

## Where FoxP3-Dependent Regulatory T Cells Impinge on the Development of Inflammatory Arthritis

Linh T. Nguyen, Jonathan Jacobs, Diane Mathis, and Christophe Benoist

**Objective.** Regulatory T cells play a suppressive role in many autoimmune diseases and can potentially affect various steps in the progression of disease. The purpose of this study was to analyze the role of Treg cells in the control of arthritis development.

**Methods.** Using crosses and cell transfers, we tested the effect of the *scurfy* loss-of-function mutation of the *Foxp3* gene in the K/BxN mouse model. In this model, arthritis develops as the result of the production of high levels of pathogenic autoantibodies.

**Results.** The absence of Treg cells in K/BxN mice led to faster and more aggressive arthritis. Strikingly, disease also spread to joints not normally affected in this model. The absence of Treg cells resulted in an acceleration of the immunologic phase of disease, with significantly earlier autoantibody production. However, the broadened spectrum of affected joints in *Foxp3*-mutant mice was not due to the earlier appearance of autoantibodies and could not be reproduced by increasing the anti-glucose-6-phosphate isomerase antibody load, which demonstrates an impact of Treg cells on effector phase manifestations. In addition, FoxP3<sup>+</sup>, CD25<sup>+</sup> Treg cells accumulated in inflamed joints, even in nontransgenic animals. This preferential localization mimics that in human arthritides and indi-

cates a preferential homing/retention of Treg cells to sites of inflammation.

**Conclusion.** These results indicate that Treg cells play a role in antibody-mediated arthritis at several levels. Treg cells are involved in constraining the immune phase of disease, as well as limiting the articular damage provoked by the pathogenic autoantibodies in terms of severity and of the range of affected joints, which may contribute to the limited distal predominance of many arthritides.

Attack of healthy tissues by all branches of the immune system is curtailed by a number of tolerance-induction mechanisms, without which autoimmune disease occurs. One of these tolerance mechanisms relies on populations of lymphocytes with dominant regulatory capacity, the best characterized of which is the CD4<sup>+</sup>, CD45RB<sup>low</sup>, CD25<sup>+</sup> regulatory T cell subset (for review, see refs. 1 and 2). Treg cells are generated during normal thymocyte differentiation and are characterized by expression of the X-linked transcription factor forkhead box P3 (FoxP3).

Several groups of investigators have demonstrated that FoxP3 is required for the differentiation and function of Treg cells (3–5). In vitro, Treg cells are able to suppress proliferation and cytokine production by effector T cells; in vivo, they can inhibit a wide variety of organ-specific autoimmune pathologies, such as manifestations induced by transfer of naive cells into lymphopenic hosts (6,7) or more targeted endocrine or neural damage due to organ-specific effector cells (8–10). Male mice that carry the *scurfy* mutation, a null mutation of the *Foxp3* gene (*Foxp3<sup>sf</sup>*), lack Treg cells and exhibit severe lymphoproliferation and infiltration of multiple organs by inflammatory cells, particularly the skin and liver (3–5,11). This requirement for FOXP3-controlled Treg cells is also true in humans, since patients with the X-linked syndrome of immune dysregulation, polyendocrinopathy, and enteropathy, who

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Linh T. Nguyen, PhD (current address: The Campbell Family Institute for Breast Cancer Research, Toronto, Ontario, Canada), Jonathan Jacobs, BSc, Diane Mathis, PhD, Christophe Benoist, MD, PhD: Joslin Diabetes Center, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Diane Mathis, PhD, or Christophe Benoist, MD, PhD, Immunology and Immunogenetics Section, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail: cbdm@joslin.harvard.edu.

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lack *FOXP3*, exhibit very severe autoimmune pathologies (12,13).

It is becoming increasingly clear that Treg cells can control immune responses by various mechanisms, such as induction of anergy in pathogenic T cells or modulation of antigen-presenting cell function (14). However, which mechanisms are dominant during different types of immune responses remains obscure.

The pathogenesis of inflammatory arthritis involves multiple branches of the immune system. In the K/BxN mouse model of spontaneous inflammatory arthritis and in collagen-induced arthritis (CIA), the production of pathogenic autoantibodies by B lymphocytes is aided by T helper cells; the pathogenic autoantibodies then initiate an inflammatory reaction in the joints that involves cellular players of the innate immune system, such as mast cells, neutrophils, or macrophages. Several studies have evaluated the impact of Treg cells on animal models of induced arthritis, using antibody-mediated depletion or supplementation with CD25+ cells as an experimental strategy (15–19). In all cases, depletion of CD25+ cells resulted in worse paw swelling, thus supporting a role of Treg cells in the control of arthritis. Yet, since CIA is based on the induction of arthritis by immunization, it was not possible to distinguish effects on the immune initiation phase versus the inflammatory effector phase.

We addressed this issue by examining the influence of Treg cells on the K/BxN mouse model of spontaneously developing inflammatory arthritis (20), in which it is experimentally straightforward to separate the upstream events of the immunologic phase from the downstream events of the inflammatory phase. The key components of the K/BxN model are the KRN transgene-encoded T cell receptor (TCR) and the class II major histocompatibility complex molecule A<sup>g7</sup>. Mice expressing the KRN-TCR transgene and A<sup>g7</sup> (for example, on the [B6 × NOD]F<sub>1</sub>, or BxN, background) spontaneously develop arthritis at ~4 weeks of age. Disease is initiated by activation of KRN-transgenic T cells upon recognition of a peptide derived from glucose-6-phosphate isomerase (GPI), which is presented by A<sup>g7</sup> (21,22). Collaboration between GPI-specific T cells and B cells then results in the production of anti-GPI autoantibodies, which eventually reach extremely high titers. Although the inciting GPI autoantigen is ubiquitously expressed, autoimmunity is limited to the joints, primarily the distal joints, where activation of the alternative pathway of complement occurs, innate cells are recruited, and synovial inflammation provokes joint destruction.

Our strategy for evaluating the role of Treg cells in controlling the development of arthritis was to perform a genetic ablation, eliminating the cells from the K/BxN mouse by introducing the *Foxp3*<sup>sf</sup>-null allele. We found that Treg cells independently affect the inflammatory phase as well as the immune phase of the arthritogenic process.

