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Monoclonal Anti-CD8 Therapy Induces Disease Amelioration in the K/BxN Mouse Model of Spontaneous Chronic Polyarthritis

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Objective. **CD8+ T cells are part of the T cell pool infiltrating the synovium in rheumatoid arthritis (RA). However, their role in the pathogenesis of RA has not been fully delineated. Using the K/BxN mouse model of spontaneous chronic arthritis, which shares many similarities with RA, we studied the potential of CD8+ T cell depletion with monoclonal antibodies (mAb) to stop and reverse the progression of experimental arthritis.**

Methods. **CD8+ T cells from the blood and articular infiltrate of K/BxN mice were characterized for cell surface phenotypic markers and for cytokine production. Additionally, mice were treated with specific anti-CD8 mAb (YTS105 and YTS169.4), with and without thymectomy.**

Results. **CD8+ T cells from the peripheral blood and joints of K/BxN mice were mainly CD69+ and CD62L−CD27+ T cells expressing proinflammatory cytokines (interferon-γ [IFNγ], tumor necrosis factor α [TNFα], interleukin-17a [IL-17A], and IL-4), and granzyme B. In mice receiving anti-CD8 mAb, the arthritis score improved 5 days after treatment. Recovery of the CD8+ T cells was associated with a new increase in the arthritis score after 20 days. In thymectomized and anti-CD8 mAb–treated mice, the arthritis score improved permanently. Histologic analysis showed an absence of inflammatory infiltrate in the anti-CD8 mAb–treated mice. In anti-CD8 mAb–treated mice, the serologic levels of TNFα, IFNγ, IL-6, and IL-5 normalized. The levels of the disease-related anti–glucose-6-phosphate isomerase antibodies did not change.**

Conclusion. **These results indicate that synovial activated effector CD8+ T cells locally synthesize proinflammatory cytokines (IFNγ, TNFα, IL-17, IL-6) and granzyme B in the arthritic joint, thus playing a pivotal role in maintaining chronic synovitis in the K/BxN mouse model of arthritis.**

Approximately 40% of the T cells infiltrating the rheumatoid synovial membrane are CD8+ T cells (1). However, their importance in the pathogenesis of rheumatoid arthritis (RA) remains to be fully elucidated.

The primary function of CD8+ T cells is the killing of virus- or cytosolic bacteria–infected cells. Moreover, they seem to play several important roles in autoimmune diseases, either protecting against or enhancing the disease. In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, CD8+ T cells have been shown to be crucial for resistance to a second induction of the disease (2). Recently, a particular subset of CD8+ T cells (CD8+CD122+) was shown to accelerate the recovery of animals with EAE after CD8+ T cells were trans-

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ferred (3). In contrast, insulitis failed to develop in NOD mice treated with anti-CD8 monoclonal antibodies (mAb) (4). This experimental treatment also inhibited the transfer of insulin-dependent diabetes mellitus (IDDM) and the development of spontaneous IDDM (5).

In RA, some patients show CD8+ T cell clonal expansions with a memory phenotype that are correlated with rheumatoid factor (RF) levels (6–10). This is most likely attributable to the important role of CD8+ T cells in maintaining ectopic germinal center structures in RA synovium (11,12).

In different animal models of collagen-induced arthritis (CIA), the absence of CD8+ T cells resulted in a reduced incidence (13,14) and severity (15) of the disease. However, higher susceptibility was observed when animals were rechallenged (14,15). Additionally, in $CD8^{+/-}$ and $CD8^{-/-}$ mice, a trend toward a delayed onset of CIA was observed, without a significant impact on disease susceptibility (16). More recently, CD8+ T cell clones generated from the arthritic joints of SKG mice transferred to histocompatible athymic nude mice led to joint swelling and synovitis with destruction of cartilage and bone (17).

In order to assess the role of CD8+ T cells in experimental chronic polyarthritis, the clinical phenotype and cytokine production of articular and peripheral blood CD8+ T cells from K/BxN mice were studied. Arthritis in these mice results from the simultaneous expression of the class II major histocompatibility complex A^{g7} molecule and a transgenic T cell receptor (TCR), followed by the production of autoantibodies against glucose-6-phosphate isomerase (GPI) (18–20). Subsequently, we assessed whether treatment with specific anti-CD8 mAb, with and without thymectomy, improved the course of established arthritis in K/BxN mice. Our results showed, for the first time, that K/BxN mouse–activated and effector memory CD8+ T cells are present in the peripheral blood and joints and that they play an important role in arthritis maintenance, because treatment with specific anti-CD8 mAb significantly improved the disease signs. These results document that CD8+ T cells should be regarded as major players in the K/BxN mouse model of experimental arthritis, along with CD4+ T cells and B cells.

MATERIALS AND METHODS

Mice. TCR-transgenic KRN mice were a kind gift from Dr. C. Benoist (Harvard University, Boston, MA) and were maintained on a C57BL/6 background (K/B). Progeny bearing the $V_\beta 6$-transgenic TCR were identified at 3–4 weeks of age by cytofluorometry of peripheral blood lymphocytes, using phycoerythrin (PE)–labeled anti-CD8 (clone YTS169.4; Instituto Gulbenkian de Ciência [IGC] Cell Imaging Unit) and fluorescein isothiocyanate (FITC)–labeled anti-$V_\beta 6$ (PharMingen) antibodies. Arthritic mice were obtained by crossing K/B mice with NOD mice (K/BxN). C57BL/6 and NOD mice were provided by the IGC Animal Facility. The mice were bred and maintained under standard conditions, with food and water available ad libitum, in a specific pathogen–free environment. All animal studies were approved by the internal IGC Ethics Committee.

