Mobilization of natural killer cells inhibits development of collagen-induced arthritis

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Although natural killer (NK) cells have been implicated in regulating immune responses, their ability to modulate disease development in autoimmune arthritis has not been analyzed. Here we investigate the contribution of NK cells to regulating collagen-induced arthritis, a well-characterized preclinical model of human rheumatoid arthritis. We find that the disease is induced by the combined action of two CD4+ T helper (Th) subsets: follicular Th and Th17 cells. Both CD4+ Th subsets are highly susceptible to lysis by NK cells after activation. Administration of antibody that activates NK cells through blockade of its inhibitory CD94/NKG2A receptor allows enhanced elimination of pathogenic follicular Th and Th17 cells and arrest of disease progression. These results suggest that antibody-dependent enhancement of NK activity may yield effective, previously undescribed therapeutic approaches to this autoimmune disorder.

antibody therapy | Qa-1 | T helper subsets

The prevalence of rheumatoid arthritis (RA) has increased markedly over the last decade, along with the increased size of the aging population (1). New therapeutic approaches have come from studies of animal models of RA. Induction of arthritis in mice after immunization with type II collagen (CII), termed collagen-induced arthritis (CIA), shares several similarities with human RA, including breach of self-tolerance, generation of autoimmune responses, and the development of inflammatory changes in multiple joints (2). This animal model has been used to evaluate the prevalence of rheumatoid arthritis (RA). Induction of arthritis in mice after immunization with type II collagen (CII), termed collagen-induced arthritis (CIA), shares several similarities with human RA, including breach of self-tolerance, generation of autoimmune responses, and the development of inflammatory changes in multiple joints (2). This animal model has been used to evaluate several FDA-approved therapies for RA, including anti-TNF Ab, IL-1 antagonists, and methotrexate (3, 4). Clinically relevant arthritis in mice after immunization with type II collagen (CII), termed collagen-induced arthritis (CIA), shares several similarities with human RA, including breach of self-tolerance, generation of autoimmune responses, and the development of inflammatory changes in multiple joints (2). This animal model has been used to evaluate several FDA-approved therapies for RA, including anti-TNF Ab, IL-1 antagonists, and methotrexate (3, 4). Clinically relevant arthritis in mice after immunization with type II collagen (CII), termed collagen-induced arthritis (CIA), shares several similarities with human RA, including breach of self-tolerance, generation of autoimmune responses, and the development of inflammatory changes in multiple joints (2). This animal model has been used to evaluate several FDA-approved therapies for RA, including anti-TNF Ab, IL-1 antagonists, and methotrexate (3, 4). Clinically relevant arthritis in mice after immunization with type II collagen (CII), termed collagen-induced arthritis (CIA), shares several similarities with human RA, including breach of self-tolerance, generation of autoimmune responses, and the development of inflammatory changes in multiple joints (2). This animal model has been used to evaluate several FDA-approved therapies for RA, including anti-TNF Ab, IL-1 antagonists, and methotrexate (3, 4). Clinically relevant arthritis in mice after immunization with type II collagen (CII), termed collagen-induced arthritis (CIA), shares several similarities with human RA, including breach of self-tolerance, generation of autoimmune responses, and the development of inflammatory changes in multiple joints (2). This animal model has been used to evaluate several FDA-approved therapies for RA, including anti-TNF Ab, IL-1 antagonists, and methotrexate (3, 4). Clinically relevant arthritis in mice after immunization with type II collagen (CII), termed collagen-induced arthritis (CIA), shares several similarities with human RA, including breach of self-tolerance, generation of autoimmune responses, and the development of inflammatory changes in multiple joints (2). This animal model has been used to evaluate several FDA-approved therapies for RA, including anti-TNF Ab, IL-1 antagonists, and methotrexate (3, 4).}
inhibit CIA initially came from findings that adoptive transfer of CII-immune CD4+ T cells and B cells into Rag2−/− Prf1−/−, but not Rag2−/−, mice resulted in robust arthritis and autoantibody development (Fig. 2B and C). The latter Rag2−/− recipients, but not the former, harbor perforin+ NK cells.