## MATERIALS AND METHODS

**Mice and arthritis monitoring.** K/BxN and K/BxN.*Foxp3*<sup>sf</sup> mice were generated in the Joslin Diabetes Center barrier facility (Institutional Animal Care and Use Committee protocols 99-18, 99-19, and 99-20) by crossing male KRN-TCR-transgenic mice on the C57BL/6 (B6) background (20) with female *Foxp3*<sup>sf</sup> mice on the NOD background carrying the *Foxp3*<sup>sf</sup> mutation on 1 of the X chromosomes (10). KRN+ male mice harboring the *Foxp3*<sup>sf</sup> mutation on their X chromosome (K/BxN.*Foxp3*<sup>sf</sup> mice) were compared with their KRN+ littermates with wild-type *Foxp3*. *Foxp3*<sup>sf</sup> male mice and their controls on the B6 background or B6.*Rag2*<sup>o/o</sup> background were also bred in our animal facility. Clinical arthritis was monitored by measuring ankle thickness using a Kafer dial micrometer (Precision Graphic Instruments, Spokane, WA). The average thickness of both hind paws from each mouse was plotted. For the induction of arthritis in non-TCR-transgenic mice, serum from arthritic donors (100 μl for BALB/c recipients; 150 μl for all other recipients) was injected intraperitoneally on days 0 and 2.

**Histologic analysis of mouse joints.** Joints were assessed histologically as described elsewhere (23). The severity of leukocyte infiltration was scored on a scale of 0–3, where 0 = no infiltration, 1 = mild infiltration, 2 = moderate infiltration, and 3 = severe infiltration.

**Flow cytometry for FoxP3.** Intracellular staining for FoxP3 was performed using antibodies obtained from eBioscience (San Diego, CA) and following the manufacturer's instructions. Phycoerythrin-conjugated rat IgG2a (PharMingen, San Diego, CA) was used as the isotype control for FoxP3 staining.

**Enzyme-linked immunosorbent assay (ELISA) for anti-GPI antibody.** Titers of anti-GPI antibody were determined by ELISA as previously described (23) and were expressed as the dilution factor that resulted in an optical density at 405 nm (OD<sub>405</sub>) that was 5-fold higher than the background value for anti-GPI IgG and IgG1 or was 2-fold higher than the background value for anti-GPI IgG2a. The relative affinities of serum antibodies were determined using a competition ELISA that was previously established for this purpose (24). First, serum dilutions (either 1:1,000, 1:10,000, or 1:100,000 such that the titer, as determined by conventional ELISA, was the same for each sample) were incubated for 30 minutes at room temperature with the following concentrations of soluble recombinant GPI/glutathione S-transferase (GST): 10, 5, 2.5, or 0 μg/ml. This mixture was then transferred to 96-well flat-bottomed plates that had been precoated with GPI/GST. Incubation for 1 hour at room temperature allowed for the capture of any free anti-GPI Ig from the serum/soluble GPI mixture by the plate-bound GPI. After washing, this fraction of

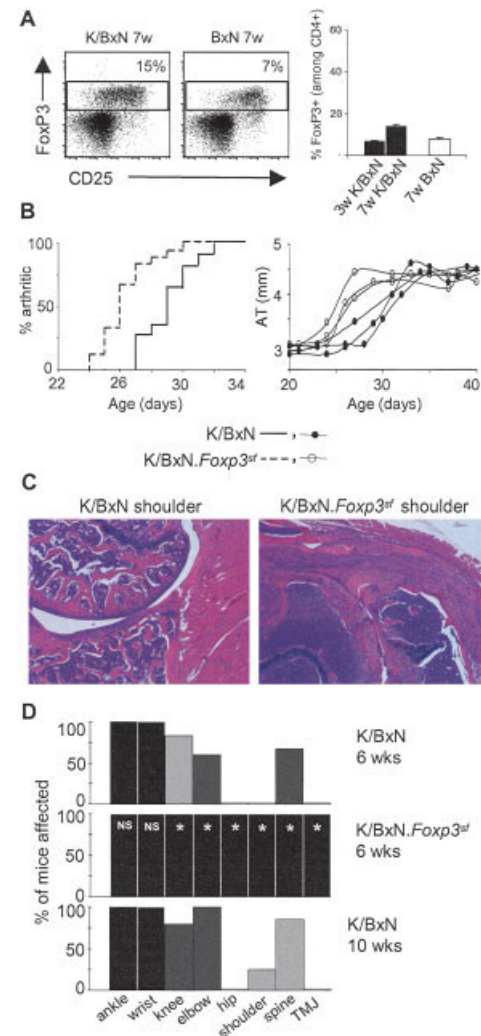
anti-GPI IgG was detected with alkaline phosphatase-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and alkaline phosphatase substrate. The OD<sub>405</sub> values were plotted as a percentage of the maximum OD<sub>405</sub> (maximum OD represents the OD of sera preincubated with 0  $\mu$ g/ml of soluble GPI) for each sample.

**Statistical analysis.** Rates of appearance of anti-GPI IgG titers were compared using survival analysis (incidence of anti-GPI titers versus time), using the Survdiff S-Plus software function (Insightful, Seattle, WA), which tests for a difference between 2 or more survival curves using the G-rho test. Histology scores were compared with Student's 2-tailed *t*-test.

## RESULTS

**Exacerbation of arthritis by the *Foxp3<sup>sf</sup>* mutation in the K/BxN model.** The generation of Treg cells can be influenced by TCR transgenes, particularly when self reactive (25,26), and we first sought to determine whether Treg cells were present in K/BxN mice. Flow cytometric analysis of K/BxN mice revealed that their regulatory compartment was indeed intact. Young K/BxN mice showed no deficit in the proportion of CD4+ T cells expressing FoxP3 in spleen or lymph nodes as compared with their nontransgenic littermates (Figure 1A). The proportion of FoxP3+ cells among CD4+ cells from prearthritic K/BxN mice (mean  $\pm$  SD 6.0  $\pm$  0.4%) is in the range of values usually observed in normal B6 and NOD mice. K/BxN mice also exhibited an increase in the proportion of FoxP3+ cells after the onset of arthritis (26  $\pm$  7% at 7 weeks), as if the inflammatory conditions that accompany the unfolding of arthritis increased Treg cell maturation or survival.