Antibodies and immunization in mice with established arthritis. Depleting anti-CD8 (clone YTS169.4), nondepleting anti-CD8 (clone YTS105), and rat IgG2a isotype control (clone YKIX302) mAb were a kind donation from Prof. H. Waldmann (Oxford University, Oxford, UK). The aim of combining nondepleting YTS105 mAb (21) and depleting YTS169.4 mAb (22) was to reduce the immunogenic potential of the antibodies (and their subsequent neutralization) that could be created after repeated injections. Mice ages 8–10 weeks old with an arthritis score >8 were injected intraperitoneally with either 150 μg of nondepleting anti-CD8 (n = 20) or anti-dog IgG isotype control (n = 19) on day 0. A second and third dose of 150 μg of depleting anti-CD8 or anti-dog IgG isotype control antibodies were injected intraperitoneally on days 7 and 16 after the first injection.

Thymectomy and CD8 depletion. Five-week-old K/BxN mice with established arthritis were subjected to total thymectomy (n = 5) or a sham operation (n = 3). Nine days after surgery, the mice were immunized intraperitoneally with 300 μg of depleting anti-CD8 antibody.

Arthritis scoring. Each swollen fore paw or hind paw was given a score of 1 point, each swollen wrist or ankle was given a score of 1 point, and each swollen finger or toe was given a score of 0.5 point, resulting in a maximum of 17 points per mouse. Scoring was performed every second day for the first 3 weeks and then once weekly for the remaining observation period.

Histochemical analysis. Skinless whole knee joints and front and hind paws were fixed in 5% formalin, decalcified in 5% formic acid, and embedded in paraffin. Sections (10 μm) were prepared from the tissue blocks and stained with either hematoxylin and eosin (H&E), MNF116 (anticytokeratin antibody), Herovici's stain, or Alcian blue–periodic acid−Schiff and observed on an Olympus IMT-2 microscope. Images were analyzed with ImageJ 1.38x software (National Institutes of Health).

Enzyme-linked immunosorbent assay (ELISA) for GPI. High-affinity Maxisorb 96-well ELISA plates (Nunc) were coated with 10 n*M Saccharomyces cerevisiae* GPI (Sigma) in potassium phosphate buffer. Plates were blocked with phosphate buffered saline/Tween/1% gelatin. Anti-GPI antibodies in sera were detected with horseradish peroxidase–labeled goat anti-mouse IgG (Southern Biotechnology) followed by incubation with tetramethylbenzidine solution (Sigma). Absorption was measured at an optical density of 450 nm.

Flow cytometric analysis. Peripheral blood samples were collected from the base of the tails of arthritic K/BxN mice on days 0, 7, 14, 21, and 35 after the first treatment with either anti-CD8 or control mAb. Mononuclear cells were isolated through a Ficoll gradient (Amersham) and stained

with anti-mouse mAb as follows: fluorescein isothiocyanate (FITC)–labeled anti-CD3 (clone 145.2C11), PE-labeled anti-CD8 (clone YTS169.4), and allophycocyanin (APC)–labeled anti-CD4 (clone GK1.5-8) (all from the IGC Cell Imaging Unit). To determine the differences in CXCR4 and CXCR5 expression and the frequency of CD8+ T cell subsets, peripheral blood samples were collected from the tails of 8–10-week-old untreated arthritic K/BxN mice (n = 9) and their nonarthritic littermates (n = 10). Mononuclear cells were isolated through a Ficoll gradient (Amersham) and stained with anti-mouse mAb, as follows: FITC-labeled anti-CD19 (clone 1D3; IGC Cell Imaging Unit), PE-labeled anti-CXCR4 (eBioscience), peridinin chlorophyll A protein (PerCP)–labeled anti-CD4 (eBioscience), APC-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit), FITC-labeled anti-CD3 (clone 145.2C11; IGC Cell Imaging Unit), PE-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit), biotinylated anti-CD62 ligand plus streptavidin–PerCP, and APC-labeled anti-CD27 (all from eBioscience). For determination of intracellular cytokine production, saponin-permeabilized peripheral blood mononuclear cells (PBMCs) were stained with APC-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit), PerCP-Cy5.5–labeled anti-CD3, FITC-labeled anti-TNFα, and PE-labeled anti–IL-6 (all from eBioscience). All samples were analyzed on a 4-color FACSCalibur system (Becton Dickinson), and data were analyzed with FlowJo 7.5.5 software (Tree Star).

Assessment of intracellular cytokine production by reverse transcription–polymerase chain reaction (RT-PCR). PBMCs and mononuclear cells from articular tissue were collected from untreated arthritic mice (n = 4), and PBMCs were collected from their healthy control littermates (n = 4). CD8+ T cells were isolated by magnetic cell separation using the CD8a+ T Cell Isolation kit (Miltenyi Biotech).

Total RNA from sorted CD8+ T cells was isolated using an RNeasy Micro kit (Qiagen). RNA integrity and quantification were analyzed using a 6000 Nano Chip kit in an Agilent 2100 Bioanalyzer. RNA was reverse transcribed with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo(dT) plus random hexamers according to the manufacturer's instructions.

Relative quantification of gene expression by real-time PCR was performed using a thermocycler LightCycler 480 II (Roche). Normalization for gene expression quantification was performed with geNorm Housekeeping Gene Selection Mouse kit (PrimerDesign) and geNorm software (Ghent University Hospital, Center for Medical Genetics) to select optimal reference genes for this study (23).

Real-time PCRs used specific *Mus musculus* QuantiTect Primer Assays (Qiagen) with optimized primers for the genes of interest, *Gzmb* (QT00114590), *Ifn* (QT01038821), *Il10* (QT00106169), *Il17a* (QT00103278), *Il2* (QT00112315), *Il4* (QT00160678), *Tnf* (QT00104006), and the reference genes *Ywhaz* (QT00105350) and *Rn18s* (QT01036875), together with a QuantiTect SYBR Green PCR Gene Expression kit (Qiagen), according to the manufacturer's instructions. Reactions were performed with the following thermal profile: 10 minutes at 95°C plus 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. Quantitative real-time PCR results were analyzed using LightCycler 480 software (Roche) and quantified using the qBasePlus software package (Biogazelle).

