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From Systemic T Cell Self-Reactivity to Organ-Specific Autoimmune Disease via Immunoglobulins

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Summary

Rheumatoid arthritis is a common and debilitating autoimmune disease whose cause and mechanism remain a mystery. We recently described a T cell receptor transgenic mouse model that spontaneously develops a disease with most of the clinical, histological, and immunological features of rheumatoid arthritis in humans. Disease development in K/BxN mice is initiated by systemic T cell self-reactivity; it requires T cells, as expected, but B cells are also needed, more surprisingly. Here, we have identified the role of B cells as the secretion of arthritogenic immunoglobulins. We suggest that a similar scenario may unfold in some other arthritis models and in human patients, beginning with pervasive T cell autoreactivity and ending in immunoglobulin-provoked joint destruction.

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Introduction

Rheumatoid arthritis (RA) is a frequent and incapacitating autoimmune disorder (Feldmann et al., 1996b). It is a chronic, progressive joint disease, characterized by leukocyte invasion of the synovial lining and hyperplasia of the resident synoviocytes. The ensuing overproduction of cytokines and other soluble mediators results in neovascularization, cartilage destruction, bone erosion, and anarchic remodeling of joint structures. Systemic manifestations can also occur—in particular, the production of autoantibodies.

The etiology and pathogenesis of RA remain controversial (Feldmann et al., 1996b). It is not known whether disease is initiated by an unrestrained inflammatory reaction to a microbial antigen (Ag), an inappropriate autoimmune response to a self-constituent, or both. An important role for T cells (Panayi et al., 1992; Fox, 1997), B cells (Zvaifler, 1973), and other leukocytes such as dendritic cells, macrophages, and neutrophils (Firestein and Zvaifler, 1990; Feldmann et al., 1996a; Thomas et al., 1996; Burmester et al., 1997) has been argued and disputed. The lack of a consensus largely reflects two factors. RA is a heterogeneous syndrome, different patients exhibiting widely variant ages of onset, disease courses, genetic profiles, and responses to therapeutic intervention. In addition, there has been a dearth of small animal models of RA, particularly those spontaneously succumbing to disease.

Recently, we reported a mouse model that spontaneously develops a disease with many of the characteristics of rheumatoid arthritis in humans (Kouskoff et al., 1996). All KRN T cell receptor (TCR) transgenic (tg) mice on the C57Bl/6xNOD genetic background (K/BxN mice) develop a joint disorder, starting at 3 to 4 weeks of age and rapidly evolving until the animal's mobility is severely compromised; as in patients, the disease is chronic, progressive, symmetrical, and has a proximal to distal gradient of severity. The murine disease exhibits all of the major histological features of the human one: leukocyte invasion, synovitis, pannus formation, cartilage and bone destruction, and anarchic remodeling. The mouse model, like patients, shows several immunological abnormalities, including cytokine (TNF-α, IL-6) imbalances and B cell hyperactivity, the latter manifest as an increase in B cell numbers, hypergammaglobulinemia, and autoantibody production. Despite these marked similarities, the murine and human disorders do diverge in certain details: a more aggressive disease in the mice, a few differences in the particular joints affected, a different spectrum of autoantibodies produced—in particular, absence in the mice of immunoglobulin (Ig) M isotype rheumatoid factor (RF: antibody [Ab] directed against the Fc portion of IgG). Nonetheless, given the similarities to the human disease, a 100% incidence, and a highly reproducible course of pathogenesis, we felt that the K/BxN model would be valuable for elucidating mechanisms of RA pathogenesis.

The mouse model was generated when the KRN/C57Bl/6 TCR tg line was fortuitously crossed with the

Table 1. KRN Arthritis Transfer

Donor	Recipient	APC	B	Fraction with Arthritis	Day of Onset
K/B	BxN-C$\alpha^{o/o}$			**12/12**	6.4 ± 0.7
B	BxN-C$\alpha^{o/o}$			0/5	—
K/BxN	BxN-RAG$^{o/o}$			**7/7**	8.3 ± 2.3
K/BxN	B-RAG$^{o/o}$			**48/49**	11.8 ± 2.4
K/B	B-RAG$^{o/o}$			0/4	—
T^- K/BxN	B-RAG$^{o/o}$			1/6	transient
B^- K/BxN	B-RAG$^{o/o}$			0/6	—
(T^- K/BxN) + (B^- K/BxN)	B-RAG$^{o/o}$			**5/6**	17.8 ± 3
(K/B)$CD4^+$ + (BxN-C$\alpha^{o/o}$)$B220^+$	BxN-RAG$^{o/o}$			**4/4**	14,19,21,21
(K/B)$CD4^+$ + (B-C$\alpha^{o/o}$)$B220^+$	BxN-RAG$^{o/o}$			0/4	—
K/B	(BxN-RAG$^{o/o}$) + $B220^+$BM(BxN-C$\alpha^{o/o}$)	bxg^7	bxg^7	**2/2**	7,10
K/B	(BxN-RAG$^{o/o}$) + $B220^+$BM(B-C$\alpha^{o/o}$)	bxg^7	b	0/2	—

NOD strain (Kouskoff et al., 1996). This was a surprising result given that the KRN receptor was known to recognize a peptide of bovine ribonuclease in the context of major histocompatibility complex (MHC) class II A^k molecules, both absent from the F1 offspring, but we eventually determined that the disease is initiated by cross-reactive recognition of NOD-derived A^{g7} molecules by the KRN TCR. Thus, in K/BxN animals, a situation of systemic autoreactivity is generated, raising two central issues—how T cells expressing the KRN specificity escape tolerance induction and how organ-specific autoimmune disease develops in the presence of systemic autoreactivity. Concerning the first issue, we found evidence for multiple levels of tolerance induction—thymocyte clonal deletion, diminished levels of TCR clonotype due to selection of cells exhibiting incomplete allelic exclusion, clonal anergy of peripheral T cells—but none of these was completely effective. Concerning the second, we began to dissect pathogenetic mechanisms by identifying the critical cellular players. Not surprisingly, T cells were required for arthritis development, although they appeared dispensable at the later stages of disease. Rather unexpectedly, B cells were also critical. Here, after evaluating a number of possibilities, we have identified the role of B cells in the development of arthritis in K/BxN mice.

