

THE ARTHRITOGENIC T CELL RECEPTOR AND ITS LIGAND IN A MODEL OF SPONTANEOUS ARTHRITIS

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Objective. Spontaneous arthritis in the KRN transgenic mouse model is due to the autoreactivity of the transgenic T cell receptor (TCR) against A^{g7} major histocompatibility complex (MHC) molecules, which leads to strong but incomplete clonal deletion. We sought to determine whether other stimuli triggering this receptor might provoke arthritis, whether the apparently systemic reactivity might have some joint-preferential component explaining the paradoxical arthritic phenotype, and whether the transgenic receptor was the only one required or whether other TCRs might be ferried along in a leaky tolerance process.

Methods. Crosses and radiation chimeras involving a panel of transgenic and knockout mouse lines were used. The reactivity of the KRN TCR was tested in carboxyfluorescein diacetate succinimidyl ester-transfer experiments and in crosses with transgenic or inbred mice expressing other molecules that stimulate the KRN receptor (the mls-1^a superantigen, the A α^{k69} A β^k mutant MHC molecule). The arthritogenic capacity of T cells expressing only the KRN TCR was tested by crossing to recombination-activating gene-

knockout mice, and constructing bone marrow chimeras with precursors to these strictly monoclonal T cells.

Results. The data show that the KRN TCR itself is the only receptor needed. It needs to be triggered by the A^{g7} molecule loaded with self-peptides in order to provoke arthritis, but there is no indication of preferential presentation of joint-derived peptides.

Conclusion. Arthritis can be generated by systemic recognition of self-MHC-peptide complexes by autoreactive T cells. This triggers B lymphocytes to produce arthritogenic antibodies, without the involvement of joint-specific T cell targets.

The hallmark of rheumatoid arthritis (RA) is inflammation and, ultimately, destruction of the joints (for review, see refs. 1 and 2). RA is thought to be autoimmune in nature, involving both T cells and B cells (1,2). Given the striking localization of disease to the joints, it would be logical to suppose that it is initiated by an antigen that is solely, or at least preferentially, expressed in joints. Yet, whether human RA is initiated by a joint-specific antigen has been a contentious issue for years.

The view that it is initiated by a joint-specific antigen has been espoused by many ever since injection of type II collagen into rodents was found to provoke arthritis (3), and some have extrapolated that it is actually collagen that initiates the disease in humans. However, there is no compelling evidence that collagen or, for that matter, any joint-specific antigen is a central player in RA. In particular, there is no evidence of pathogenic antigen-driven T cell clones (for review, see refs. 1 and 4–6). Alternatively, RA could be initiated by an exogenous antigen, via systemic stimulation by an antigen of endogenous origin, or by localized infection or stress.

We have recently described a murine model of RA in which disease is confined to the joints, but is initiated by an antigen expressed in an apparently sys-

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temic manner (7). K/BxN T cell receptor (TCR) transgenic mice spontaneously develop an aggressive arthritis with most of the characteristics of human RA. As in humans, the disease in K/BxN mice is symmetric and has a proximal-to-distal gradient of severity, with little or no involvement of the spine or sacroiliac joints. The murine disorder shares all of the major histologic features of the human disorder: leukocyte invasion, synovocyte proliferation, pannus formation, cartilage and bone destruction, and anarchic remodeling of joint structures. Finally, K/BxN mice, like humans with RA, show several immunologic abnormalities, in particular, polyclonal B cell stimulation, which is manifested as an increase in B cell numbers; hypergammaglobulinemia; and autoantibody production, although not IgM anti-IgG immunoglobulins (rheumatoid factor).

The K/BxN model was generated serendipitously when KRN TCR transgenic mice on the C57Bl/6 background were crossed with mice of the NOD strain. The key to disease lies in the autoreactive specificity of the transgene-encoded TCR. Although the KRN receptor was initially identified as recognizing a peptide of bovine ribonuclease in the context of class II major histocompatibility complex (MHC) A^k molecules, we eventually determined that it shows cross-reactive recognition of NOD-derived A^{g7} molecules loaded with an unknown self-peptide or set of self-peptides. As might have been expected (8), this situation of systemic self-reactivity results in multiple levels of tolerance induction in the T cell compartment (thymocyte clonal deletion, diminished levels of TCR clonotype due to selection of cells exhibiting incomplete allelic exclusion, clonal inactivation of peripheral cells). However, none of these is completely effective; a low level of KRN T cell reactivity persists and eventually provokes arthritis.

A major conclusion of our initial analyses was that disease in K/BxN mice—and by extension, perhaps also in humans with RA—has its root in an incompletely contained T cell reactivity to systemically expressed self-MHC-peptide complexes. However, 3 important questions were often raised. First, is the KRN TCR itself responsible for arthritis development in K/BxN mice? As mentioned above, and detailed previously (7), we noted that cells that had escaped clonal deletion in the thymus emerged into the periphery with diminished levels of KRN clonotype on their surface. This was due to coexpression of endogenously encoded TCR chains consequent to incomplete allelic exclusion at the TCR α or TCR β locus, or both. It could be that these “second” receptors include the truly arthritogenic specificities. Cells expressing them might be inefficiently tolerized

due to their reduced level of expression, and mobilized through stimulation via the KRN receptor. This scenario reflects previous hypotheses concerning the potential role of “dual” TCRs in autoimmune disease (9,10).

Second, is the KRN TCR somehow inherently arthritogenic, in that T cells expressing it and stimulated by any of its MHC-peptide ligands will engender arthritis? A related question is whether any “allogeneic” TCR/MHC-antigen combination will promote arthritis, an issue raised by the observation of arthritic syndromes in some animals undergoing a graft-versus-host reaction (11). We already knew that the A^{g7} target brought by another genetic background—the B6.H2g7 congenic strain (7)—was as effective in triggering arthritis in this model as that derived from NOD mice. Would non-A^{g7}-related targets also be effective?