Serum cytokine quantification. Serum samples from arthritic K/BxN mice before (n = 11) and after (n = 11) anti-CD8 treatment and from their nonarthritic control littermates (n = 7) were obtained from whole blood after centrifugation. TNFα, IFNγ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and monocyte chemoattractant protein 1 (MCP-1) titers in the sera were quantified using the cytometric bead arrays, a Mouse Th1/Th2 Cytokine kit and a Mouse Inflammation kit (Becton Dickinson) according to the manufacturer's instructions, and analyzed with BD Cytometric Bead Array Software (Becton Dickinson). IL-17a titers were determined using a Mouse IL-17A ELISA kit (Invitrogen). Test sensitivity thresholds for the different cytokines were as follows: for TNFα, 6.3 pg/ml; for IFNγ, 2.5 pg/ml; for IL-2, 5.0 pg/ml; for IL-4, 5.0 pg/ml; for IL-5, 5.0 pg/ml; for IL-6, 5.0 pg/ml; for IL-10, 17.5 pg/ml; for IL-12p70, 10.7 pg/ml; for IL-17a, 5.0 pg/ml; and for MCP-1, 52.7 pg/ml. Mean titers below those thresholds were considered undetectable.

Statistical analysis. Data were checked for normality, in order to decide whether to use the parametric one-way analysis of variance and post hoc Tukey's test or the nonparametric Kolmogorov-Smirnov test. Data were analyzed using StatView 5.0 software (Abacus Concepts). *P* values less than 0.05 were considered significant.

RESULTS

Activation of K/BxN mouse CD8+ T cells in the articular infiltrate. In an effort to characterize the CD8+ T cells in K/BxN mice, mononuclear cells were isolated from the peripheral blood and from the articular inflammatory infiltrate and analyzed for the expression of surface markers and cytokine production. In contrast to the circulating CD4+ T cell pool, a significantly higher ($P < 0.05$) percentage of circulating CD8+ T cells from K/BxN mice expressed the V_β6-transgenic TCR (mean ± SD 32 ± 10% and 84 ± 7% for CD4 and CD8, respectively) at 3 weeks after birth, before any external clinical signs of arthritis could be detected.

The frequencies of CD8+ T cell subsets defined by the expression of CD27 and CD62L in the peripheral blood and articular infiltrate from arthritic K/BxN mice were compared with those in the peripheral blood of healthy mice (Figure 1A). The frequency of CD27−CD62L− short-lived effector CD8+ T cells (T_{se}) was similar in both K/BxN mouse tissue and peripheral blood from healthy mice. However, the peripheral blood of arthritic K/BxN mice presented a significantly ($P = 0.019$) higher frequency of CD27+CD62L− effector memory CD8+ T cells (T_{em}) than the peripheral blood of healthy mice. Moreover, the frequency of this T_{em} subset was higher, although not reaching statistical significance ($P = 0.0791$), in the articular infiltrate than in the peripheral blood of K/BxN mice. The frequency of CD27+CD62L+ central memory CD8+ T cells (T_{cm}) was comparable in the peripheral blood of K/BxN mice