Engagement of Inhibitory CD94/NKG2A Receptor on NK Cells Regulates CIA Development. Elimination of pathogenic T cells by NK cells in vivo is normally held in check through engagement of the CD94/NKG2A inhibitory receptor on NK cells by the Qa-1/Qdm ligand expressed on activated T cells (8). Consistent with findings that NKG2A+ NK cells accumulate in human arthritic synovia (11, 12), the numbers of NKG2A+ NK cells increased systemically in all lymph nodes (LN)s with disease progression (Fig. 3A). We asked whether NK cells might inhibit the development of CIA in animals given CII-immune CD4+ T cells and determined whether the inhibitory CD94/NKG2A receptor might represent a suitable molecular target for therapeutic intervention. To this end, we evaluated arthritis development in adoptive hosts (Rag2−/− Prf1−/−) given CD4+ T cells from B6.WT or B6.Qa-1 R72A knock-in mice along with (B6) NK cells. CD4+ T cells from Qa-1 R72A mice express a mutant Qa-1 protein that prevents binding of Qa-1 to the inhibitory CD94/NKG2A receptor and increases their susceptibility to NK lysis (8). Infusion of either Qa-1 WT or Qa-1 mutant CD4 cells and B cells promoted arthritis development (Fig. 3B). Cotransfer of NK cells reduced disease in hosts given Qa-1

**Fig. 1.** The contribution of Th17 and Tfh cells to CIA. ICOS+CXCR5+BTLA+CD25−CD4+ Tfh cells were FACS-sorted from arthritic mice (score = 12) and transferred into Rag2−/−Prf1−/− mice. (A) Serum Ig and autoantibody titers (anti-mouse CII) were measured at d 21 and 40. (A) Arthritis scores of three mice per group are shown. Arrows indicate CIA immunization and boosting. (C) Representative images of joint histology along with the arthritis score are shown for mice given the indicated Tfh subsets. Data represent one of three identical experiments.

**Fig. 2.** The contribution of NK cells to regulation of CIA. (A) IL-2-expanded NK cells were incubated for 4 h with CII-specific Th1 and Th17 cell subsets (induced in vitro) at the indicated E:T ratios. Percent lysis is shown. (B) Purified CD4 cells (lacking CD25+CD4+ Treg) and B cells from arthritic mice with indicated disease severity were transferred into Rag2−/−Prf1−/− recipients. Arthritis was induced as described and scores are shown. (C) Serum anti-chicken CII IgG and IgG1 titers were measured at d 45 and represent one of three identical experiments for B and C.
WT CD4 cells and abolished arthritis development in hosts coinfected with QA-1 R72A CD4 cells (Fig. 3B). These findings suggest that genetic interruption of the inhibitory CD94/NKG2A–QA-1 interaction resulted in increased levels of NK cell-dependent inhibition of CIA.

**Anti-NKG2A F(ab)2 Treatment Inhibits Arthritis.** In vitro cytotoxicity assays indicated that addition of anti-NKG2A F(ab)2 Ab to block the inhibitory CD94/NKG2A receptor enhanced NK lysis of collagen-specific TFH and TH17 target cells (Fig. 3C). These findings suggested that Ab-dependent interruption of the inhibitory interaction between the QA-1/Qdm ligand and the CD94/NKG2A receptor might enhance NK lysis of arthrogenic TFH and TH17 cells. We therefore evaluated the impact of administration of anti-NKG2A F(ab)2 Ab on CIA development in vivo.

Injection of anti-NKG2A F(ab)2 Ab after CIA immunization resulted in prolonged suppression of CIA that persisted after cessation of treatment (Fig. 4A). Anti-NKG2A F(ab)2 Ab treatment ameliorated joint inflammation/destruction, prevented pannus formation—a bona fide histological feature of severe arthritis (Fig. 4B)—and reduced collagen-specific recall responses of LN cells (Fig. S2A). Further analysis revealed that this treatment decreased autoantibody titers (Fig. 4C) and reduced the numbers of Tfh cells (ICOS+CXCR5+ or ICOS+BTLA+ CD4 cells and IL-21+ CD4 cells), but not B cells (Fig. 4D and E and Fig. S2B), similar to findings in vitro (Fig. 3C). Moreover, numbers of Tfh17 (IL-17+), but not Tfh1 (IFN-γ+) or Th12 (IL-4+), cells were also reduced, consistent with selective in vivo sensitivity of Tfh17 and Tfh17 cells to NK lysis (Fig. 4E and Fig. S2C).

Finally, we investigated the impact of anti-NKG2A F(ab)2 Ab treatment on joint infiltration by activated NK cells and CD4+ Tfh cells. Treatment with anti-NKG2A resulted in increased proportions of perforin+ NK cells (Fig. 5A) as well as a substantial reduction in the numbers of CD4+ Tfh cells (Fig. 5B) and a prominent decrease in ICOS+CXCR5+ Tfh cells (Fig. 5C).