To examine the effect of eliminating these Treg cells from K/BxN mice, we crossed KRN-TCR-transgenic males with NOD females harboring the X-linked *Foxp3<sup>sf</sup>* mutation (10). Besides examining clinical parameters of arthritis and histologic features of the joints that are usually affected in this model, we looked at a variety of additional organs to see whether the range of tissue targets was extended when Treg cells were eliminated. As has been observed in mice of other genetic backgrounds (11), non-TCR-transgenic BxN.*Foxp3<sup>sf</sup>* mice showed a severe wasting disease by 3 weeks of age, with infiltration of multiple organs (liver, salivary glands, lung, exocrine pancreas, and dermis) (Table 1). In contrast, but as observed in other TCR-transgenic systems (10,27), introduction of the KRN-TCR transgene greatly attenuated the fulminant autoimmune response: K/BxN.*Foxp3<sup>sf</sup>* mice had only mild-to-moderate liver infiltrates at 6 weeks of age (Table 1). The protection from widespread autoimmune pathology



**Figure 1.** Exacerbation of arthritis in K/BxN.*Foxp3<sup>sf</sup>* mice. **A**, Treg cells in 7-week-old K/BxN and BxN mice were identified by staining lymph node cells for CD25 and forkhead box P3 (FoxP3). Profiles were gated on CD4+ cells (left). Data (mean and SD) from 3 independent experiments are shown graphically (right). **B**, Clinical analyses, showing percentages of arthritic (average ankle thickness 3.5 mm) male K/BxN and K/BxN.*Foxp3<sup>sf</sup>* mice as a function of age (left) and average thicknesses of hind paws from individual mice (right). Curves are representative of 10 K/BxN and 15 K/BxN.*Foxp3<sup>sf</sup>* mice. **C**, Shoulder abnormalities in hematoxylin and eosin-stained sections from 6-week-old K/BxN and K/BxN.*Foxp3<sup>sf</sup>* mice (original magnification  $\times$  50). **D**, Penetration and severity of arthritis in various joints from 6-week-old and 10-week-old K/BxN mice and from 6-week-old K/BxN.*Foxp3<sup>sf</sup>* mice. Shown are the percentages of mice with arthritis in each joint and the average severity of infiltration in each joint (0 = none [open bars], 0.1–1.0 = mild [lightly shaded bars], 1.1–2.0 = moderate [darkly shaded bars], and 2.1–3.0 = severe [solid bars]). Data represent 3–7 mice per joint per group. \* = *P* < 0.05 versus 6-week-old K/BxN mice, by Student's *t*-test. NS = not significant; TMJ = temporomandibular joint.

**Table 1.** Infiltration of organs by regulatory T cells in BxN.*Foxp3<sup>sf</sup>* and K/BxN.*Foxp3<sup>sf</sup>* mice\*

	3-week-old BxN. <i>Foxp3<sup>sf</sup></i> mice		3-week-old K/BxN. <i>Foxp3<sup>sf</sup></i> mice		6-week-old K/BxN. <i>Foxp3<sup>sf</sup></i> mice	
	No. affected/ no. examined	Mean severity of infiltration	No. affected/ no. examined	Mean severity of infiltration	No. affected/ no. examined	Mean severity of infiltration
Liver	3/3	2	2/4	0.5	3/3	1.7
Salivary gland	3/3	2	1/4	0.25	0/3	0
Pancreas	3/3	2	0/4	0	0/3	0
Dermis (paws)	4/4	2	0/3	0	0/4	0
Lung	3/3	1	0/4	0	0/3	0
Kidney	0/3	0	0/4	0	0/3	0

\* The severity of infiltration was scored on a scale of 0–3, where 0 = none, 1 = mild, 2 = moderate, and 3 = severe. The mean severity of infiltration was then calculated for all mice examined in each group. At 6 weeks of age, 0 of 3 BxN mice and 0 of 3 K/BxN mice were affected (all organs).

is particularly striking in this case of a TCR that is capable of responding to a ubiquitously expressed self antigen.

K/BxN.*Foxp3<sup>sf</sup>* mice developed a markedly more aggressive arthritis than did their K/BxN littermates. Clinical evaluation revealed the onset of disease and the kinetics of its progression to be accelerated in mice lacking Treg cells (Figure 1B). They also exhibited much more severe joint destruction and deformity in the distal paws, as demonstrated by external examination of the limbs (data not shown) as well as histologic sections of the skeleton.

A detailed histologic comparison of joints from K/BxN and K/BxN.*Foxp3<sup>sf</sup>* mice at 6 weeks of age revealed that the spectrum of affected joints differed. In K/BxN mice, the affected joints were mainly distal, and the knees, elbows, and spine were only sporadically and mildly affected (20). The spread of affected joints was much broader in K/BxN.*Foxp3<sup>sf</sup>* mice, with all individuals exhibiting severe infiltration in all joints examined, including the knees, elbows, shoulders, hips, temporomandibular joints, and a majority of the articular facet joints in the spine (Figures 1C and D). This difference was not just one of kinetics, since older (10 weeks) K/BxN mice did not show an expanded range of affected joints (Figure 1D).

Thus, K/BxN mice lacking Treg cells exhibited an exacerbated arthritis compared with that characteristic of ordinary K/BxN mice in 2 respects. First, there was acceleration of the kinetics of disease and enhancement of the severity of the inflammation and bone destruction in the joints usually affected. Second, there was a broadening of the spectrum of joints involved.

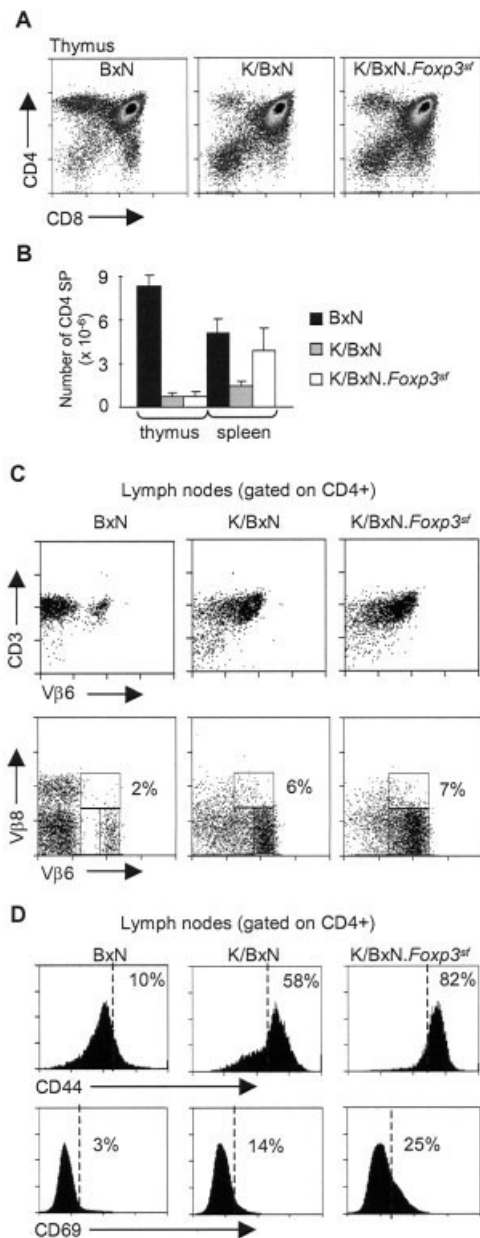
**Acceleration of the immunologic phase of arthritis by a deficiency of Treg cells.** We next sought to pinpoint the reason for the more aggressive disease in K/BxN.*Foxp3<sup>sf</sup>* mice. As discussed above, arthritogenesis

in the K/BxN model involves 2 phases: an immunologic initiation phase that promotes the production of pathogenic autoantibodies and an inflammatory effector phase induced by these antibodies. The absence of FoxP3 might affect either of these phases.