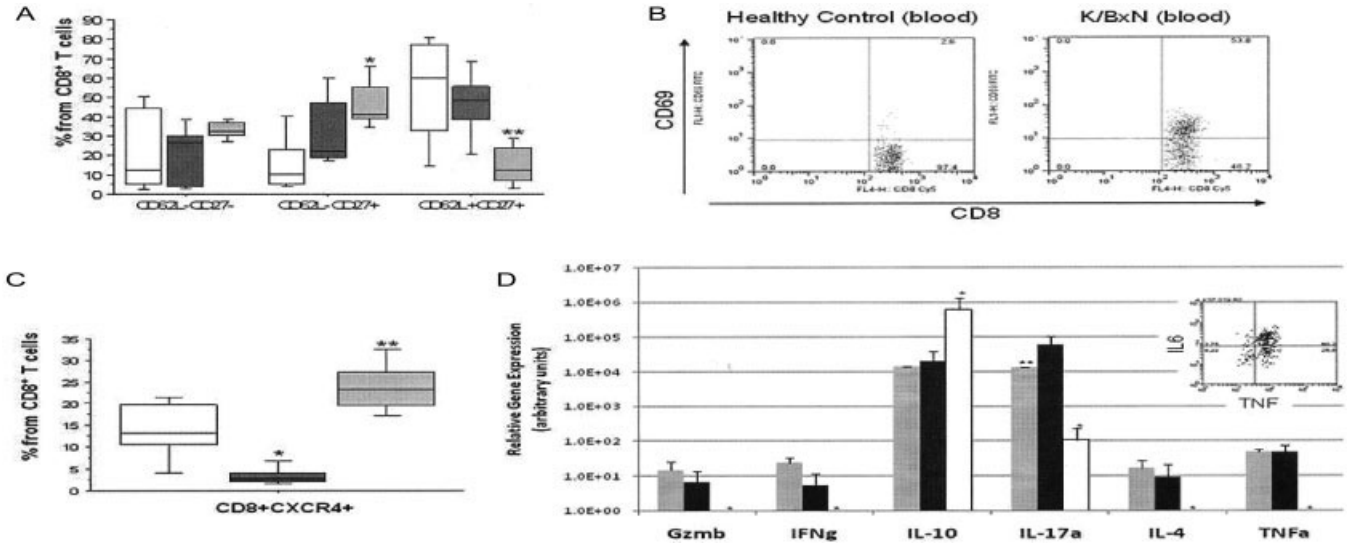

Figure 1. CD8+ T cells of K/BxN mice present an activated effector memory phenotype, homing preferentially to the articular tissue where they produce proinflammatory cytokines. **A** and **C,** Frequency of CD62L−CD27−, CD62L+CD27+, and CD62L−CD27+ CD8+ T cells (**A**) and frequency of CD8+CXCR4+ CD8+ T cells in the blood of healthy control mice (open boxes; n = 10), the blood of K/BxN mice (darkly shaded boxes; n = 9), and the articular tissue of arthritic K/BxN mice (lightly shaded boxes; n = 9). Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. * = $P < 0.05$ versus control; ** = $P < 0.05$ versus K/BxN mouse blood. **B,** Dot plots of CD69 versus CD8 in the blood of a representative healthy control mouse and an arthritic K/BxN mouse. **D,** Relative expression of several cytokine genes in unstimulated CD8+ T cells isolated from the articular tissue (lightly shaded bars; n = 4) and peripheral blood (darkly shaded bars; n = 4) of arthritic K/BxN mice and from control peripheral blood (open bars; n = 4). Bars show the mean and SD. **Inset,** Intracellular production of tumor necrosis factor α (TNF) and interleukin-6 (IL-6) in CD8+ T cells from the articular tissue of a representative K/BxN mouse. * = $P < 0.01$ versus K/BxN mouse blood and articular tissue; ** = $P < 0.05$ versus K/BxN mouse blood. IFNγ = interferon-γ; Gzmb = granzyme B.

and that of healthy mice. However, the frequency of T_{cm} was significantly lower ($P = 0.008$) in the articular infiltrate of K/BxN mice than in the peripheral blood of K/BxN mice.

In contrast to what was observed in healthy mice, the majority of CD8+ T cells circulating in K/BxN mouse peripheral blood expressed the early activation marker CD69 (Figure 1B), and this increased expression of CD69 was also observed on the surface of CD8+ T cells infiltrating the joints.

To assess whether the expression of chemokine receptors by K/BxN mouse articular CD8+ T cells could contribute to the skewed distribution of the different CD8+ T cell subsets in the articular infiltrate, the frequencies of CD8+ T cells expressing specific chemokine receptors were determined (Figure 1C). Interestingly, the frequency of CD8+CXCR4+ was significantly increased in the articular tissue when compared with the peripheral blood ($P = 0.002$) of K/BxN mice. Moreover, the peripheral blood of K/BxN mice had a significantly ($P = 0.0007$) decreased frequency of CD8+CXCR4+ T cells when compared with that of controls. In contrast to what has been reported in humans (24), we were not able to clearly identify a circulating CXCR5-expressing CD8+ T cell population in either the healthy mice or the K/BxN mice (data not shown).

To determine whether CD8+ T cells infiltrating the joints of arthritic K/BxN mice had the potential to actively participate in the inflammatory and joint destruction process by producing proinflammatory cytokines and cytolytic enzymes, we quantified the relative gene expression of several cytokines and granzyme B in unstimulated CD8+ T cells. As depicted in Figure 1D, both articular tissue and peripheral blood CD8+ T cells from arthritic K/BxN mice had similar expression of the genes coding for granzyme B, IFNγ, IL-4, and TNFα, while no expression of these genes was detected in CD8+ T cells isolated from control peripheral blood. Interestingly, more than half of the articular CD8+ T cells producing TNFα also produced IL-6 (inset in Figure 1D). However, expression of the gene coding for IL-17a was significantly higher ($P < 0.01$) in K/BxN mouse peripheral blood CD8+ T cells than in articular tissue or control peripheral blood. Nevertheless, the CD8+ T cells from the articular tissue still expressed significantly higher levels of *Il17a* than did the control