**Discussion**

Clinical approaches to the treatment of arthritis have focused mainly on downstream events in this autoimmune disease process and have yielded drugs that inhibit TNF and IL-1 cytokine expression by macrophages and synovial cells (13, 14). Here we describe a strategy based on targeting these pathogenic Tfh subsets responsible for induction of the two primary components of CIA: autoantibodies (anti-collagen and rheumatoid factor) and intraarticular inflammatory responses.

Previous findings have indicated that Tfh cells are particularly well equipped to induce autoantibody responses in collaboration with GC B cells (7, 15). The contribution of Tfh cells to the autoantibody response was confirmed by adoptive transfer studies revealing induction of strong anti-collagen Ab responses. Moreover, mild disease was observed in mice supplied with Tfh1 or Tfh17 cells alone, whereas severe arthritic disease reflected cooperation between Tfh1 and Tfh17 cells (Fig. 1). These observations and others suggest that the disease may proceed along two cellular pathways: one that depends on Tfh1-induced autoantibodies and a second that depends mainly on induction of inflammatory responses by Tfh17 cells. We propose that binding of autoantibodies to collagen in joints activates and triggers local inflammatory cell infiltration (16) and that Tfh17-dependent cytokine responses amplify this response, resulting in robust and destructive intraarticular disease (e.g., Fig. 1). An important caveat to this model comes from the potential plasticity of these Tfh subsets after expansion in vivo (17). Additional studies are needed to address the stability of Tfh17 and Tfh cells after long-term sojourn in adoptive hosts.

The contribution of NK cells to arthritis has been unclear, in part because of the diverse activities of this lymphocyte subset (18). Our experimental approach has revealed the importance of NK cells in regulating arthritis in an animal model. The regulatory role of NK cells may depend largely on its cytotoxic function, as judged from its dependence on perforin expression in these studies. Interestingly, NK cells in RA synovium that display impaired cytotoxic activity may contribute to dysregulated immune responses in arthritic patients (19, 20). The susceptibility of pathogenic Tfh1 and Tfh17 subsets to NK-dependent lysis in vitro prompted us to test the impact of F(ab)2 anti-NKG2A-dependent up-regulation of NK activity in vivo. Anti-NKG2A F(ab)2 mAb treatment did not deplete NKG2A+ NK cells or render NK cells hyporesponsive. Instead, blockade of the inhibitory CD94/NKG2A receptor for QA-1/Qdm enhanced NK cell lysis of Tfh1 and Tfh target cells in vitro and inhibited disease progression.

![Fig. 3. Regulation of NK cell activity through a CD94/NKG2A–QA-1 interaction. (A) Numbers of NKG2A+ NK cells from draining LN (dLN) and non-draining LN (nondLN) during CIA development are shown for groups of three mice for each time point. (B) Purified CD4+ T cells (depleted of CD25+CD4+ Treg) from arthritic B6.WT or B6.Qa-1 R72A mice were transferred into Rag2−/− hosts along with B6.WT B cells. In some cases, sorted B6.WT NK cells were transferred into hosts before infusion of CD4 and B cells. Mice were immunized and boosted with CII at d 21 and 36 (black arrows). **p < 0.01. Arthritis scores are shown for adoptive hosts (three per transfer). (C) IL-2–expanded NK cells were preincubated with 10 μg mL−1 20d5 F(ab)2 for 1 h at 37 °C followed by incubation for 4 h with in vitro-differentiated CII-specific Tfh subsets at the indicated E:T ratios. Percent lysis is shown.](https://www.pnas.org/doi/10.1073/pnas.1112188108)
in vivo. These studies indicate that anti-NKG2A-dependent interruption of the inhibitory interaction between Qa-1/Qdm on activated CD4 TH cells and NKG2A+ NK cells "released the brakes" on NK cells, resulting in increased elimination of pathogenic CD4+ TH cells and durable inhibition of arthritis. The basis for preferential NK cell targeting of TH17 and TFH cells, rather than TH1 or TH2 cells, in these studies is unclear. Increased levels of NKG2D ligands by TH17/TFH cells probably do not play a decisive role in the susceptibility of these TH subsets (Fig. S3C), and further studies are required to define the selective sensitivity of the TH17–TFH pair to NK lysis. In sum, these studies indicate that amelioration of arthritis can be achieved through mobilization of NK cells by anti-NKG2A F(ab)′2 treatment and consequent elimination of pathogenic autoreactive T cells. Because CD94/NKG2A and its ligands are highly conserved in rodents and humans, this anti-NKG2A–based approach may be translated into an effective, previously undescribed therapy for human RA.