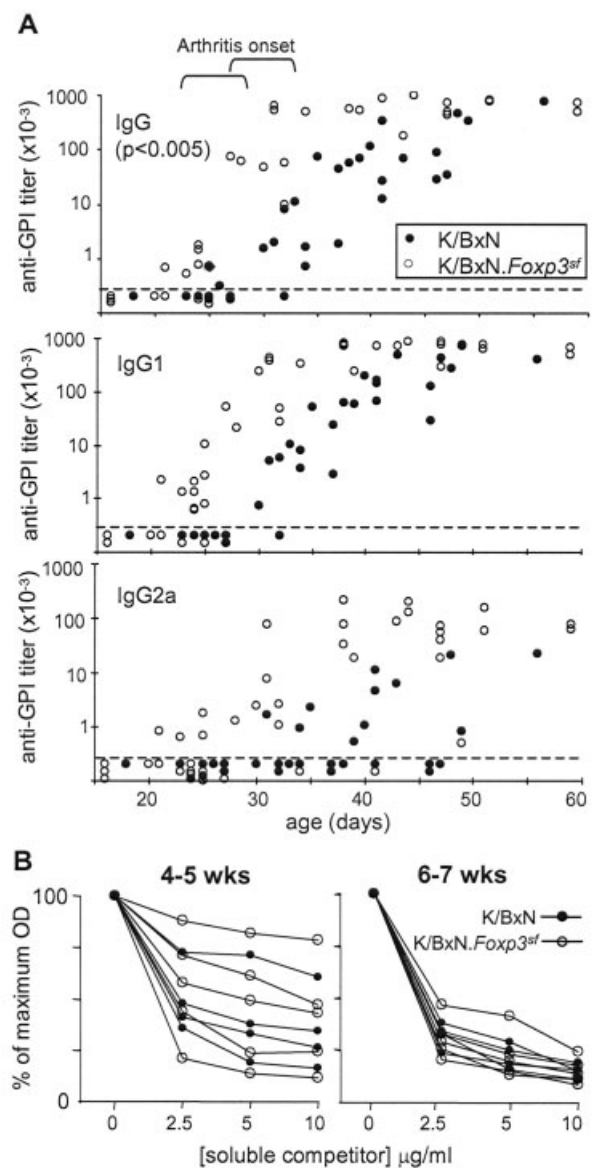
Since the requirement for FoxP3 in the generation and functioning of Treg cells is well established, as is the role of Treg cells in constraining the behavior of effector T cells, we first examined whether the T cell phenotype of K/BxN mice might be influenced by the *scurfy* mutation. The main peculiarities of the K/BxN T cell compartment were present: strong clonal deletion in the thymus (Figures 2A and B), incomplete expression of the clonotypical V $\beta$ 6 chain in peripheral CD4<sup>+</sup> T cells (Figure 2C, top) and perturbed allelic exclusion resulting in V $\beta$ 6/V $\beta$ 8 double-positive T cells (Figure 2C, bottom). Consistent with a role of Treg cells in the suppression of effector T cells, the peripheral T cell compartment of K/BxN.*Foxp3<sup>sf</sup>* mice showed an expansion of CD4<sup>+</sup> T cells (Figure 2B), which were more activated compared with those of K/BxN mice, based on expression levels of the activation markers CD44 and CD69 (Figure 2D).

One of the key characteristics of the K/BxN model is the GPI-focused T cell help that provokes massive production of pathogenic anti-GPI autoantibodies. Since T cell activation was enhanced in K/BxN.*Foxp3<sup>sf</sup>* mice, we tested whether autoantibody production was in turn augmented. A comparison of the time course of anti-GPI titers in K/BxN mice with or without Treg cells revealed that the rise in anti-GPI IgG titers occurred faster in K/BxN.*Foxp3<sup>sf</sup>* mice (Figure 3A). This acceleration was evident for anti-GPI antibodies of the IgG1 isotype, the predominant isotype in K/BxN mice (28) and for the less abundant IgG2a isotype as well (Figure 3A).

We also compared the affinity of anti-GPI autoantibodies from the 2 strains using a competition ELISA



**Figure 2.** Flow cytometric analyses of the T cell compartment in BxN, K/BxN, and K/BxN.*Foxp3<sup>sf</sup>* mice. BxN mice are KRN-T cell receptor (TCR)-transgene-negative (B6 × NOD)<sub>F1</sub> mice. Data represent 6–7 mice (ages 4–6 weeks) per group. **A**, Analyses of thymi from 6-week-old mice stained for CD4 and CD8. **B**, Numbers of CD4 single-positive (SP) T cells in the thymus and spleen of the 3 mouse strains. Values are the mean and SD. **C**, Expression patterns of the KRN transgene-encoded TCR ( $V_{\beta 6}$ ) and CD3 in K/BxN.*Foxp3<sup>sf</sup>* mice compared with K/BxN mice (top). Note that incomplete expression of the KRN TCR is indicated by cells falling off the diagonal. Expression patterns of the KRN TCR ( $V_{\beta 6}$ ) and an endogenous TCR ( $V_{\beta 8}$ ) in K/BxN.*Foxp3<sup>sf</sup>* compared with K/BxN mice are also shown (bottom). **D**, Comparison of T cell activation in K/BxN.*Foxp3<sup>sf</sup>*, K/BxN, and BxN mice, based on expression levels of the activation markers CD44 and CD69.



**Figure 3.** The autoantibody response in K/BxN and K/BxN.*Foxp3<sup>sf</sup>* mice. **A**, Sera were assayed for anti-glucose-6-phosphate isomerase (anti-GPI) IgG (top), anti-GPI IgG1 (middle), and anti-GPI IgG2a (bottom) by enzyme-linked immunosorbent assay (ELISA). Titers were determined as described in Materials and Methods. Each data point represents an individual mouse. Broken line indicates the lower limit of detection. *P* values were determined by survival analysis (presence of anti-GPI IgG >10,000 between days 20 and 35). **B**, The relative affinity of anti-GPI IgG in sera from mice ages 4–5 weeks (left) and 6–7 weeks (right) was evaluated by a modified ELISA as described in Material and Methods. Values are percentages of the maximum optical density (OD) for each sample (see Materials and Methods for details). The ODs shown reflect the amount of “unbound” antibodies after the anti-GPI/soluble GPI preincubation and, thus, are inversely related to the affinity of the anti-GPI IgG for soluble GPI.