Results

B cells could play several possible roles during the unfolding of arthritis in K/BxN mice. First, it could be that B cells exert only an indirect effect on disease development influencing, for example, the genesis of lymphoid organs or the composition of the cytokine/chemokine milieu. Indeed, it is known that B cell-deficient mice have aberrant lymph node architecture (Fu et al., 1998), and such indirect effects may explain a critical B cell influence on prion disease (Klein et al., 1998). Second, it could be that the Ag recognized by KRN T cells to initiate disease is synthesized only by B cells. Igs are obvious candidates, and their particular specificities might or might not be important, but there are plenty of other possibilities. The arthritogenic Ag might be presented by B cells themselves or be shed and then taken up and presented by other antigen-presenting cells (APCs). Third, B cells could play their critical role in Ag presentation. This might be independent of their Ig specificities, as B cells may process some Ags differently from other APCs (Vidard et al., 1992) and may elicit different effector functions in responding T cells (Stockinger et al., 1996). Or it might be critically dependent on Ig specificity: Ags internalized after binding to surface Igs on B cells are processed and presented by class II molecules with significantly enhanced efficiency, as are Ags complexed with secreted Igs and internalized by other APCs via binding to Fc receptors (Amigorena and Bonnerot, 1998). Finally, it could be that a particular B cell product plays a directly pathogenic role. Again, likely candidates are Igs, which might promote arthritis because of their particular specificities or simply due to overproduction. Other possibilities include soluble mediators like cytokines.

Arthritis Transfer Systems

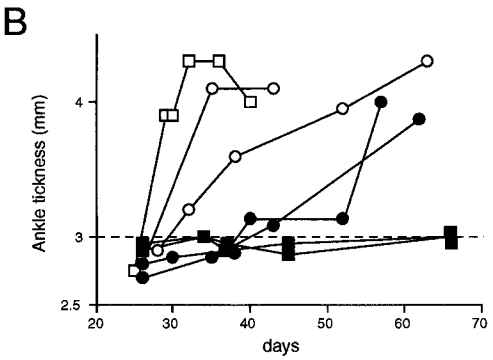
Disease transfer systems have been useful tools for unraveling mechanisms of autoimmune pathogenesis. Thus, as a first step in identifying the role of B cells in arthritis development in K/BxN mice, we established two transfer protocols.

In the first (Table 1), 2×10^7 splenocytes from healthy K/B donors were injected intravenously (i.v.) into BxN-$Ca^{o/o}$ hosts devoid of α:β T cells. Within 8 days, all recipients had developed an arthritis clinically and histologically indistinguishable from that of K/BxN mice. None of the control recipients injected with B6 splenocytes showed signs of disease. This system permits convenient analysis of the K/BxN disease in its entirety, from initiation through all stages of pathogenesis.

The second system (Table 1) focuses on the effector phases of disease, after the KRN T cells have already been confronted with Ag. BxN-$RAG^{o/o}$ hosts, lacking all mature lymphocyte subsets, were injected i.v. with 2×10^7 splenocytes from arthritic K/BxN donors; by 11 days, all recipients had succumbed to the typical arthritis. Interestingly, this protocol also worked, after a slight delay, with B-$RAG^{o/o}$ recipients, indicating that arthritis development does not require A^{g7}-mediated presentation of the Ag on resident synovial fibroblasts or chondrocytes, at least in the effector phases.

A

μ	κ	Normal onset	Delayed onset	Protected
		1/1 (30)		
		3/6 (26)	2/6 (38)	1/6
		3/9 (29)	6/9 (40)	
			4/5 (40)	1/5
			2/2 (57)	
			1/1 (59)	
				5/5



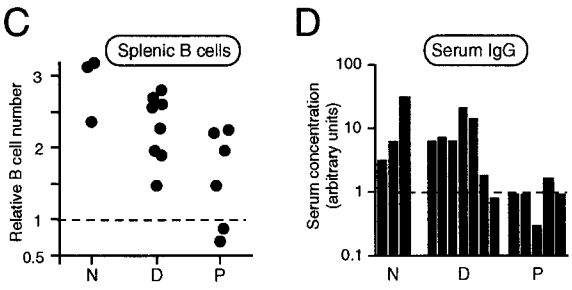


Figure 1. Disease Development in K/BxN Mice with a Restricted B Cell Repertoire

(A) Arthritis incidence in mice carrying various Ig knockin alleles. Genotypes at the μ and κ loci are indicated on the left: black square, homozygosity for the knockin allele; half-black square, heterozygosity; white square, homozygosity for the normal allele. Arthritis was evaluated by inspection of the joints and measurement of ankle thickness, and the mice were categorized as presenting the usual K/BxN arthritis, arthritis with delayed onset (onset >35 days), or being arthritis free, the absence of disease being confirmed by histology.

(B) Representative plots of ankle thickness in K/BxN mice carrying different Ig knockin alleles: only wild-type alleles (open squares); heterozygous for either the μ or κ knockin allele or both (open circles); homozygous for either the μ or κ knockin allele (black circles); homozygous for both (closed squares). The dashed line shows the maximum value for healthy control mice at these ages.

(C) Numbers of splenic IgM^+B220^+ B cells, determined by cytofluorimetry, shown relative to non-TCR tg littermates to standardize for variations in the ages of the mice. Each point represents a single tg:non-TCR tg littermate pair.

(D) Serum IgG levels, measured by ELISA, in several of the knockin mice grouped as described above. N, normal onset; D, delayed onset; P, arthritis free. Each bar represents an individual mouse.

Although B cell–deficient K/BxN-$\mu M^{o/o}$ mice do not develop arthritis, and we confirmed that BxN-$\mu M^{o/o}$ hosts transferred with K/B splenocytes do not either (S. M., unpublished data), it was not known whether B cells are still required during the effector phases of disease. According to the results depicted in Table 1, they are: K/BxN splenocytes depleted of either T or B cells were unable to transfer arthritis into B-$RAG^{o/o}$ recipients.

These two transfer systems, both B cell dependent, should facilitate the dissection of pathogenetic mechanisms. Their advantages vis a vis the regular model are their relative synchrony, rapidity, and manipulability.

An Indirect Role?

To rule out the possibility that B cells play only an indirect role in the K/BxN model, we demonstrated that an A^{g7}-restricted B cell function is critical. The data presented in Table 1 show that arthritis development requires more than the mere presence of B cells—rather, B cells expressing A^{g7} molecules are needed. In the first set of experiments, purified $CD4^+$ T cells from K/B mice, when complemented with purified $B220^+$ B cells displaying A^b plus A^{g7} (BxN-$C\alpha^{o/o}$) but not just A^b (B-$C\alpha^{o/o}$) molecules, precipitated disease in BxN-$RAG^{o/o}$ hosts devoid of lymphocytes. In the second experiment, B cells expressing either A^b plus A^{g7} or A^b alone were generated in $RAG^{o/o}$ mice by transfer of bone marrow cells harboring B cell progenitors, and 6 weeks later, once the B cell compartment had been reconstituted, the mice were injected with K/B splenocytes. Only those animals whose B cells displayed A^{g7} molecules became arthritic. Clearly, then, the role of B cells in the K/BxN model is not merely an indirect influence on the general development or functioning of the immune system. An A^{g7}-restricted B cell function is required, most likely a B/T cell interaction as occurs during B cell delivery of Ag to T cells, either to stimulate them or in eliciting their help for Ig production.