Materials and Methods

Mice. C57BL/6 (B6), Rag2−/−, Rag2−/−Prf1−/− (Taconic Farms), and B6.Qa-1 R72A mice (back-crossed for 11 generations; ref. 8) were housed in pathogen-free conditions. All experiments were performed in compliance with...
CIA Induction and Assessment. Chicken CII (MD Bioscience) was dissolved in 0.01 M acetic acid at a concentration of 4 mg mL⁻¹ by stirring overnight at 4 °C. All mice were males between the age of 8 and 12 wk. To induce CIA, C57BL/6 (B6.WT) mice were injected intradermally at the base of the tail with 100 μg of chicken CII emulsified in complete Freund’s adjuvant (supplemented with 4 mg mL⁻¹ Mycobacterium tuberculosis) and boosted at the indicated days with 100 μg of chicken CII emulsified in incomplete Freund’s adjuvant. For adoptive transfers, purified CD4 (CD25⁺CD4⁺ Treg depleted) and B cells from arthritic mice with differing severity as noted were transferred into Rag2⁻⁻Prf1⁻⁻ or Rag2⁻⁻ hosts, immunized at d 0, and boosted at the indicated days as described above. Clinical assessment of CIA was performed every 2–3 d each week, and scoring was as follows: 0, normal; 1, mild swelling and/or erythema confined to the midfoot or ankle joint; 2, moderate edematous swelling extending from the ankle to the metatarsal joints; and 3, pronounced swelling encompassing the ankle, foot, and digits. Each limb was graded thus, allowing a maximum score of 12 per mouse.

Measurement of Abs Against CII. Serum levels of anti-chicken or anti-mouse CII IgG or IgG subclasses were measured by ELISA. Serum was collected at indicated days after first chicken CII immunization. Briefly, 96-well ELISA microplates were coated with chicken or mouse CII (Chondrex) at 5 μg mL⁻¹ dissolved in dilution buffer (Chondrex) at 100 μL per well at 4 °C overnight. 100 μL of diluted serum sample was incubated for 2 h at room temperature. The plates were washed with PBST (0.05% Tween-20 in PBS) five times, followed by addition of peroxidase-conjugated goat anti-mouse IgG at 1:50,000 concentration (Sigma) or peroxidase-conjugated goat anti-mouse IgG1 or anti-mouse IgG2a at 1:1,000,000 concentration (BD Bioscience) and incubated further for 6 h. Intracellular staining of perforin in intraarticular cells, paw pieces were isolated and digested with collagenase/dispase (Roche) for 1 h at 37 °C followed by filtration to yield single-cell suspensions. Cells were incubated with Fc block for 15 min followed by staining with relevant Abs against surface markers. CD45 marker was included to gate leukocytes from joints for further analysis. To detect levels of surface NKGA2A, cells from control and F(ab)⁻2-treated groups were first incubated with rat20DSF(ab)⁻2 Ab (1 μg mL⁻¹) (Novo Nordisk A/S) for 30 min to saturate surface NKGA2A and further stained with FITC anti-rat Ig κ light chain monoclonal Ab. For intracellular cytokine staining, cells were restimulated with leukocyte activation mixture (BD Bioscience) for 5 h, stained with surface markers, fixed, and permeabilized, followed by incubation with indicated Abs. To stimulate NK cells, cells from each organ (2 × 10⁶ cells per mL) were stimulated with plate-bound anti-NK1.1 (20 μg mL⁻¹) for 1 h followed by the addition of BD GolgiPlug (BD Bioscience) and incubated further for 6–7 h. Intracellular staining of perforin in NK cells was performed as described above. Cells were acquired on a FACS Canto II by using FACS Diva software (BD Biosciences) and analyzed with FlowJo software (Tristar).