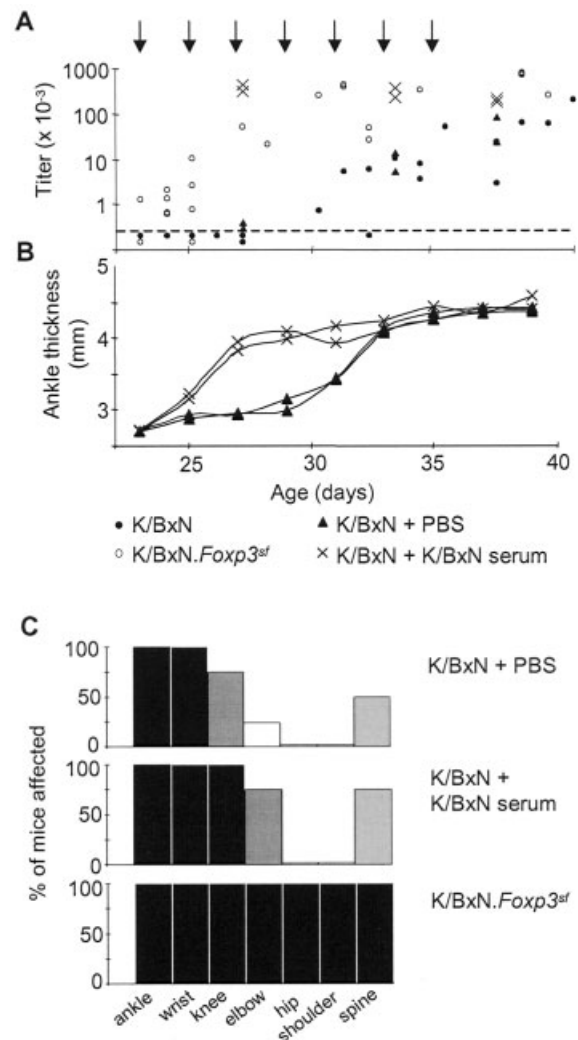
in which titrated amounts of soluble GPI were used as fluid-phase competitors (24). As shown in Figure 3B, the affinity maturation of anti-GPI antibodies was largely comparable in K/BxN.*Foxp3*<sup>sf</sup> mice and K/BxN mice, with both showing the clear shift in affinity that occurs between 4 and 7 weeks of age (28). Thus, the absence of FoxP3 and of FoxP3-dependent Treg cells promoted greater T cell help and a faster buildup of anti-GPI autoantibodies, but did not drastically affect their isotype bias or affinity maturation.

**Worsening of arthritis not fully explained by accelerated anti-GPI titers in K/BxN.*Foxp3*<sup>sf</sup> mice.** Next, we wanted to determine whether the faster appearance of anti-GPI antibodies in the K/BxN.*Foxp3*<sup>sf</sup> mice could, alone, account for the dramatic worsening of joint inflammation/destruction and the broadening of the range of affected joints (Figure 1). Since anti-GPI antibodies eventually reached similar titers and affinities in K/BxN and K/BxN.*Foxp3*<sup>sf</sup> mice beyond the age of 50 days, we hypothesized that there exists a particular window of disease susceptibility between 20 and 40 days of age during which arthritis spreads more extensively than in more mature animals. This hypothesis predicted that young K/BxN mice given frequent injections of high-titer sera from older animals would exhibit an arthritis that was identical in severity and spectrum to that seen in K/BxN.*Foxp3*<sup>sf</sup> mice.

Repeated transfer of K/BxN sera (400  $\mu$ l every 2 days between ages 21 and 35 days) elicited high circulating titers in young K/BxN mice that were equal to or greater than those in the K/BxN.*Foxp3*<sup>sf</sup> mice (Figure 4A). This serum supplementation led to an acceleration of the disease, as evidenced by increases in ankle thickness (Figure 4B). In contrast, histologic examination of the joints at 6 weeks of age demonstrated that the serum-treated K/BxN mice did not show any significant increase in the spectrum of joints affected: the profile remained very different from that in K/BxN.*Foxp3*<sup>sf</sup> mice, substantially more circumspect (Figure 4C).

Thus, while the accelerated ankle thickening in K/BxN.*Foxp3*<sup>sf</sup> mice can be explained by the acceleration of anti-GPI kinetics, it is clear that the impact of *Foxp3* deficiency on the immunologic phase of disease in this regard is not responsible for the severity and spectrum of joint infiltration in these animals. Rather, it appears that some element other than autoantibody titers is responsible for this phenotype.

**The other element?** To identify other elements contributing to the exacerbation of disease that occurs in K/BxN.*Foxp3*<sup>sf</sup> mice, we first considered that FoxP3 might play a role not only in Treg cells, but also in one or more cell types involved in generating arthritis, such



**Figure 4.** Role of accelerated titers of anti-glucose-6-phosphate isomerase (anti-GPI) in worsening arthritis. K/BxN mice were given 400  $\mu$ l of K/BxN mouse sera (K/BxN + K/BxN serum) or phosphate buffered saline (K/BxN + PBS) every other day from age 21 days to age 35 days (arrows). **A**, Serum titers of anti-GPI IgG1 were determined and compared with titers from untreated K/BxN and K/BxN.*Foxp3*<sup>sf</sup> mice (see Figure 3A). **B**, Changes in ankle thicknesses. **C**, Penetration and severity of arthritis in various joints from 6-week-old mice stained with hematoxylin and eosin. Shown are the percentages of mice with arthritis in each joint and the average severity of infiltration in each joint (0 = none [open bars], 0.1–1.0 = mild [lightly shaded bars], 1.1–2.0 = moderate [darkly shaded bars], and 2.1–3.0 = severe [solid bars]). Data are from 3 independent experiments ( $n = 2$  mice per group per experiment). Histologic findings in K/BxN.*Foxp3*<sup>sf</sup> mice are included for reference.

as synoviocytes, mast cells, or neutrophils. To test whether FoxP3 plays a cell-autonomous role in the cells involved in the effector phase of disease, we transferred K/BxN serum into alymphoid recipients that carried