Dependence on Ig Specificity?

As detailed above, each of the three putative roles involving a more direct input from B cells—Ag synthesis, Ag presentation, and synthesis of a particular product—could rely on a mechanism dependent on or independent of Ig specificities. Thus, establishing whether arthritis development in K/BxN mice is influenced by the Ig repertoire would expeditiously rule out a number of possible mechanisms.

To do this, we analyzed K/BxN animals with a restricted B cell repertoire. Due to the dictates of allelic exclusion, it is possible to substantially reduce an animal's Ig heavy chain repertoire by inserting a rearranged $V_HD_HJ_H$ segment into the germline J_H locus, and the repertoire is further constrained if both chromosomes carry the "knockin" allele (Taki et al., 1993). Similarly, though less drastically, the Ig light chain repertoire can be reduced by introducing a rearranged V_KJ_K segment into the germline J_K locus, again most effectively when the alteration is present on both chromosomes (Zou et al., 1993). We made use of two existing mouse lines, one carrying a knockin of the VHB1-8-DH-JH2 segment encoding the heavy chain variable region of an anti-(4-hydroxy-3 nitrophenyl)-acetyl (NP) Ab (Sonoda et al.,

1997), and the other a Vk3-83-Jk2 segment specifying the light chain variable region of an anti-MHC class I (H-2K^b, D^{k,b}) Ab (Pelanda et al., 1996). These were crossed appropriately to produce K/BxN animals carrying knockins in the heterozygous or homozygous state at the heavy and/or light chain locus.

Figure 1A summarizes the genotypes and phenotypes of these mice, and some typical plots of disease course are presented in Figure 1B. The major point to emerge from these data is that, as the Ig repertoire becomes more and more restricted, arthritis is delayed and eventually abrogated. Almost all animals with at least one wild-type allele at both the heavy and light chain loci developed arthritis, a substantial number at the normal time of onset. A large majority of animals with homozygous knockin alleles at either one or the other Ig locus also succumbed to arthritis, but in all cases disease onset was delayed. The delayed disease often showed diminished severity, with mild or asymmetrical joint swelling for several days; nonetheless, in most cases, all four paws were eventually afflicted and became deformed. Finally, none of the mice with homozygous knockin alleles at both Ig loci developed arthritis, at least until 100 days of age.

We also analyzed the B and T cell compartments of K/BxN mice with the various knockin genotypes. In all cases, even disease-free animals with a highly restricted Ig repertoire, there were reduced numbers of mature CD4$^+$ T cells and a diminished fraction of cells expressing high levels of the transgene-encoded TCR (data not shown). There was also an enrichment for T cells displaying late activation markers (data not shown). These observations are reminiscent of findings on the T cell compartment of regular K/BxN mice (Kouskoff et al., 1996) and indicate that the first step of disease, KRN T cell recognition of the potentially arthritogenic antigen, takes place in all of the knockin animals. Again in all cases, B cell differentiation appeared essentially normal, according to cytofluorimetric analysis of spleen and peritoneal cells with anti-B220, -IgM, -IgD, -Mac-1, and -CD5 reagents (data not shown). As expected from previous results on regular K/BxN mice, B lymphocyte numbers were elevated almost 3-fold in arthritic knockin animals with partially restricted Ig repertoires; more surprisingly, they were also higher, although only about 1.5-fold, in disease-protected animals with a highly restricted Ig repertoire (Figure 1C). While almost all arthritic animals also exhibited hypergammaglobulinemia, those that were disease-free because of a constrained Ig repertoire did not, although they did show Ig class switching (Figure 1D).

Thus, arthritis development in K/BxN mice relies critically on B cells with particular Ig specificities, although certain of the early disease manifestations appear less dependent. Several of the possible mechanisms evoked above are thereby ruled out.

A B Cell–Specific Antigen?

To evaluate the possibility that the K/BxN model requires B cells simply because the Ag recognized by the KRN receptor is synthesized only by that cell type, we asked whether KRN T cells see their Ag in mice devoid of B cells.

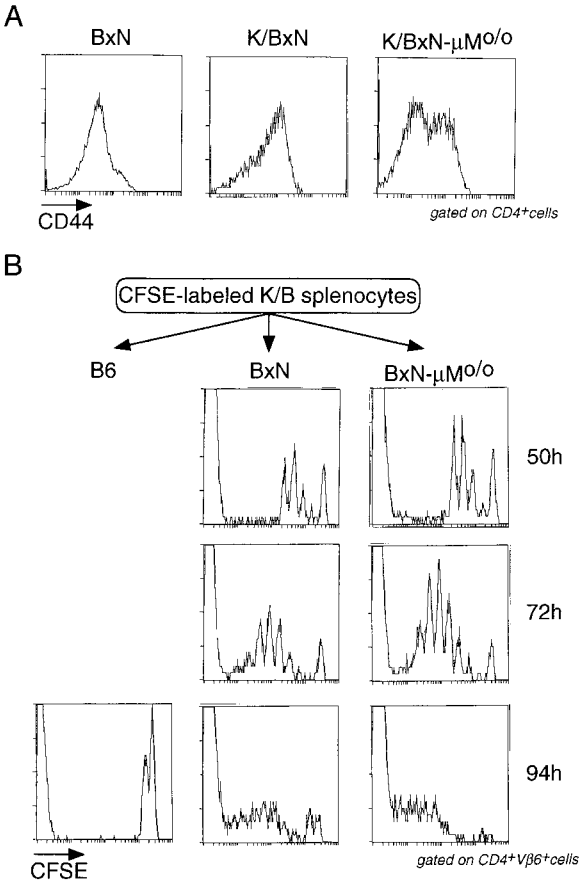


Figure 2. Activation of KRN T Cells in the Absence of B Cells

(A) Surface expression of the CD44 late activation marker on gated CD4$^+$ lymph node cells, in non-TCRtg BxN littermates, and in TCR tg K/BxN mice heterozygous (with a normal B cell complement) or homozygous (B cell deficient) for the μM^o mutation.

(B) Division of CFSE-labeled K/B splenocytes after injection into non-TCR tg recipients, having B cells (BxN) or not (BxN-μM$^{o/o}$). Division was measured as dilution of CFSE staining intensity in CD4$^+$Vβ6$^+$ cells that had homed to the axillary lymph nodes, measured by cytofluorimetry at different times after transfer.

First, we analyzed the activation status of KRN T cells residing in B cell–deficient K/BxN-μM$^{o/o}$ mice by quantitating surface levels of the late activation marker CD44. As described previously (Kouskoff et al., 1996) and illustrated in Figure 2A, elevated numbers of actived T cells are found in arthritic K/BxN mice vis a vis healthy non-TCRtg BxN littermates. More activated T cells were also observed in K/BxN-μM$^{o/o}$ animals (Figure 2A), despite the fact that they will never develop arthritis. In addition, as in regular K/BxN mice, KRN thymocytes were subject to clonal deletion in K/BxN-μM$^{o/o}$ animals (data not shown).

