice with CIA and control mice were prepared for intracellular staining of IFNγ and gated on CD3− cells. Results are representative of 2 independent experiments. **C,** Frequency of CD4+IL-17+IFNγ− Th17 cells in the presence of NK cells. CD4+ T cells from mice with CIA were cultured with gamma-irradiated antigen-presenting cells, T cell expander (1:5 dilution), IL-6 (20 ng/ml), IL-23 (20 ng/ml), and TGFβ (2 ng/ml) with or without NK cells isolated from adjuvant-treated mice. Intracellular staining of IL-17 and IFNγ was performed and analyzed on the CD4+ T cells. Results are representative of 3 independent experiments. **D,** Participation of IFNγ in suppressing the generation of Th17 cells by NK cells. Purified splenic CD4+ T cells were cultured for 3 days as described in **C**, but with or without the addition of anti-IFNγ (10 μg/ml) or anti–IL-4 (10 μg/ml). IL-17–secreting cells were detected by ELISPot assay. Results are representative of 3 independent experiments. Values in **A** and **D** are the mean and SD. $* = P \leq 0.05$; $** = P \leq 0.01$.

evidence that NK cells might play a role in modulating IL-17 production during the development of CIA.

Suppressed Th17 cell generation by NK cells through IFNγ. The development of Th17 cells is largely controlled by the cytokine milieu in the microenvironment. NK cells are known to play a role in regulating immune responses by producing both Th1 and Th2 cytokines. To examine possible changes in the cytokine profile in NK cells during autoimmune conditions, we used quantitative PCR analysis to measure mRNA levels of IFNγ, TNFα, TGFβ, IL-10, IL-13, and CSF-1 in splenic DX5+CD3− NK cells from mice with CIA and control mice. The IFNγ mRNA level was significantly reduced (by >4-fold), whereas IL-10 transcripts were markedly up-regulated (by almost 5-fold) (Figure 6A). To further confirm these data, we used flow cytometry to examine the frequency of IFNγ-producing DX5+CD3− NK cells. As shown in Figure 6B, the frequency of

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IFNγ-producing NK cells was markedly reduced by >3-fold in the spleens of mice with CIA. Previous studies have demonstrated that IFNγ actively suppresses the generation of Th17 cells (38) and plays a critical role in the pathogenesis of CIA through the suppression of Th17 cell development (32).

AQ: 14 Because NK cells are known to be one of the major and earliest sources for IFNγ production in immune responses (39,40), our findings suggest a link of defective IFNγ production by NK cells to unrestrain Th17 cell generation during the pathogenesis of arthritis. In order to verify this, we analyzed whether or not NK cells can directly regulate Th17 cell generation. We cultured CD4+ T cells with NK cells prepared from adjuvant-treated mice and analyzed the frequency of Th17 cells. As shown in Figure 6C, flow cytometric analysis detected a markedly reduced frequency of CD4+IL-17+IFNγ− Th17 cells in the presence of NK cells, indicating that NK cells can inhibit the generation of Th17 cells. The addition of blocking antibodies for IFNγ reversed the inhibitory effects of NK cells on IL-17 production, suggesting that IFNγ participates in suppressing the generation of Th17 cells by NK cells (Figure 6D). Taken together with the markedly reduced IFNγ production in NK cells from mice with CIA, our data support the notion that NK cells modulate the development of Th17 cells under conditions of autoimmunity.

DISCUSSION

In this study, we observed substantially reduced frequencies of NK cells in the peripheral blood and spleens of mice with CIA. Splenic NK cells displayed increased apoptosis with reduced cytotoxicity in arthritic mice. Moreover, depletion of NK cells in vivo accelerated the onset of disease and enhanced the clinical severity of arthritis in type II collagen–immunized mice, together with markedly increased autoantibody and IL-17 production. NK cells isolated from mice with CIA expressed low levels of IFNγ. We further showed that IFNγ mediates the suppressive effect of NK cells on the generation of Th17 cells. These results suggest that NK cells play a protective role in the development of experimental arthritis, possibly by modulating IL-17 production via their IFNγ production.