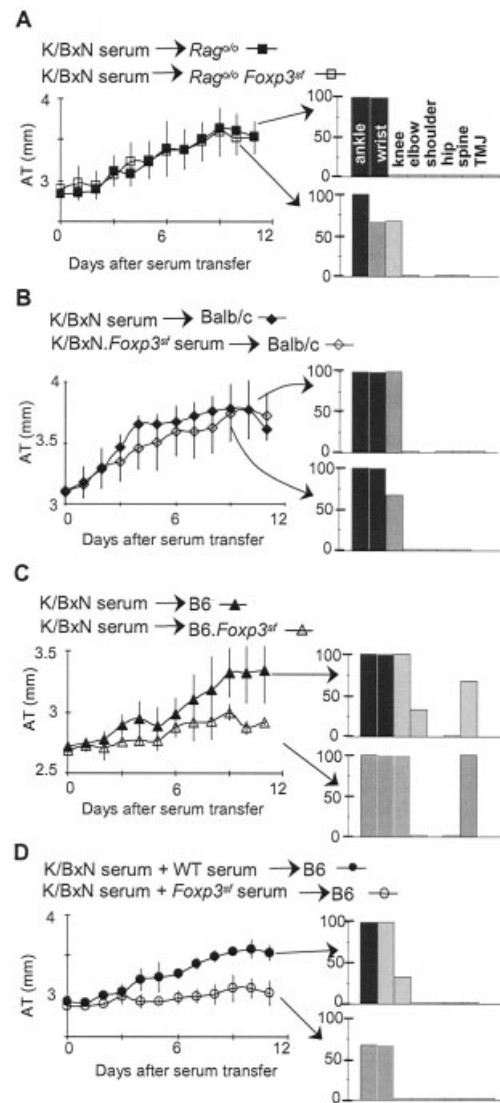
either wild-type *Foxp3* or the *Foxp3<sup>sf</sup>* mutation and were also deficient in T cells and B cells (carrying a knockout mutation of the *Rag* locus). This experimental setup tested for a possible role of FoxP3 that was independent of Treg cells, and the absence of T cells also meant that these mice did not succumb to the autoimmune manifestations imparted by the *scurfy* mutation.

Figure 5A shows that the *Foxp3* deficiency in these alymphoid hosts had little impact on the course of arthritis or the spectrum of affected joints. We did find evidence of arthritis in the knee joints of *Foxp3*-deficient alymphoid hosts; however, the degree of infiltration was mild. Thus, any effect that FoxP3 may have outside of lymphocyte populations is extremely limited.

Our next hypothesis was that the worsened arthritis might be induced by an unknown factor in K/BxN.*Foxp3<sup>sf</sup>* serum, an element distinct from anti-GPI antibodies, but one that synergized with the antibodies to produce very aggressive disease. This idea was tested by transferring sera from either K/BxN or K/BxN.*Foxp3<sup>sf</sup>* mice, matched for anti-GPI titer, into wild-type recipients (Figure 5B). The joint phenotype was the same in mice receiving serum from the 2 strains. These results rule out the existence of a transferable factor(s) in the blood of K/BxN.*Foxp3<sup>sf</sup>* mice that would explain the enhanced arthritis severity and spectrum of affected joints. The findings, together with the data shown in Figure 3, also confirm that there are no unique characteristics of the autoantibodies produced in K/BxN.*Foxp3<sup>sf</sup>* mice that may confer the hyperaggressive phenotype.

As discussed above, *Foxp3<sup>sf</sup>* mice present with devastating autoimmune infiltration in multiple organs, which is largely, but not quite completely, blocked by TCR transgenes through a reduction of the T cell repertoire (10,27). Thus, another possible explanation for the heightened arthritis in K/BxN.*Foxp3<sup>sf</sup>* mice was that the multiple-organ autoimmunity, progressing at low grade because of incomplete suppression by the KRN-TCR transgenes, might synergize with the arthritic process induced by anti-GPI antibodies, either by increasing the numbers of cells infiltrating the tissues or by generating additional proinflammatory cytokines. If this were correct, it should be possible to reproduce the hyperactive arthritis seen in K/BxN.*Foxp3<sup>sf</sup>* mice by transferring serum from standard K/BxN mice into *scurfy* recipients.

This hypothesis, however, also proved to be wrong. B6.*Foxp3<sup>sf</sup>* mice were actually more resistant to arthritis than their wild-type counterparts (Figure 5C), with a lesser degree of ankle swelling and reduced joint infiltration. This lower sensitivity of B6.*Foxp3<sup>sf</sup>* hosts was



**Figure 5.** Evaluation of the mechanisms underlying disease exacerbation. Shown are changes in ankle thickness (AT) over time (left) (mean and SD), and the penetrance and severity of arthritis in various joints (right), indicating the percentages of mice with arthritis in each joint and the average severity of infiltration in each joint (0 = none [open bars], 0.1–1.0 = mild [lightly shaded bars], 1.1–2.0 = moderate [darkly shaded bars], and 2.1–3.0 = severe [solid bars]). TMJ = temporomandibular joint. **A**, Transfer of K/BxN mouse serum into *Rag<sup>0/0</sup>* or *Rag<sup>0/0</sup>.Foxp3<sup>sf</sup>* recipients. Data are from 3 independent experiments ( $n = 8$ –9 mice per recipient genotype). **B**, Transfer of autoantibody titer-matched serum from K/BxN mice or K/BxN.*Foxp3<sup>sf</sup>* mice into BALB/c recipients, which are more sensitive to serum-transferred arthritis than B6 mice, were used. Data are from 2 independent experiments ( $n = 9$  mice per serum donor genotype). **C**, Transfer of K/BxN serum along with serum from either wild-type mice or *Foxp3<sup>sf</sup>* mice into B6 recipients. Data are from 2 independent experiments ( $n = 5$  mice per serum donor genotype). **D**, Transfer of K/BxN serum into B6 or B6.*Foxp3<sup>sf</sup>* recipients. Data are from 3 independent experiments ( $n = 6$ –7 mice per recipient genotype).

confirmed in parallel experiments in which the cotransfer of serum from B6.*Foxp3<sup>3f</sup>* and K/BxN mice impaired the ability of the K/BxN mice to induce arthritis, indicating that the factor that makes mice more resistant to arthritis is present in serum (Figure 5D).