We also looked more directly at KRN T cell priming. Splenocytes isolated from K/B animals were labeled with 5, 6 carboxyfluorescein diacetate succinimidyl ester (CFSE). This fluorescent dye is incorporated in the cytosol and stably maintained, so that with subsequent cell divisions its concentration is successively halved, a reduction that is readily apparent upon cytofluorimetric analysis. The labeled splenocytes were transferred into

various recipients, and at progressively later times, the axillary lymph nodes were removed and CFSE levels in $CD4^+$ $V\beta6^+$T cells assessed. As illustrated in Figure 2B, while the cells did not divide after transfer into control B6 recipients, substantial division took place when they were introduced into F1 animals containing (BxN) or lacking (BxN-$mMT^{o/o}$) B cells. The parallel kinetics and similar degree of division in the two cases indicate that T cell stimulation was not just due to B cells in the innoculum.

According to these two criteria—long-term stimulation of resident cells and priming of naive cells—T cells expressing the KRN receptor recognize their Ag in the absence of B cells. Thus, synthesis of the arthritogenic Ag cannot be confined uniquely to B cells.

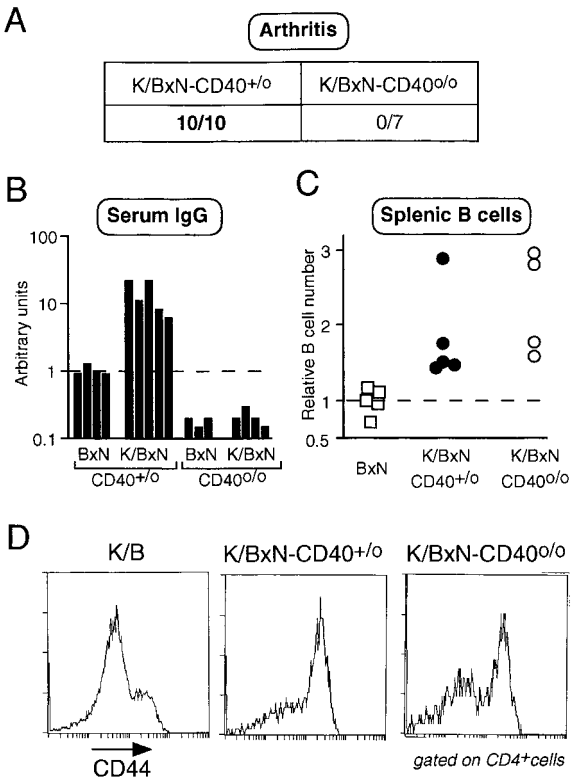
Ag Presentation?

That arthritis development in K/BxN mice depends on an A^{g7}-restricted B cell function (Table 1) argues for the importance of a cognate interaction between the KRN TCR and MHC molecules on B cells, either in promoting T cell stimulation or in eliciting T help for Ig production. The fact that KRN T cells can be efficiently primed and maintained in an activated state in the complete absence of B cells (Figure 2) indicates that B cells are not solely responsible for T cell stimulation. To evaluate the necessity for T help-dependent Ig production, we determined whether and when the K/BxN disease depends on CD40 expression by B cells, given that CD40/CD40L interactions are known to be critically involved in the delivery of T cell help (Clark et al., 1996).

The data presented in Figure 3 indicate that arthritis development requires CD40 display on B cells. K/BxN mice carrying a null mutation at the CD40 locus did not develop arthritis (A). Nor did they exhibit the hypergammaglobulinemia characteristic of regular K/BxN animals (B). Yet they did show some early signs of the disease process, such as increased B cell numbers (C) and activation of KRN T cells (D). (In addition, the usual clonal deletion of KRN thymocytes was detected [data not shown]).

Given that CD40 can be expressed on cell types other than B cells (e.g., macrophages, dendritic cells, and endothelial cells), it was important to establish that the absence of disease in the mutant animals was due to the absence of CD40 on B cells, and this was done via a series of transfer experiments (Figure 3E). Splenocytes from K/BxN-$CD40^{o/o}$ mice were unable to provoke arthritis in CD40-positive BxN-$RAG^{o/o}$ recipients, suggesting that expression of CD40 on non-B APCs does not suffice for disease development. That the T cells from CD40-negative mice were not somehow incompetent was ruled out because purified $CD4^+$ cells from K/BxN-$CD40^{o/o}$ animals could provoke disease in CD40-positive BxN-$C\alpha^{o/o}$ recipients. Rather, the problem lies with the B cells lacking CD40: they could not interact with purified $CD4^+$ cells from CD40-positive K/B6 mice to precipitate disease in BxN-$RAG^{o/o}$ recipients, also CD40-positive.

Taken together, the data in Table 1 and Figures 2 and 3 argue that arthritis development in K/BxN mice needs an A^{g7}-restricted, CD40-dependent T/B cell interaction downstream of initial T cell stimulation. A likely possibility is a requirement for T/B collaboration in the production of Igs.



E

Donor	Recipient	Arthritis	Onset
K/BxN-$CD40^{o/o}$	RAG^{o}/BxN	0/4	–
K/BxN-$CD40^{+/o}$	RAG^{o}/BxN	**4/4**	11,13,13,16
($CD4^+$ + K/BxN-$CD40^{o/o}$)$B220^+$	$C\alpha^{o}$/BxN	**2/2**	7,9
($CD4^+$ + K/BxN-$CD40^{+/o}$)$B220^+$	$C\alpha^{o}$/BxN	**2/2**	7,7
($CD4^+$ + B6)$CD4^+$ + (BxN-$CD40^{o/o}$)$B220^+$	RAG^{o}/BxN	0/4	–
($CD4^+$ + B6)$CD4^+$ + (BxN-$CD40^{+/o}$)$B220^+$	RAG^{o}/BxN	**4/4**	13,13,13,13

Figure 3. Dependence of the K/BxN Model on CD40 Expression by B Cells

(A) Arthritis incidence in K/BxN littermates, heterozygous (phenotypically wild-type) or homozygous (CD40-deficient) for the CD40 knockout mutation. Arthritis was evaluated by inspection of the joints and measurement of ankle thickness, as in Figure 2A. The time of onset and severity of arthritis in K/BxN-$CD40^{+/o}$ mice were indistinguishable from those in regular K/BxN mice.

(B and C) B cells in KRN TCR tg mice and non-TCRtg littermates on the BxN background, with or without a CD40 deficiency. (B) Serum IgG levels as determined by ELISA; (C) numbers of splenic IgM+B220+ cells. Standardization as for Figure 2; each point or bar represents an individual mouse.

(D) T cell activation, assessed as surface expression of CD44 on gated $CD4^+$ lymph node cells.