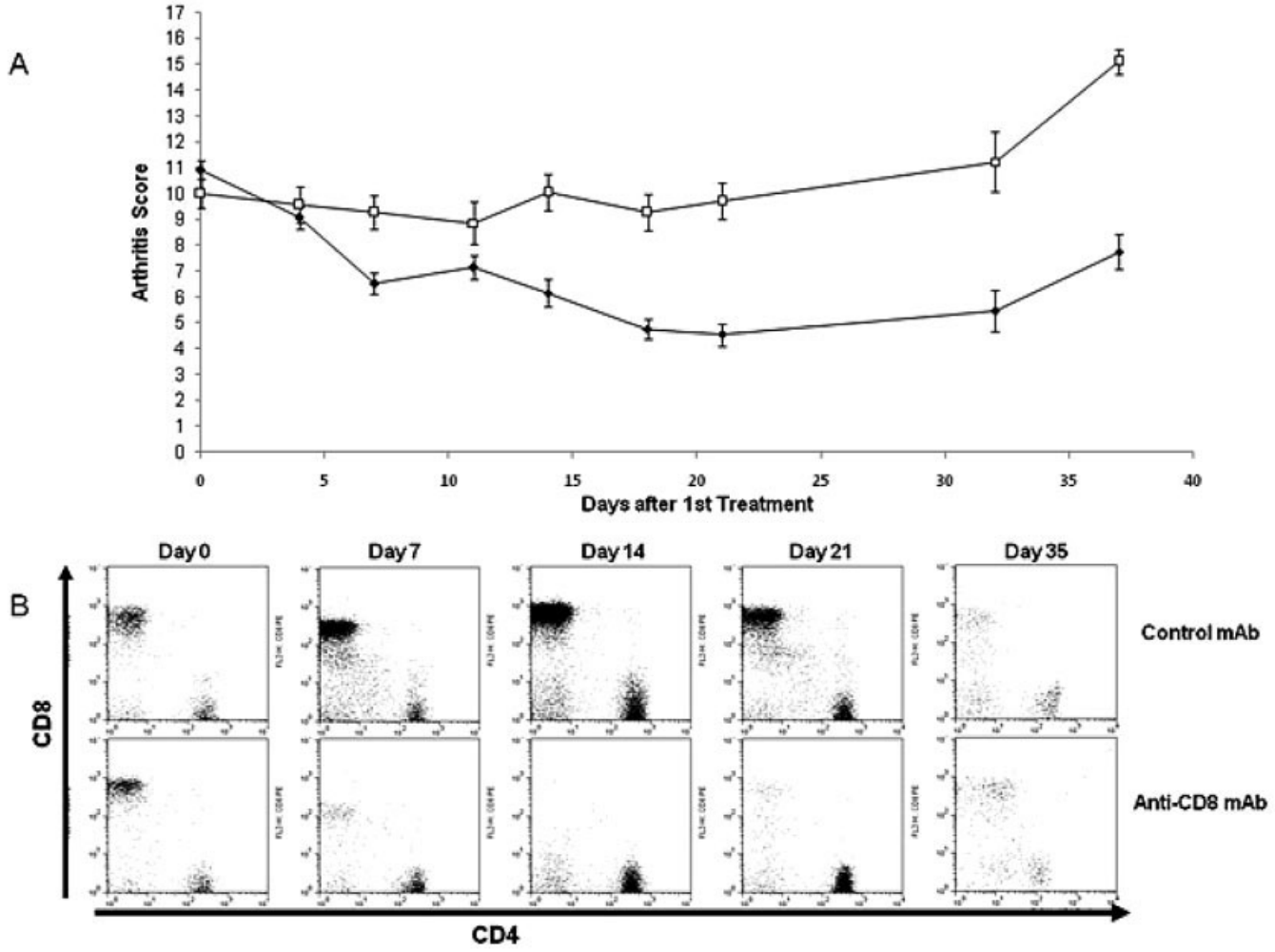

Figure 2. Treatment with anti-CD8 monoclonal antibodies (mAb) after polyarthritis is established ameliorates disease signs in K/BxN mice, and disease relapse occurs with CD8+ T cell recovery. **A,** Evolution of the disease score over 40 days in the control mAb–treated group (squares; n = 19) and the anti-CD8 mAb–treated group (diamonds; n = 20). Mice received an injection of YTS105 (blocking) or mock antibody on day 0 and an injection of YTS169.4 (depleting) or mock antibody on days 7 and 16. Values are the mean ± SEM. **B,** Representative dot plots of CD4 versus CD8 in CD3+ peripheral blood T cells on days 0, 7, 14, 21, and 35 after treatment with anti-CD8 mAb or control mAb.

peripheral blood. As expected, the expression of *Il10* was increased in the CD8+ T cells of all 3 tissue types, with the control peripheral blood presenting a significantly higher expression ($P < 0.05$), while no *Il2* gene expression was detected in any of the CD8+ T cells isolated from the different tissues.

Improvement in macroscopic and microscopic signs of disease by depletion of CD8+ T cells with mAb. To assess the importance of CD8+ T cells in the maintenance of chronic polyarticular inflammation in K/BxN mice, specific mAb that either blocked (YTS105) or depleted (YTS169.4) CD8+ T cells were administered after arthritis was established (arthritis score >9). As shown in Figure 2A, the arthritis scores for the mice treated with anti-CD8 mAb began to improve starting 5 days after the initial treatment, as compared with the groups receiving control mAb. Furthermore, a lower arthritis score was maintained for more than a month thereafter in the anti-CD8–treated group. The increase in the arthritis score in the anti-CD8–treated mice observed from day 21 onward corresponded to a recovery of the CD8+ T cell pool (Figure 2B).

Histologic analysis of the hind paw ankle joints revealed an absence of inflammatory infiltrate accompanied by new bone formation and normal synovial bursae in arthritic K/BxN mice 30 days after receiving

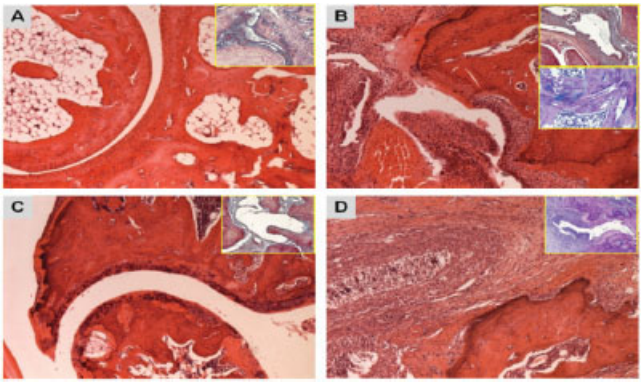

Figure 3. Histologic assessment of articular tissue shows clearance of the inflammatory infiltrate in anti-CD8 monoclonal antibody–treated K/BxN mice. **A,** Joint section from a healthy control mouse (H&E stained; original magnification × 10). **Inset,** Normal synovial bursae (Herovici stained; original magnification × 200). **B,** Joint section from a K/BxN mouse before treatment, showing massive inflammation and cartilage/bone destruction (H&E stained; original magnification × 10). **Upper inset,** Inflamed hyperplastic synovium (Herovici stained; original magnification × 100). **Lower inset,** Chondral sclerosis and fibrous ankylosis (Alcian blue–periodic acid−Schiff stained; original magnification × 100). **C,** Joint section from an anti-CD8 monoclonal antibody–treated K/BxN mouse 30 days after treatment, showing complete clearance of the inflammatory infiltrate and normalization of the articular architecture (H&E stained; original magnification × 10). **Inset,** Normal synovial bursae (Herovici stained; original magnification × 100). **D,** Joint section from a control monoclonal antibody–treated K/BxN mouse 30 days after treatment, showing complete destruction of the joint structure by massive infiltration of inflammatory cells and fibrosis (H&E stained; original magnification × 10). **Inset,** Proliferative synovitis with destruction of articular cartilage (Alcian blue–periodic acid–Schiff stained; original magnification × 100).

anti-CD8 mAb (Figure 3C), as opposed to mice receiving control mAb, which presented an inflamed hyperplastic synovium and articular erosions (Figure 3D).