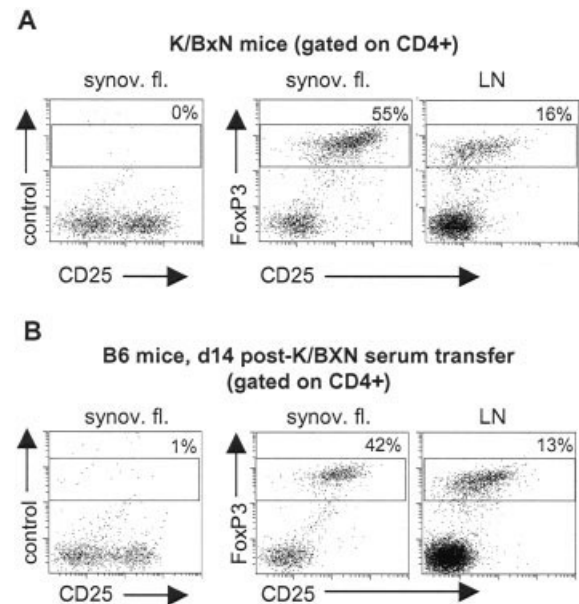
Thus, serum factors associated with generalized autoimmunity because of a mutation of *Foxp3* did not increase the severity or spectrum of affected joints. Instead, there was protective activity, operating likely through antiinflammatory factors elicited as negative feedback by the raging inflammation in the *scurfy* mice.

**FoxP3 positivity of a high proportion of synovial fluid T cells.** Thus, our search for the mechanism underlying the exacerbated severity and broadened spectrum of affected joints in K/BxN.*Foxp3<sup>3f</sup>* mice successively eliminated a number of potential explanations. The remaining hypothesis was that Treg cells normally dampen the inflammatory response during the effector phase of arthritis.

Indeed, the model of inflammatory bowel disease provided precedence for a direct action of Treg cells on nonlymphoid cells (29). This hypothesis predicts that Treg cells are present in the joints of arthritic K/BxN mice. We already knew that CD4+ T cells are present in the synovial fluid of K/BxN mice, albeit as a minor component (20), and so, we examined how many of them were of a Treg cell phenotype. As illustrated in Figure 6A, an extraordinary proportion of these CD4+ T cells had such a phenotype. Most displayed high levels of CD25, and the majority expressed FoxP3 (45–70% in different experiments). In addition, quantitative reverse transcription–polymerase chain reaction confirmed that the synovial fluid CD4+,CD25+ cells expressed high levels of *Foxp3* mRNA, comparable to or even higher than the expression in the corresponding lymph node populations (data not shown).

Enrichment for cells of the Treg phenotype was very specific to the joints of K/BxN mice, since their lymph node (Figure 6A) and splenic (data not shown) CD4+ T cell populations were dominated by FoxP3–cells. To address the basis of this enrichment, we evaluated FoxP3 expression in lymph nodes and synovial fluid after transfer of K/BxN serum into B6 recipients. Interestingly, arthritis induced by the serum-transfer system also resulted in a higher proportion of FoxP3+ cells in synovial fluid compared with lymph nodes (Figure 6B).

This finding indicates that the Treg cell population in the arthritic joint is not the result of a “joint-centered” activation of KRN+ T cells by antigen, but arises because of joint inflammation (through either induction or recruitment). This may be related to the



**Figure 6.** Enrichment of synovial fluid forkhead box P3 (FoxP3)-positive CD4+ cells induced by synovial inflammation. **A**, Cells from lymph nodes (LN) or synovial fluid (synov. fl.) from 7-week-old K/BxN mice were stained for surface markers and intracellular FoxP3 and then analyzed by flow cytometry. Profiles were gated on CD4+ T cells. Results are representative of 5 independent experiments. **B**, Arthritis was induced in B6 mice by transfer of K/BxN serum. On day 14 after serum transfer, popliteal lymph node and synovial fluid cells were stained for surface markers and intracellular FoxP3. Results are representative of 2 independent experiments ( $n = 4$  pooled mice per experiment).

more modest increase in the proportion of Treg cells in non-joint-related lymphoid organs of K/BxN mice, as noted above (Figure 1A). The finding that cells with the typical Treg cell phenotype preferentially accumulate in the joint cavity of arthritic K/BxN mice parallels the findings of several studies in patients with rheumatoid arthritis (30–32) and is consistent with a role in local dampening of the joint inflammation process.

## DISCUSSION

By examining arthritis in the absence of *Foxp3*, and thereby the absence of Treg cells, we found that the regulatory population exerted a suppressive effect at more than one point of disease pathogenesis. In the immunologic initiation phase, they dampened the interaction between T and B lymphocytes that results in high-level production of pathogenic autoantibodies: in the absence of Treg cells, the increase in anti-GPI autoantibody titers was accelerated. In the inflammatory



effector phase, Treg cells also exerted a strong influence on the spectrum of affected joints: in their absence, the degree of local destruction became more severe, and joints that are usually unaffected in the K/BxN model became targets. This effect was not attributable to the acceleration of autoantibody titers and, thus, was indicative of a previously unrecognized, additional role of Treg cells in inflammatory arthritis.

The K/BxN model has already yielded insights into the issue of why joints, but not other tissues, are affected in inflammatory arthritis. GPI-anti-GPI immune complexes are not limited to the articulations, which indicates that joint specificity is not solely determined at the level of immune complex deposition (23). However, colocalization of immune complexes with C3 occurs only in joints, which suggests that the specificity is regulated, at least in part, after immune complex deposition and before complement activation. Recent studies using intravital imaging to examine vascular leakage have shown that vessel permeability can be induced by immune complexes and that this effect is preferential at the joints affected by K/BxN arthritis, and not at other areas, such as the skin or mesenteric vessels (33). Thus, joint specificity is also promoted by differential susceptibility to vascular permeability.

In this study, we tested whether Treg cells represented another level of control over joint specificity. Histologic examination of various organs in K/BxN.*Foxp3<sup>sf</sup>* mice revealed that they were largely spared from any autoimmune manifestations. This finding is evidence against a role of Treg cells in limiting autoimmune destruction to the joints, although as discussed above, there was an increase in the range of joints affected.

Although the predominant function thus far ascribed to Treg cells is their ability to constrain the behavior of effector T cells, an additional role in suppressing cells of the innate immune system is beginning to emerge. Evidence supporting this role includes studies in a T cell-independent, bacterially induced model of intestinal inflammation, where transfer of CD4<sup>+</sup>,CD25<sup>+</sup> Treg cells resulted in suppression of innate immune pathology (29). Second, in a T cell-deficient burn injury model of innate immune reactivity, the production of inflammatory cytokines *ex vivo* in response to Toll-like receptor agonists was dampened if CD4<sup>+</sup>,CD25<sup>+</sup> Treg cells were administered *in vivo* at the time of injury (34). As was the case in the present study, these models were independent of T cells. It will be important to determine whether Treg cells dampen the activation of cells of the innate immune system that are essential to the inflammatory process, such as mast cells, macrophages, or

neutrophils, and/or whether they influence the stromal elements that contribute to the process, such as fibroblast-like synoviocytes in the case of arthritis.