In Vitro Differentiation of T₈ Subsets. Cells from spleen and LN were collected from B6 mice preimmunized with 150 μg of chicken CII/CFA. CD4⁺CD25⁻ cells were purified and enriched by negative selection. 2 × 10⁵ cells per mL CD4 cells were stimulated with 100 μg mL⁻¹ chicken CII in the presence of 2 × 10⁶ irradiated total splenocytes. For the differentiation of CD4 cells to each T₈ cell phenotype, the following cytokine mixtures were added into the culture: T₈₁: 5 ng mL⁻¹ rmIL-12 and 10 μg mL⁻¹ anti-IFN-γ Ab; T₈₂: 10 ng mL⁻¹ rmIL-4, 10 μg mL⁻¹ anti-IL-12 Ab, 10 μg mL⁻¹ anti-IL-4 Ab; T₈₁/T₈₂: 3 ng mL⁻¹ TGF-β, 20 ng mL⁻¹ rmIL-6, 20 ng mL⁻¹ rmIL-23, 10 μg mL⁻¹ anti-IL-12 Ab, 10 μg mL⁻¹ anti-IFN-γ Ab, 10 μg mL⁻¹ anti-IL-4 Ab; T₈₁/T₈₂/T₈₁7: 50 ng mL⁻¹ IL-1, 10 μg mL⁻¹ anti-IFN-γ Ab, 10 μg mL⁻¹ anti-IL-4 Ab, 20 ng mL⁻¹ rmIL-6, and 20 μg mL⁻¹ anti-TGF-β (1D11) Ab. At d 5, live CD4⁺ cells were harvested from cultures by percoll gradient centrifugation. Total RNA was extracted, and quantitative RT-PCR was performed to measure levels of transcription factors by using specific TaqMan probes (Applied Biosystems) as indicated.

Proliferation Assay. NondLN cells were incubated for 48 h with different concentrations of chicken CII in DMEM supplemented with 10% FCS and 2% FBS.
50 μM 3H-mercaptoethanol. 3H-thymidine was incorporated in the last 18 h before quantification with a Micro-Bi counter (Wallac).

**NK Cytotoxicity Assay.** Purified NK cells were incubated with 1,000 U mL−1 IL-2 (Peprotech) for 5 d and used as effector cells. Target cells were labeled with 50 μCi of Na2[^51]CrO4 for 1 h at 37 °C and washed three times with PBS before mixing (1 × 10⁵ per well) with effector cells in U-bottomed 96-well plates at different E:T ratios as indicated, in triplicate. After 4 h of incubation, cell-free supernatants were collected, and radioactivity was measured by Micro-Bi counter (Wallac). Percent lysis is calculated by (sample release − spontaneous release)/(maximum release − spontaneous release) × 100.


**Statistical Analyses.** Statistical analyses were performed by using Student’s t test or Mann–Whitney test as indicated. Error bars denote mean ± SE. P < 0.05 was considered to be statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

**ACKNOWLEDGMENTS.** This work was supported in part by National Institutes of Health Research Grant AI 037562 (to H.C.), a Collaborative Sponsored Research Agreement with Novo Nordisk (H.C.), a gift from The Leroy Schecter Research Foundation (H.C.), a Swedish Research Council Award (X.W.), and a grant from the National Natural Science Foundation of China (to X.W.). J.W.L. is a National Research Service Award Fellow (T32 CA070083).

**Supporting Information**

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Fig. S1. Generation of type II collagen (CII)-specific TH subsets. (A) Levels of mRNA Tbx21, Rorc, and Bcl6 mRNA were measured by quantitative RT-PCR to define TH subsets generated in vitro. (B) Specificity of TH subsets for CII. In vitro-generated TH subsets were incubated with different concentrations of chicken CII or OTII peptide for 48 h and pulsed with $^3$H-thymidine for the last 18 h before proliferation assays were performed.
Fig. S2. Administration of anti-NKG2A F(ab)′2 and CIA. (A) Nondraining lymph node (nondLN) cells from treated or untreated mice (Fig. 4) were incubated with different concentrations of chicken CII for 48 h and pulsed with \(^{3}H\)-thymidine for the last 18 h before proliferation assays were performed. (B) Numbers of GC B cells (IgM^+^Fas^+^B220^+^) from each organ (Fig. 4) are shown; no significant differences between isotype control and 20d5 F(ab)′2 treated groups. (C) Levels of intracellular IL-4 and IFN-γ in CD4^+^ T cells from nondLNs (Fig. 4) are shown. No significant differences in IL-4 and IFN-γ levels were observed between isotype control and 20d5 F(ab)′2 treated groups. n = 5 (isotype control) and n = 4 (F(ab)′2); Student's t test: *P < 0.05.
Enhanced NKG2D ligand expression does not account for differential susceptibility of T_{h} subsets. T_{h} subsets were stimulated with leukocyte activation mixture for 4 h before surface staining with anti-CD4, anti-BTLA, and NKG2D-Fc (revealed with goat anti-human APC) followed by intracellular staining with Abs to indicated cytokines. Levels of NKG2D-Fc-dependent fluorescence were measured for IFN-γ^{+}, IL-10^{+}, IL-17^{+}, and BTLA^{+} CD4 cells. Representative histogram overlays are shown with mean fluorescence intensity indicated.