(E) Cell transfers establishing the need for CD40 expression on B cells. Donor cells were: top experiment, total splenocytes; middle, 1×10^6 positively enriched $CD4^+$ T cells; bottom, 2×10^6 $CD4^+$ T cells plus 5×10^6 $B220^+$ B cells, both positively enriched.

A B Cell Product?

To explore the possibility that arthritis development in this model depends critically on a particular B cell product, we tried to provoke disease in B cell–deficient K/BxN-$\mu M^{o/o}$ mice by serum transfer. Severe joint swelling appeared in the animals injected with serum from

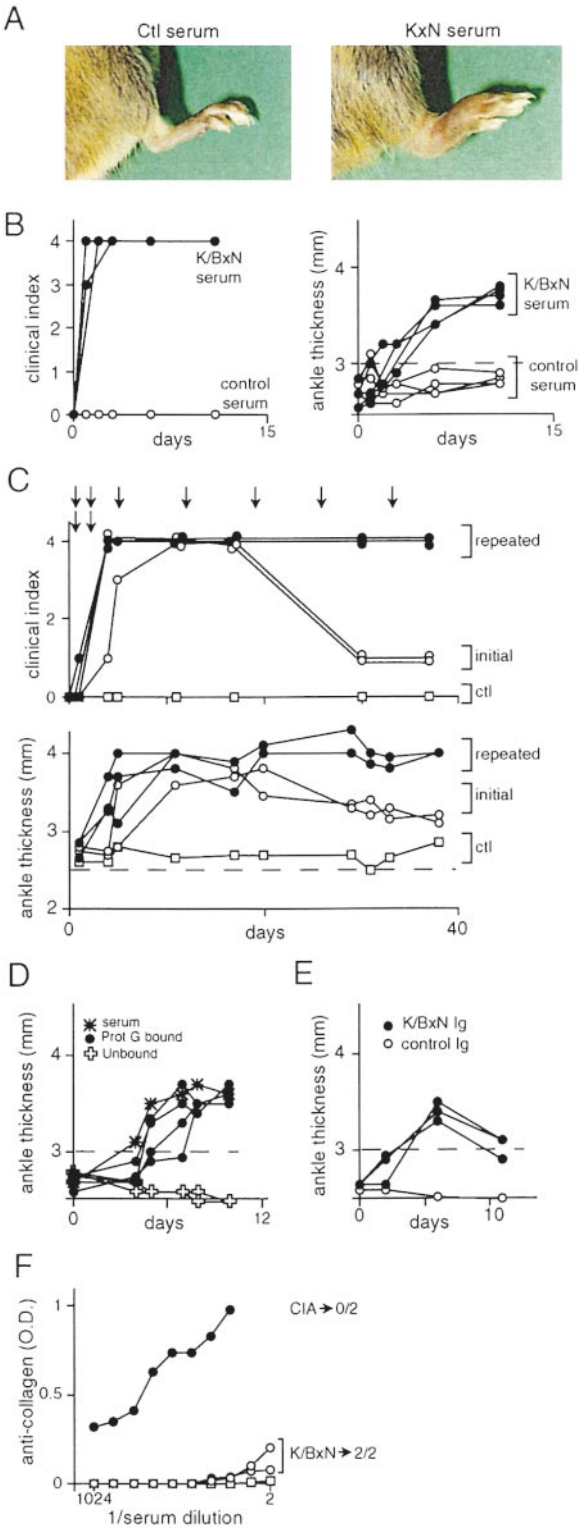


Figure 4. Arthritis Provoked by Transfer of IgG from Arthritic K/BxN Mice

(A) Hind limbs of 6-week-old K/BxN-μM$^{o/o}$ mice 7 days after injection of 2 × 250 μl serum from an arthritic K/BxN mouse or from a BxN control littermate.

(B) Clinical index and ankle thickness in several RAG$^{o/o}$ mice injected with serum from arthritic K/BxN mice or with control serum. The clinical sore is defined as: 0, normal; 1, doubt; 2, two paws affected; 3, three paws affected; and 4, all limbs affected.

arthritic K/BxN donors but not in those receiving serum from nonarthritic BxN controls (Figure 4A). Similar results were obtained upon injecting sera from arthritic and nonarthritic donors into lymphocyte-deficient RAG$^{o/o}$ hosts (see below). Disease could be induced with as little as 100 μl of K/BxN serum and showed up in all 32 of the B cell–deficient or lymphocyte-deficient hosts injected. Disease was provoked very rapidly, measured by either clinical score or ankle thickness, evident by the former already within 2 days after serum injection (Figure 4B).

The arthritis provoked by serum transfer presented all of the histological features of the disease in regular K/BxN mice, including invasion of inflammatory cells, hyperplasia of synoviocytes, pannus formation, and cartilage destruction (Figures 5A and 5B). However, the transfered disease was somewhat more heterogeneous, in that neighboring joints could differ greatly in the severity of their histological lesions—compare for example Figures 5C and 5D. This variability probably reflects the one striking difference between the serum-induced and spontaneous diseases—that is, the induced arthritis was transient. In mice that had received a single pair of injections, joint inflammation began to subside after about 15 days (Figure 4C); after 30 days, some of the joints appeared quite normal, even in the animals that had initially been fully arthritic. Histology of such joints (Figures 5E and 5F) revealed minimal leukocyte infiltration and synoviocyte hyperplasia and intact cartilage showing clear evidence of regeneration. Interestingly, disease transience could be overcome by repeated injections of serum from arthritic mice, as measured by either clinical score or ankle thickness (Figure 4C). That this was true even for RAG$^{o/o}$ recipients suggests that Ig instability is the explanation for the transient nature of the disease, rather than the absence of a critical input from disease-specific T or B cells.

To test whether the arthritogenic serum factor was an Ig, we fractionated serum from K/BxN mice into IgG and non-IgG components and tested their disease-inducing capacity. Only the IgG fraction was capable of provoking arthritis in RAG$^{o/o}$ hosts, and its potency was similar to that of whole serum (relative to the starting volume) (Figure 4D), as were the histological features of the disease it induced (data not shown). The arthritogenic property of K/BxN Igs could be due to either their elevated concentration, about 10-fold higher than in serum from normal mice, or to their particular specificities. However,

(C) Wane of arthritis, read out as clinical index or ankle thickness, after a short course of K/BxN serum administration (open circles), but persistence after repeated injection (black circles). Recipients were RAG$^{o/o}$ mice.

(D and E) Arthritogenic activity of the serum IgG fraction. (D) RAG$^{o/o}$ mice were injected with similar amounts (relative to starting volumes) of serum from arthritic K/BxN mice (stars), the flow-through (crosses) or the fraction eluted from a protein-G column (closed circles). (E) Twice the amount of IgG from non-TCR tg littermates was incapable of inducing arthritis.