To investigate whether the arthritis improvement after anti-CD8 therapy was associated with changes in the levels of circulating cytokines, the concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17a, MCP-1, TNFα, and IFNγ were measured in the serum of healthy control and K/BxN mice at baseline and 20 days after the initial treatment (Figures 4A–D). Arthritic K/BxN mice that were assessed before treatment had significantly higher ($P < 0.02$) serologic titers of IL-5, IL-6, and TNFα than healthy controls. Twenty days after anti-CD8 therapy, the serologic levels of all 3 cytokines and IFNγ had significantly dropped ($P < 0.04$) from their baseline values and were comparable with the ones present in healthy control mice. The serologic levels of all other cytokines did not pass the minimum test threshold.

Prevention of arthritis relapse by complete thymectomy followed by depletion of CD8+ T cells. In an effort to verify whether a permanent absence of CD8+ T cells could protect K/BxN mice from arthritis relapse, 5–6-week-old K/BxN mice with established polyarthritis (arthritis score >8) underwent total thymectomy followed by the injection of a high dose of depleting (YTS169.4) anti-CD8 mAb 9 days later. Control mice underwent sham operations and received an equal dose of depleting anti-CD8 mAb 9 days later, after which arthritis evolution was monitored for a further 90 days.

Thymectomy alone did not seem to induce a short-term alteration of the course of the disease, since no significant changes in the arthritis score could be observed between day 0 and day 9. Administration of anti-CD8 mAb led to an amelioration of the clinical signs of arthritis in both the thymectomized and control K/BxN mice. However, although the control mice had a relapse of the disease 43 days after having received the depleting anti-CD8 mAb (the longer time before relapse observed in these mice compared with those in Figure 2A is attributable to the higher dose of anti-CD8 mAb they received), the thymectomized mice experienced further arthritis improvement, which lasted until the end of the 90-day followup (Figure 5A). The absence of complete clinical remission in the thymectomized mice (arthritis score 0) is attributable to the effects of residual deformities on the scoring system. In fact, the histologic sections obtained on day 90 from the hind paws of the thymectomized mice showed an absence of inflammatory infiltrate in the synovial membrane and preservation of the articular and bone structure (Figure 5B), as opposed to the expanded inflammatory infiltration and extensive arthrosis observed in the sham-operated controls (Figure 5C).

Effect of disease amelioration on anti-GPI antibody titers. The development and maintenance of polyarthritis in K/BxN mice has been linked to the production of anti-GPI autoantibodies, the serum concentration of which increases with age and disease progression (20). Therefore, we determined the serologic titers of anti-GPI IgG in K/BxN mice at baseline (before mAb treatment or thymectomy was started) and after 30 days of YTS105 followed by YTS169.4 anti-CD8 treatment or control anti-dog IgG treatment. Similar assessments were performed 90 days after thymectomy or sham operation and YTS169.4 treatment in K/BxN mice and age-matched nonarthritic control littermates (treated either with YTS105 followed by YTS169.4 anti-CD8 or control anti-dog IgG), but no significant effects of treatment on the anti-GPI IgG titers were