While it is clear from this study that removal of Treg cells from an otherwise lympho-replete animal can profoundly increase the severity of inflammatory arthritis, the interplay of factors that regulate this process can be very complex. For example, transfer of K/BxN serum into mice with a null mutation at the *Rag* or *TCR-C $\alpha$*  locus, and lacking both conventional and regulatory T cells, did not result in the severe arthritis that affected the K/BxN.*Foxp3<sup>sf</sup>* mice (35). It is possible that serum transfer is simply too mild a stimulus to allow for broadening of the spectrum of affected joints (as compared with spontaneous disease in the KRN-transgenic line). Alternatively, it might suggest that the presence of conventional T cells during the effector phase of disease is somehow required in order to reveal the effect of removing Treg cells on the spectrum of affected joints.

This direct action of Treg cells in the joint, independently of their influence on the adaptive immune system, extends the results of recent studies of the CIA model, where it is more difficult to rule out an effect on immunologic initiation events. Recent studies reported a correlation between CIA intensity and Treg cell manipulation, without observable suppression of autoantibody titers. Kelchtermans et al (19) made this observation by depleting mice of CD25<sup>+</sup> cells 2 weeks after immunization with type II collagen in Freund's complete adjuvant, whereas Morgan et al (36) transferred additional CD4<sup>+</sup>,CD25<sup>+</sup> cells during early stages of arthritis.

A prerequisite for Treg cells to be able to directly suppress innate immune cells in the joint is that they be present at that location. Our data show that a large proportion, ~50%, of the synovial fluid T cells in K/BxN mice expressed FoxP3 (Figure 6). This preponderance is reminiscent of the situation with synovial fluid from patients with rheumatoid arthritis, where a high proportion of CD4<sup>+</sup>,CD25<sup>+</sup> cells with suppressive activity *in vitro* has been reported (30–32,37). Clearly, the inflamed joint is a preferred location for Treg cells, which raises 2 key questions: How is this predominance achieved? And, what are Treg cells actually achieving in the joint?

The predominance of Treg cells in synovial fluid may result from preferential homing to, or retention in, the inflamed joints. Indeed, Frey et al (17) have provided some evidence, through cell transfers in the CIA system, that there is a relative preference for Treg cells to settle in the joint. This preference may be the result of particular anatomic cues (such as the particular redox

conditions in the joint), antigenic stimulation, or chemokine gradients. Indeed, analysis of gene-expression signatures in Treg cells compared with conventional T cells (ref. 38, and Nguyen LT, et al: unpublished observations) shows that the CXCR2, CCR2, and other chemokine receptors are preferentially expressed by Treg cells. Interestingly, microarray analysis of K/BxN mice with serum-transferred arthritis demonstrated that the corresponding ligands (CXCLs 1, 2, and 5 and CCLs 2, 7, and 8) are up-regulated in the ankle joints during the course of arthritis (Jacobs J, et al: unpublished observations). These expression patterns suggest attractive explanations for the prevalence of Treg cells in inflamed joints.

Alternatively, these cells may be generated de novo in the particular conditions of the inflamed joint. Transforming growth factor  $\beta$  (TGF $\beta$ ) is a very potent inducer of Treg cell differentiation in vitro, and naive CD4+ cells are converted to the FoxP3+ Treg cell phenotype after activation in the presence of TGF $\beta$  (39–41). Thus, it is also quite plausible that the TGF $\beta$  elicited by the inflammatory process in the joint (42,43) also promotes phenotypic conversion of conventional FoxP3– T cells that might have entered in the joint.

Whether preferentially retained or differentiated in the joint, the question arises as to what Treg cells actually achieve in the joint and how they do it. It has been speculated that their action might be quite limited, since the inflammation burns on despite this large number of Treg cells (44). Yet, there are clear indications of a beneficial action in our study, as well as in the previous complementation experiments in other models (17,36). In addition, successful treatment of rheumatoid arthritis patients with tumor necrosis factor–blocking agents was shown to correlate with heightened activity of CD4+,CD25+ cells (45).

By what mechanisms do Treg cells suppress the inflammatory phase of arthritis in the K/BxN mouse? Some of the same Treg cell–produced molecular mediators responsible for dampening effector T cell responses may also mitigate the inflammation promoted by innate cells and/or synovial cells. For example, interleukin-10 (IL-10) has suppressive effects on T cell responses via down-regulation of various antigen-presenting cell functions as well as through direct suppression of T cell–derived cytokine production (46). In addition, this cytokine can inhibit the production of proinflammatory cytokines by innate cells, such as neutrophils. Indeed, IL-10 mediates in part the suppressive effect of Treg cells on innate cell types in a T cell–independent, bacterially induced model of intestinal inflammation (29). Studies in arthritis models have

shown that IL-10 can have suppressive effects on disease (47–49). Thus, it would be interesting to test which of the known soluble mediators of Treg cell activity can suppress the arthritis in K/BxN mice.

According to our results, Treg cells do seem to dampen the activity of the inflammatory lesions, resulting in far less destruction and remodeling of the joints that are usually affected. But, perhaps more importantly, the action of Treg cells may also be to protect those joints that are normally refractory to arthritis: in the absence of Treg cells, the spine, elbow, or hip joints emerge as frequent targets. If this is true, it remains to be explained why Treg cells are effective at “sterilizing” the process in proximal joints, but act only as partially effective dampers in distal joints. The explanation may be quantitative, in that Treg cells are simply unable to contain an inherently more aggressive inflammation in the distal joints. Alternatively, it may be that the numbers or activity of Treg cells in proximal joints is higher or that their action is less inhibited by counterregulatory factors in that location. Consistent with this line of interpretation, our results raise the intriguing possibility that the geographic distribution of lesions in diseases such as the arthritis in K/BxN mice or rheumatoid arthritis in humans is due in part to the differential action of Treg cells.

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## AUTHOR CONTRIBUTIONS

Drs. Mathis and Benoist had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Dr. Nguyen, Mr. Jacobs, and Drs. Mathis and Benoist.  
**Acquisition of data.** Dr. Nguyen, Mr. Jacobs, and Drs. Mathis and Benoist.

**Analysis and interpretation of data.** Dr. Nguyen, Mr. Jacobs, and Drs. Mathis and Benoist.

**Manuscript preparation.** Dr. Nguyen, Mr. Jacobs, and Drs. Mathis and Benoist.

**Statistical analysis.** Dr. Nguyen.

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