(F) Inactivity of anti-cII Abs. The low level of anti-cII Abs detected at the lowest dilution of one of the K/BxN sera cannot account for the disease transfer, as a 30-fold excess of the CIA serum—matched relative to cII IgG—could not provoke disease in two recipients.

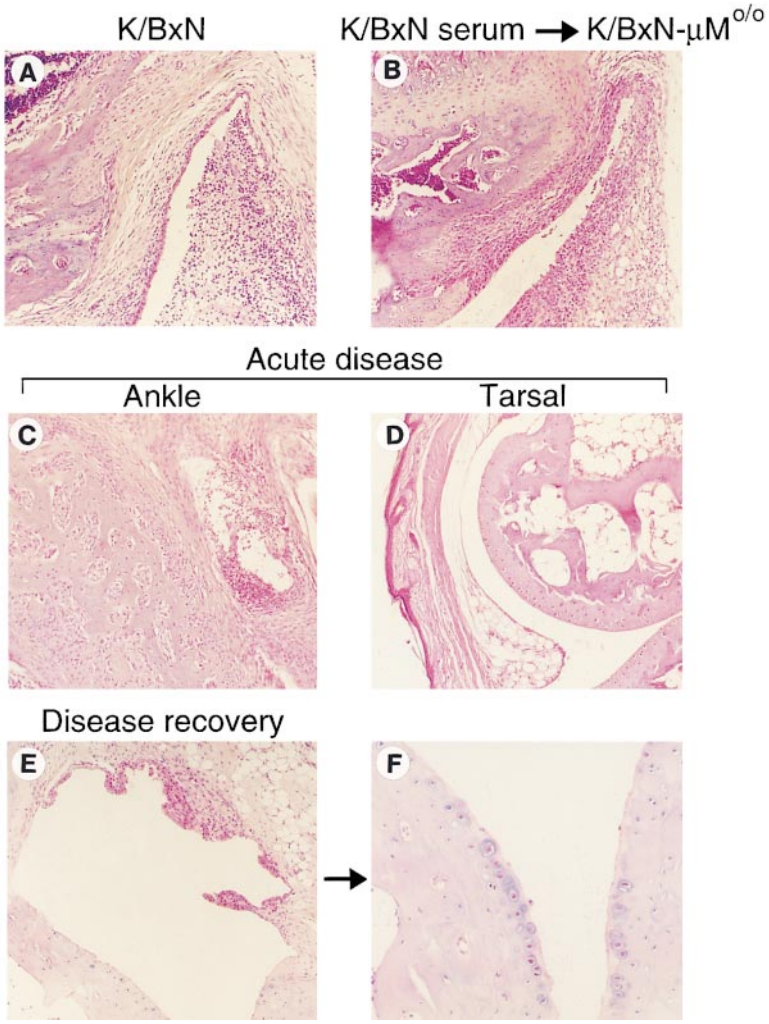


Figure 5. Histological Features of Ig-Induced Arthritis

(A and B) Knee sections from a typical K/BxN mouse (A) or a K/BxN-μM$^{o/o}$ mouse 10 days after two injections of 200 μl serum from an arthritic K/BxN animal (B). Note in both cases the thickened synovial lining, the massive underlying inflammatory infiltration, which extends over the cartilage and begins its destruction and the presence of polymorphonuclear cells in the articular cavity. Hematoxylin and eosin (H+E) staining, 10× objective.

(C and D) Heterogeneity of serum-induced disease. Two sections from the same RAG$^{o/o}$ mouse 8 days after transfer of serum from arthritic K/BxN mice. The ankle (C) presents a very agressive form of disease with the same histological picture as described above, while the tarsal joint (D) is intact. H+E staining, 10× objective.

(E and F) Recovery from joint histopathology after a single administration of serum. (E) Section of an ankle, 35 days after administration of serum from arthritic K/BxN mice. The articular cavity is now clear of neutrophils; the synovial lining is still slightly thickened, but the underlying inflammatory infiltrate has almost completely subsided—save for a small area (star). (F) The articular cartilage appears to be regenerating, showing a rough but free surface, and images evoking dividing chondrocytes (juxtaposed chondrocyte lacunae, two cells per lacuna; arrows; 40× objective).

when we directly compared disease induction with Igs from K/BxN mice versus double the amount from control animals, we found the former and never the latter to be effective (Figure 4E).

Type II collagen (cII) is a popular candidate autoantigen for rheumatoid arthritis. It seemed plausible, then, that the arthritogenic Igs might be anti-cII Abs, and so we assayed sera from arthritic K/BxN mice for reactivity to bovine or rat type II collagen, the latter being highly homologous to its mouse counterpart. We did detect marginal titers of anti-cII Abs in sera from K/BxN animals, about 10^3-fold lower than in sera from positive-control mice with collagen-induced arthritis (CIA) and not significantly higher than in sera from negative-control BxN mice when corrected for Ig concentration. Nonetheless, we directly tested the pathological relevance. In parallel, RAG$^{o/o}$ mice were injected with serum from arthritic K/BxN donors or from donors with CIA, both sera having been titred for anti-cII Abs. Only in the former case was arthritis provoked in the recipients, even though in the latter over 30-fold more anti-cII Abs were transferred (Figure 4F).

Thus, a B cell product is key to arthritis development in K/BxN mice. The arthritogenic product is one or more Igs of a particular specificity, which is not anti-cII. The product provokes arthritis reproducibly, amazingly rapidly—though transiently—and in the complete absence of lymphocytes.

A Secondary Role for T Cells

Injection of serum (or Igs) from arthritic K/BxN mice into RAG$^{o/o}$ animals precipitated arthritis indicating that, once produced, the arthritogenic Igs do not absolutely require KRN T cells, other T or B cells, or A^{g7} molecules to provoke disease (e.g., Figures 4B–4F; Figures 5C and 5D). Yet, we noted that a more severe arthritis—measured by clinical score, ankle thickness, or histology—usually developed after serum transfer into K/BxN-μM$^{o/o}$ animals. To better understand this phenomenon, we directly compared disease development in different types of recipients, all on the BxN genetic background; representative results from one such comparison of several are presented in Figure 6. K/BxN-μM$^{o/o}$ recipients almost always developed the most severe arthritis. In particular, compared with BxN-μM$^{o/o}$ animals, their disease was more persistent (left panel), more inflammatory (right panel), and more aggressive histologically (data not shown), indicating that KRN T cells still have an influence even in the late stages of disease. On the other hand,

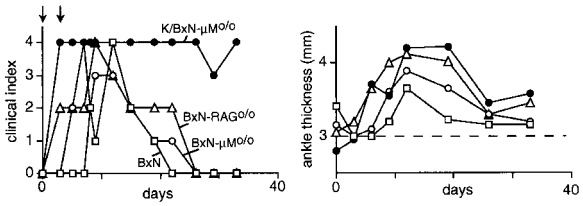


Figure 6. Secondary Role for T Cells in the Ig-Induced Disease

Arthritis was induced by transfer of serum from arthritic K/BxN mice into different recipients, all on the BxN genetic background: non-TCR tg wild-type, non-TCR tg B cell–deficient (μM$^{o/o}$), non-TCR tg lymphocyte-deficient (RAG$^{o/o}$) or KRN TCR tg B cell–deficient (K/BxN-μM$^{o/o}$).