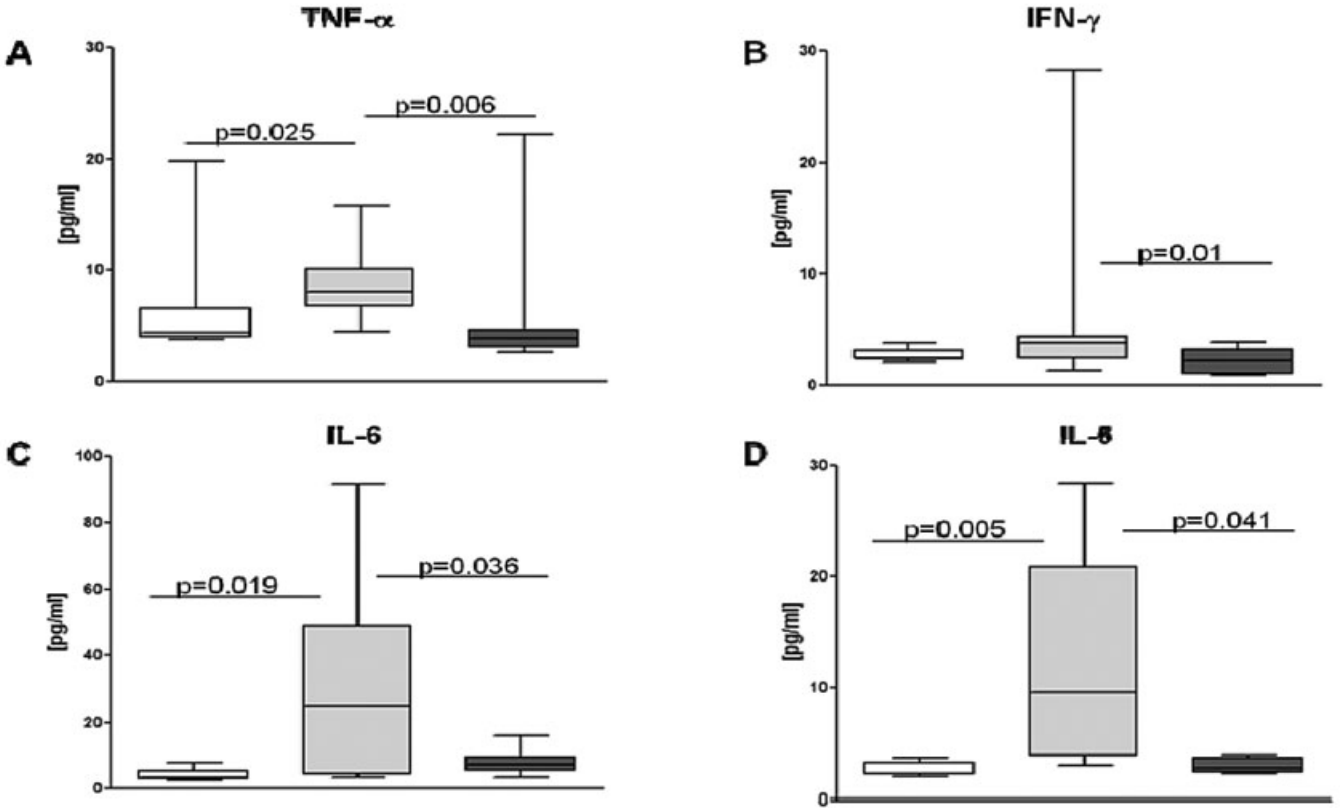

Figure 4. Treatment with anti-CD8 monoclonal antibodies normalizes the serologic levels of proinflammatory cytokines in K/BxN mice. Titers of tumor necrosis factor α (TNFα) (**A**), interferon-γ (IFNγ) (**B**), interleukin-6 (IL-6) (**C**), and IL-5 (**D**) in the blood of untreated K/BxN mice (lightly shaded boxes; n = 11), the blood of healthy control mice (open boxes; n = 7), and the blood of anti-CD8–treated K/BxN mice (darkly shaded boxes; n = 11) are shown. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles.

observed (Figure 6). No significant effects of treatment on anti-GPI IgG titers were observed (Figure 6). Actually, the titers of anti-GPI antibodies increased in the thymectomized mice even though inflammation of the joints subsided. The frequency of circulating CD138+ plasma cells was also not affected by any of the treatments (data not shown).

DISCUSSION

The role of CD8+ T cells in the pathogenesis of RA remains unclear. Nevertheless, several studies in patients with RA that associated the effector functions and the memory CD45RO+ and activated "false" memory CD29+CD45RA+CD45RO− phenotypes of CD8+ T cells with RF production and disease activity point out that the contribution of CD8+ T cells to RA should be reevaluated (6,7,9,10). This is also the case in animal models, because data from the literature on experimental arthritis are few and contradictory. Several studies on the CIA model of experimental polyarthritis focusing on the involvement of CD8+ T cells in initiating arthritis (13,14,25) revealed that anti-CD8 treatment rendered experimental animals less susceptible (14) or fully resistant (13) to the disease. Another study involving NOD/SCID mice engrafted with human rheumatoid synovium stressed the importance of CD8+ T cells for the maintenance of synovial follicular-like structures (11).

Most studies were carried out in the CIA model and manipulated the CD8+ T cell response before arthritis induction, thus focusing on the potential role of CD8+ cells in the initiation of the disease (13,14,16,25). This is consistent with current paradigms regarding the role of CD8+ cells but was also favored because of the transient nature of CIA, which would render it difficult to distinguish between the ameliorating effect of CD8 blockade and natural disease remission. Therefore, the contribution of CD8+ T cells to the chronicity of polyarthritis has not been addressed.

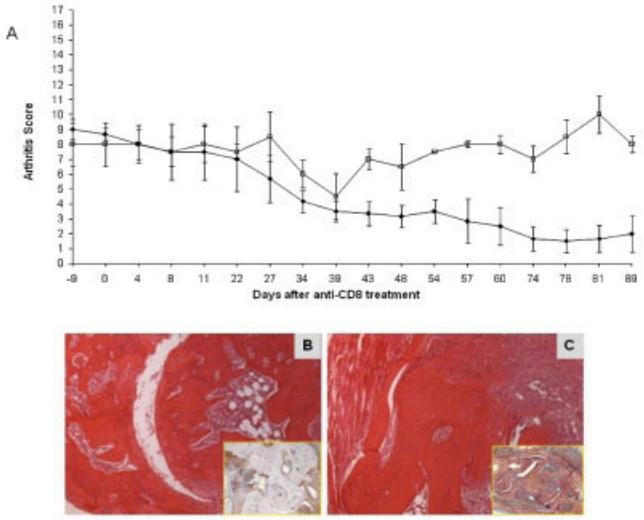

Figure 5. Thymectomy followed by CD8+ T cell depletion stops arthritis relapse, reduces the inflammatory infiltration of the joint, and preserves bone and articular integrity in K/BxN mice. **A,** Evolution of the arthritis score for 90 days after CD8+ T cell depletion in sham-operated control mice (squares; n = 3) and thymectomized mice (diamonds; n = 5). Surgery was performed on day −9, and CD8 depletion was performed on day 0. Values are the mean ± SEM. **B,** Section from the hind paw of a K/BxN mouse 90 days after thymectomy and CD8+ T cell depletion (H&E stained; original magnification × 10). **Inset,** Preserved joint and synovial proliferation without inflammation (MNF116 stained; original magnification × 100). **C,** Section from the hind paw of a K/BxN mouse 90 days after sham operation and CD8+ T cell depletion (H&E stained; original magnification ×10). **Inset,** Arthrosis of the joint (Herovici stained; original magnification × 40).

The recent development of murine models of persistent chronic polyarthritis—the SKG (26), the K/BxN (19), and the B10.Q/Ncf1* (27) models—provide new tools for studying CD8+ T cell involvement in arthritis maintenance.

In the present study, we used the K/BxN mouse model of chronic polyarthritis to show that CD8+ T cells circulating in the peripheral blood and infiltrating the joints are responsible for maintaining chronic articular inflammation. An evident decrease in articular swelling and redness a few days after the initial anti-CD8 treatment was confirmed by the absence of histologic signs of inflammatory infiltrates and evidence of de novo ossification. Moreover, normalization of the serologic levels of proinflammatory cytokines, such as TNFα, IFNγ, and IL-6, in the anti-CD8 mAb–treated mice represents evidence that the role of CD8+ T cells in arthritis maintenance is at least partially mediated through self-production of these cytokines or by (co)stimulation of production in other cells. Additionally, normalization of the serologic levels of IL-5, a cytokine involved in growth and differentiation of both B cells and eosinophils (28), after anti-CD8 treatment accompanied a reduction in joint inflammation. Further evidence for the involvement of CD8+ T cells in K/BxN mouse polyarthritis was provided by the disease relapse observed in treated mice as soon as the numbers of circulating CD8+ T cells were normalized.