Early studies have shown the recruitment of NK cells into the target organs of patients with autoimmune diseases including RA (41,42), suggesting the involvement of NK cells in the pathogenesis of autoimmunity. However, it is largely unclear how dysregulated NK cells contribute to the development of experimental arthritis. We observed that the degeneration of NK cells with defective killing capacity is associated with the onset of CIA, indicating that both numeric and functional changes of NK cells may contribute to the pathogenesis of CIA. Consistently, deficiencies in the number and function of gut NK cells have been found to precede the onset of diabetes in diabetes-prone BB rats, while NK cell depletion abrogates cyclophosphamide-induced diabetes in NOD mice (3,4). A compromised killing ability AQ: 15 of NK cells in mice with CIA might facilitate the formation of autoreactive T cells and APCs in inciting experimental arthritis (5,43). Previous studies have indicated that IL-10 provides a protective effect on the development of arthritis (14,44). Although we detected up-regulated IL-10 expression in splenic NK cells from mice with CIA, a reduced number of NK cells might minimize IL-10–mediated production.

Our data show that NK cells display elevated apoptosis during the development of CIA. It has been reported that the degeneration of NK cells in experimental autoimmune myasthenia gravis is mediated by IL-21 derived from autoreactive CD4+ T cells (45). However, we observed normal levels of IL-21 expression in the CD4+ T cells of mice with CIA (data not shown). It currently remains unclear whether other cytokines such as TNFα are involved in mediating increased apoptosis of NK cells (46). Further studies are warranted to identify the triggering factors responsible for enhanced NK cell apoptosis during CIA development.

The divergent functions of NK cells in the pathogenesis of autoimmunity are mainly dependent on the types of autoimmune diseases and animal models. Our data indicate that NK cells may play a role in regulating autoantibody-producing plasma cells during the pathogenesis of CIA. Increased numbers of follicular and germinal center B cells in the spleens of NK cell–depleted mice with CIA indicate that more mature B cells are activated, undergoing further differentiation into antibody-producing plasma cells. The regulatory mechanisms by which NK cells modulate adaptive immune response are less well understood, but several studies have suggested an important role for NK cells in inhibiting T cell activation and in modulating the survival and function of dendritic cells (11). In addition, NK cells have been shown to determine the outcome of B cell–mediated autoimmunity (8). Consistent with previous observations that anti-DNA autoantibody production correlated with the disappearance of NK cells in autoimmunity-prone *lpr* mice (47), our findings suggest that NK cells can either directly or indirectly affect B cell maturation and antibody production in vivo. Further

studies are being performed in our laboratory to elucidate the underlying mechanisms by which NK cells modulate plasma cell generation and antibody secretion.

Increasing evidence highlights the critical roles of cytokine secretion by and the killing ability of NK cells in autoimmune diseases (43,48,49). In accordance with the function of IFNγ as a potent inhibitor for Th17 cell generation, our results that NK cells from arthritic mice display reduced expression of IFNγ are consistent with the findings of increased IL-17 production and exacerbated arthritis upon NK cell depletion in vivo. Our data that NK cells suppress the generation of Th17 cells in vitro, an effect that could be reversed by blockade of IFNγ, provide strong evidence for a previously unrecognized role of NK cells in inhibiting Th17 cell generation via IFNγ. Although the possibility of reduced IFNγ production by CD4+ cells cannot be excluded (Figure 6B), it has been recently reported that CpG-induced dendritic cell–NK cell interactions inhibit the effector phase of inflammatory arthritis in the K/BxN mouse serum transfer model, while IFNγ produced mainly by NK cells blocks neutrophil recruitment to the joint (50). Thus, IFNγ produced by NK cells may protect mice from developing arthritis via diverse mechanisms. Taken together, our findings demonstrate that NK cell degeneration promotes the onset and progression of experimental arthritis, possibly through enhanced IL-17 production. Therefore, functional modulation of NK cells may provide a new therapeutic possibility for patients with RA.

AUTHOR CONTRIBUTIONS

Dr. Lu 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 design. Lo, Lam, Lu.
Acquisition of data. Lo, Sun, Ko, Wang.
Analysis and interpretation of data. Lo, Lam, Xu, Wu, Zheng, Lu.
Manuscript preparation. Lo, Lam, Lu.
Statistical analysis. Lo.

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