BxN-RAG$^{o/o}$ recipients routinely developed a more severe arthritis than either BxN-μM$^{o/o}$ or BxN mice, and BxN-μM$^{o/o}$ recipients a stronger disease than BxN animals, suggesting that non-KRN T cells and other B cells or Igs might have an inhibitory effect.

Discussion

Taking all of the data into account, we favor the following scenario. (1) T cells expressing the KRN receptor recognize a self-peptide in the context of A^{g7} molecules displayed on peripheral APCs and become activated. The critical APCs are probably dendritic cells and/or macrophages as this process does not require display of A^{g7} on B cells nor on synovial fibroblasts or chondrocytes (Figure 2; Table 1; data not shown). (2) Activated KRN T cells interact with B cells through TCR:A^{g7} and CD40L:CD40 engagements, promoting further T cell stimulation, polyclonal B cell activation, and T help-dependent Ig overproduction (Table 1; Figure 3). (3) Among the Igs produced, some bind to joint constitutents and precipitate arthritis, this process depending critically on particular Ig specificities (Figures 1, 4, and 5; data not shown). Ab/Ag complexes could be pathogenic via several mechanisms: direct perturbation of joint structures; activation of the complement cascade, resulting in cell lysis and production of inflammatory mediators; and engagement of Fc receptors, inducing activation of local cells. (4) While arthritogenic Igs can precipitate arthritis in the absence of T cells expressing the KRN receptor, indeed without any lymphocytes present at all, KRN T cells can intensify disease (Figure 6).

This proposed scenario raises three key questions. First, what are the specificities of the arthritogenic Igs? Immediate suspects were RF and anti-cII Abs. RF has been considered a hallmark of RA in humans, but it occurs in only about 70% of patients and its relevance to disease has never been established (Vaughan, 1993). Anti-cII Abs are also found in RA patients, though there seems to be little correlation with disease parameters (Claque and Moore, 1984; Rudolphi et al., 1997); more suggestively, injection of homologous or heterologous type II collagen into rodents elicits anti-cII T cell and Ab reactivity and provokes arthritis (Trentham and Dynesius-Trentham, 1995). However, we have ruled out both IgM isotype RF and anti-cII as the arthritogenic Abs in K/BxN serum (Kouskoff et al., 1996; Figure 4F). This leaves us to apply more systematic methods of characterizing Ab reactivities, which are in progress but which will be laborious given the necessity of proving the pathogenic relevance of any antigen so identified.

Second, how do these particular Ig specificities come to be produced? T cells expressing the KRN receptor can recognize essentially any cell displaying A^{g7} molecules (Kouskoff et al., 1996; data not shown). Thus, KRN T cells should be able to provide help to the entire repertoire of B cells, as they all display MHC class II molecules, as well as CD40. However, polyclonal B cell activation is too simple an explanation for disease in the K/BxN model as other polyclonal activators do not elicit the aggressive and isolated arthritis characteristic of these mice. There has to be selectivity to the stimulation, at least at the level of Ig production. Which B cells secrete Igs in K/BxN mice probably depends on a number of factors. The first is that their surface Ig receptors must be engaged, since isolated TCR:MHC and CD40L:CD40 interactions are not sufficient to push B cells to Ig production in vivo (Cooke et al., 1994). This implies that only those cells with specificities directed at self-antigens or coincidentally expressed foreign antigens should become Ig secretors. An important factor for the potentially self-reactive B cells is whether and how they are tolerized. For example, the noncognate help provided by KRN T cells may interfere with the usual deletion or inactivation of newly generated autoreactive B cells (Cooke et al., 1994; Fulcher et al., 1996), may "awaken" a fraction of cells that had been rendered anergic, in particular those recognizing polymeric or membrane-bound Ags (Cooke et al., 1994), or may activate those autoreactive B cells that are not normally tolerized themselves but remain innocuous because their T cell partners responsible for providing cognate help have been deleted or inactivated (Adelstein et al., 1991; Hannum et al., 1996). On the other hand, help from KRN T cells should not elicit Ig secretion from the fraction of anergic B cells that dies in response to subsequent encounter with Ag in the presence of T cell help (Cooke et al., 1994; Rathmell et al., 1995). Finally, a factor recently shown to influence autoantibody production is the cytokine milieu: generation of some anti-self-specificities in pristane-injected mice is regulated by IL-6, while production of others is not (Richards et al., 1998). This complexity of factors probably underlies the narrow manifestation of autoimmunity in K/BxN mice; the critical task now is to identify which "slice" of B cell tolerance is broken by KRN T cell–mediated help, and why.

Third, can the role of B cells in the K/BxN model be related to other arthritis models and to human RA patients? At first glance, an analogy might be made with CIA: both T and B cells are required for full disease development (Taylor et al., 1995); serum anti-cII Abs appear to have arthritogenic properties like the unidentified (non-anti-cII) Igs from K/BxN mice (Stuart and Dixon, 1983; Stuart and Dixon, 1982). However, serum from animals with the cII-induced disease is quantitatively and qualitatively less potent: it requires larger amounts (about 10 times more than K/BxN serum), provokes an evanescent disease (resolved in several days), induces lesions more characteristic of the early stages of arthritis (just synovial inflammation in the absence

of significant cartilage and bone destruction), and only precipitates disease in animals harboring T lymphocytes (Kadowaki et al., 1994; Taylor et al., 1995). It is also important to keep in mind that CIA and the disease in K/BxN mice are mechanistically different, the former induced by a classical Ab response centered on a single Ag, the latter by broad T and B cell stimulation. In fact, it has recently been reported that injection of cII can induce preliminary arthritic lesions even in the absence of lymphocytes, raising the complication that cII may have nonantigenic arthritogenic properties (Plows et al., 1999). Perhaps more relevant to the KRN model are the pathogenesis of adjuvant-induced or reactive arthritis. It is conceivable that the strong immune stimulation in these diseases activates T cells reactive to self-MHC complexes, and arthritis unfolds essentially as in the K/BxN model.