Nevertheless, it was important to establish whether the permanent absence of CD8+ T cells prevented arthritis relapse. Therefore, 5-week-old K/BxN mice with established polyarthritis were thymectomized and subsequently inoculated with a high dose of CD8+ T cell–depleting mAb. Amelioration of the clinical signs of arthritis was evident after 2 weeks, and no relapses were observed in the 90-day followup period. In fact, after those 90 days, normal levels of TCR-transgenic CD4+ T cells were still present in the circulation, and the levels of B cells and plasma cells did not change when compared with those in sham-operated mice. Such observations strengthen the hypothesis that CD8+ T cells, and not only CD4+ T cells and B cells (19), are essential to the maintenance and even the initiation of chronic polyarthritis in K/BxN mice. In contrast to findings with therapies involving CD40 blockade (29), no changes were observed in the serologic levels of anti-GPI autoantibodies after any of the anti-CD8 therapies, suggesting that CD8 blockade stops/reverses ar-

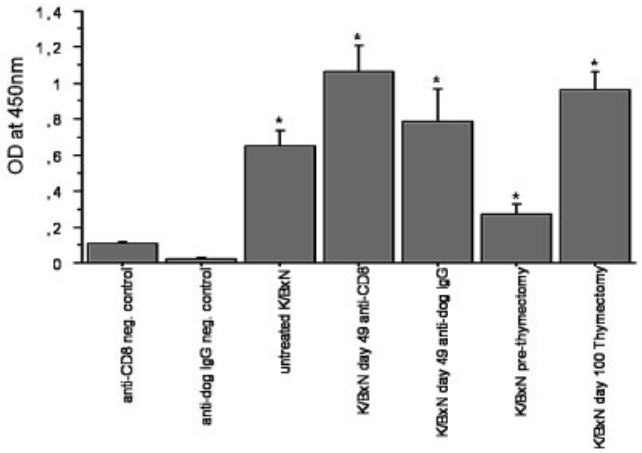

Figure 6. Blockade of CD8 does not reduce the serologic levels of anti–glucose-6-phosphate isomerase (anti-GPI) autoantibodies. Bars show the mean and SD optical density at 450 nm, measured in an anti-GPI enzyme-linked immunosorbent assay, for untreated (n = 18), anti-CD8 monoclonal antibody–treated (n = 15), or control monoclonal antibody–treated (n = 13) K/BxN mice and prethymectomized (n = 6) and postthymectomized (n = 6) K/BxN mice as well as either control monoclonal antibody–treated (n = 9) or anti-CD8 monoclonal antibody–treated (n = 6) healthy control mice. * = $P < 0.05$ versus controls. OD = optical density.

thritis progression without influencing the autoreactive B cell and plasma cell pools.

Even though the CD8+ T cells of K/BxN mice express the transgenic $V_\beta 6$ TCR, thus rendering them potentially autoreactive (as extensively described for the K/BxN mouse TCR-transgenic CD4+ T cells [19]), they have been poorly studied. A functional and phenotypic characterization of the transgenic CD8+ T cells in this mouse model is especially important in view of its larger and earlier expansion in comparison with CD4+ T cells: the CD8+ T cell pool comprised up to 85% of TCR-transgenic cells 21 days after birth and 2 weeks before arthritis was established.

CD8+ T cells are usually subdivided into particular phenotypes with characteristic effector functions, homing properties, and proliferative capacity. The expansion phase after antigen presentation is dominated by short-lived effector CD8+CD27−CD62L− T cells (T_{se}) capable of producing proinflammatory cytokines (IFNγ, TNFα, IL-2, IL-17) and cytotoxic molecules (perforin, granzyme B). These cells heavily migrate into the peripheral organs (30–32). Upon interaction with CD154 expressed on helper CD4+ T cells (33,34), subsets of effector CD8+ T cells and antigen-primed naive CD8+ T cells, respectively, develop into CD8+CD27+CD62L− effector memory cells (T_{em}) or CD8+CD27+CD62L+ central memory cells (T_{cm}) (31,35–37). While T_{em} accumulate in the peripheral organs and rapidly become effector cells upon reencounter with antigen but have poor expansion and self-renewal capacity, T_{cm} accumulate in the lymphoid organs and are capable of large expansion upon antigen reencounter and frequent self-renewal (31,37–39). Considering these functional and homing differences, it is not surprising to observe that the K/BxN mouse articular tissue showed an accumulation of the 2 effector subsets, particularly the T_{em} subset, which are more likely to participate in the local autoantigen-driven tissue destruction.

The presence of TNFα-, IL-6–, IFNγ-, IL-17–, and granzyme B–producing CD8+ T cells in the articular infiltrate and the elevated frequency of CD8+ T cells expressing the homing chemokine CXCR4 suggest that the joint-infiltrating effector CD8+ T cells might be subdivided into 2 main groups. A first group might be actively participating in joint destruction through granzyme B secretion. A second group may be involved in the recruitment and priming of other immune cells into the joint, which are the IL-17a–producing CD8+ T cells that have been described as proinflammatory but with reduced cytotoxic potential (40). Additionally, the elevated presence of both CD69-expressing CD8+ T cells, which are markers of early activation, and T_{em} in the peripheral blood of arthritic K/BxN mice suggests that there is continuous systemic activation, and eventually recruitment, of (pathogenic) CD8+ T cells in the K/BxN mouse model of spontaneous chronic polyarthritis.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Souto-Carneiro 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 conception and design. Raposo, Pereira da Silva, Graça, Souto-Carneiro.

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