The relative importance of different leukocyte subsets and their products in human RA remains the subject of considerable debate. An early emphasis on B cells and their products (Zvaifler, 1973) gave way to a "T cell-centric" view (Panayi et al., 1992), which reigned for a number of years but has recently been supplanted by an emphasis on monocytes, dendritic cells, and the cytokines they produce (Firestein and Zvaifler, 1990; Feldmann et al., 1996a; Thomas and Lipsky, 1996; Burmester et al., 1997). A role for B cells in human RA was suggested by the routine detection of autoantibodies, in particular RF (Vaughan, 1993), also sometimes anti-cII and other anti-joint reactivities (Claque and Moore, 1984; Rudolphi et al., 1997), and by the demonstration of immune complexes suggestively localized in joint lesions (Zvaifler, 1973). The emphasis on T cells derived from observations of a strong association of RA with particular MHC alleles, the detection of activated T cells in the joint, the demonstration of T cell reactivity to joint constituents (including cII), and analogies with animal models of arthritis (Firestein and Zvaifler, 1990; Fox, 1997). However, any critical T cell function should operate in the early stages of disease because the T cells in established lesions show little proliferative activity and secrete few cytokines and because anti-T cell reagents seem to have little influence on ongoing arthritis (Firestein and Zvaifler, 1990; Fox, 1997). A central role for macrophages or dendritic cells has been argued primarily on the basis of monokine imbalances in RA patients and of strong disease inhibition after treatment with anti-monokine reagents (e.g., anti-TNF-α and anti-IL-1) (Feldmann et al., 1996a).

In analogy with the K/BxN model, we propose a synthesis of the data on human RA according to the following scenario: T cell activation, perhaps pervasive, by an unknown provocateur; T-B cell collaboration resulting in broad activation of B cells and autoantibody production by a narrow "slice" of the repertoire; and Ab-precipitated joint lesions, amplified by locally produced monokines, with little input from T cells. This scenario provides a framework for experimental dissection of RA pathogenesis and for consideration of therapeutic strategies.

Experimental Procedures

Mice and Cytofluorimetry

KRN TCR tg mice have been described (Kouskoff et al., 1996). They were maintained on the C57Bl/6 (B) background and as such are asymptomatic. Crossing these K/B animals with NOD/Lt (N) mice generated arthritic K/BxN offspring. KRN TCR tg mice carrying the μM^o mutation, resulting in a lack of B cells when homozygous, have been described (Kouskoff et al., 1996). The TCR-α ($C\alpha^o$; Philpott et al., 1992) and RAG-1 (RAG^o; Mombaerts et al., 1992) knockout mutations were each crossed onto both the B and N backgrounds, and in each case the two lines intercrossed to generate homozygous mutant F1 animals. In some serum transfer experiments, $RAG^{o/o}$ mice on the standard Bx129 background were used. The $CD40^o$ knockout mutation (Kawabe et al., 1994) was backcrossed in parallel to K/B and N mice, and the second-generation offspring were intercrossed to generate K/BxN-$CD40^{o/o}$ animals or K/BxN-$CD40^{+/o}$ control littermates. Similarly, the Ig knockin mutations, B1–8i and 3–83ki, were backcrossed together to K/B and N mice in parallel; the second-generation offspring were then intercrossed to generate transgene-positive F1 animals with varying light and heavy chain genotypes; some of these were further intercrossed to generate additional animals of desired genotypes. In all backcrosses to the N background, only breeders homozygous for the g7 haplotype at the MHC were used. Typing details for all lines can be found as supplemental data at http://www.immunity.com/cgi/content/full/10/4/451/DC1. Arthritis was evaluated as described (Kouskoff et al., 1996).

Flow cytometric analysis has been described; references to monoclonal antibodies (mAbs) can be found in Cosgrove et al. (1991) and Kouskoff et al. (1996).

Cell Transfers

For splenocyte transfers, cell suspensions were made from pooled spleens, depleted of erythrocytes in 0.83% NH_4Cl for 5 min, and adjusted to 10^8/ml in RPMI. Cells (2×10^7) were injected i.v. into unirradiated recipients. In some cases, T cells were depleted by complement killing (LoTox-M, Cederlane) after incubation with a cocktail of mAbs (anti-Thy1, -CD4, and -CD8: J1-J, RL-172, and 31M). B cell–depleted splenocytes were obtained by magnetic sorting (anti-rat Ig Dynabeads, Dynal) after incubation with anti-B220 and -MHC class II mAbs (RA3-6B2, M5/114). In some cases, $CD4^+$ and $B220^+$ splenocytes were positively enriched with directly conjugated MACS beads (Miltenyi Biotech). For details of cell purifications, see http://www.immunity.com/cgi/content/full/10/4/451/DC1. To reconstitute B cells, BxN-$Rag^{o/o}$ recipients were lightly irradiated (450 R) and injected i.v. with 5×10^6 bone marrow cells, positively purified on B220-conjugated MACS beads.

For the CFSE experiments, single-cell suspensions from pooled spleens of 6- to 10-week-old K/B mice were depleted of erythrocytes in 0.83% NH_4Cl for 1 min, adjusted to 10^7/ml in PBS, and incubated for 10 min in the presence of 2.5 μM CFSE (Molecular Probes) prepared as a 5 mM stock solution in DMSO. After incubation, the cells were washed twice with RPMI supplemented with 5% FCS and adjusted to 1.5×10^8/ml. In each experiment, 300 μl (4.5×10^7 cells) were injected i.v. into adult recipients. At various times after injection, axillary lymph nodes were removed and analyzed for the presence of CFSE-labeled cells.

Serum Transfers

Sera from paired lots of K/BxN mice or control BxN littermates (40 to 60 days of age) were pooled and injected intraperitonealy, in 150–250 μl total volume (adjusted with PBS if necessary). In most cases, recipients got two injections at a 2 day interval.

Ig Purification

Pooled sera from arthritic K/BxN or control BxN mice were cleared by ultracentrifugation (30 min at 58,000 rpm, SW60). Serum (5 ml) from arthritic mice or 12 ml from control mice, diluted to 100 ml with 50 mM Na phosphate (pH 7.5), was loaded onto a protein G column (15 ml gel volume, Protein-G Sepharose, Pharmacia). The flow-through was saved, and bound IgG was eluted in 0.1 M glycine (pH 2.8) and quickly neutralized with 1/6 volume of 1 M Tris HCl (pH 8.5). The flow-through and pooled eluted fractions were concentrated and switched to PBS buffer by centrifugation (Centricon-30, Amicon).

Measurement of Total and cII-Specific IgG

Serum IgG levels were measured by ELISA as described, using reagents from Jackson Immunoresearch (Cosgrove et al., 1991); optical density readings were normalized relative to standard curves obtained with sera from non-TCRtg littermates. Levels of serum anti-cII IgG were also estimated by ELISA, on plates coated with type II collagen of bovine (Sigma C-7806) or rat origin (a gift from Dr R. Holmdhal, Lund, Sweden). Anti-cII titers were compared with a standard serum from DBA/1 mice with CIA (a gift from Dr. I. Campbell, Melbourne, Australia).

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