Third, is the arthritis in K/BxN mice truly due to systemic self-reactivity, or is there some joint specificity to the antigen, if only quantitative? The KRN TCR can be stimulated by A^{g7}-peptide complexes present in the spleen and, by inference, from the deletion phenotype, also in the thymus (7). Yet, it could be that the relevant complexes are expressed more abundantly in the synovial tissue, thereby accounting for the paradoxical joint specificity of a disease induced by systemic reactivity.

We have addressed these 3 questions in the present study. The answers we found provide important restrictions on the nature of the TCR-autoantigen encounter that provokes arthritis in the K/BxN model, and thus provide clues to possible modes of pathogenesis of RA.

MATERIALS AND METHODS

Mice. The KRN transgene (encoding TCR α/β chains) has been described (7). It was maintained by serial crossing from heterozygotes against the C57Bl/6 background. This transgene is present in several crosses used in the present study, and it is abbreviated “K” (e.g., K/B denotes KRN transgenic mice on the C57Bl/6 background).

The mice used in this study are listed in Table 1. They included normal inbred strains of hybrid mice (C57Bl/6 [B], NOD/Lt [N], DBA/2J [DBA], [C57Bl/6 \times NOD]F₁ [BxN]); the class II MHC transgenic line A β 42, which expresses the wild-type A β^k molecule in all normal class II MHC-positive cells (12); and the K69 transgenic line (VK) (unpublished), which expresses in all normally class II-positive cells the variant A α^{k69} molecule, which differs from A α^k by a single T \rightarrow A replacement at position 69 (13). Recombination-activating gene 1 (RAG-1)-knockout mutant mice (R⁰) (14) were maintained on the (129 \times B6)F₂ background. They were crossed with K/B, and the progeny were crossed with R⁰ to generate homozygous RAG-negative transgene-positive mice (K/B/R⁰). The TCR α

Table 1. Mice used in this study*

Acronym	Description	Feature of interest
B	Normal C57Bl/6J inbred mouse	–
BxN	Normal (C57Bl/6J × NOD/Lt)F ₁ hybrid	–
Aβ42AαK69	Class II MHC transgenic mice (Aα ^{k69} Aβ ^k)	Mutant class II MHC
Cα ⁰ /BxN	TCRα knockout mutation on the BxN background	T cell deficient
K/B	KRN+ transgenic on the C57Bl/6 background	Tg+
K/BxN	KRN+ transgenic on the BxN background	Tg+, arthritic
K/BxDBA	KRN+ transgenic on the C57Bl/6 × DBA/2 background	Tg+, mls-1 ^a
K/B/R ⁰	KRN+ transgenic on the BxN background, deficient in RAG-1 genes	Tg+, no other TCRs

* MHC = major histocompatibility complex; TCR = T cell receptor; Tg = transgene; RAG-1 = recombination-activating gene 1.

knockout mutation (Cα⁰) (15) was crossed onto both the B and N backgrounds, and the 2 lines were intercrossed to generate homozygous mutant F₁ (Cα⁰/BxN) animals.

All the mice were kept at the Institut de Génétique et de Biologie Moléculaire et Cellulaire animal facility in Strasbourg, following European Economic Community guidelines (Agrément Ministère de l'Agriculture). Typing details for all lines can be found at our Internet site (<http://biblio-igbmc.u-strasbg.fr/cbdlm>). Arthritis was evaluated as described previously (7,16). It was scored as a clinical index (16) or by caliper measurement of ankle thickness.

Antibodies and flow cytometry. The monoclonal antibodies (mAb) used for flow cytometric analysis of T populations in KRN transgenic mice have been described elsewhere (7).

Adoptive cell transfers. For bone marrow transfers, T cells were eliminated by complement killing (Low-Tox-M Rabbit; Cedarlane, Hornby, Ontario, Canada) after incubation with a cocktail of mAb (anti-Thy1, anti-CD4, and anti-CD8: J1J, RL-172, 31M [17–19]). Cells (2–4 × 10⁶) were adjusted to 200 μl in RPMI and injected intravenously into Cα⁰/BxN recipients 24 hours after lethal irradiation (800 rads).

For carboxyfluorescein diacetate succinimidyl ester (CFSE) experiments, single-cell suspensions from pooled spleens of adult K/B or B mice were depleted of erythrocytes in 0.83% NH₄Cl for 1 minute, adjusted to 10⁷ cells/ml in phosphate buffered saline, and incubated for 10 minutes at 37°C in the presence of 2.5 μM CFSE (Molecular Probes, Eugene, OR) diluted from a 5-mM stock solution of DMSO. After incubation, the cells were washed twice with Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and adjusted to 10⁸ cells/ml. In each experiment, 300 μl (3 × 10⁷ cells) was injected intravenously into adult recipients. At various times after injection, lymphoid organs were removed and analyzed by flow cytometry for the presence of CFSE-labeled cells.

RESULTS

The KRN TCR responsibility for arthritis in K/BxN mice. In the K/BxN arthritis model, two genetic elements must be brought together in the same mouse for disease to appear: 1) the KRN TCR transgenes, normally carried on the C57Bl/6 background, where they

are innocuous; and 2) the NOD-derived MHC, which provides the stimulating A^g7 molecule. Since combining these two elements engenders an autoreactive state, it would seem logical that they are the root of any autoimmune disease that develops. However, T cells expressing the KRN TCR appear to be poorly reactive when stimulated through this receptor (7), and essentially all of them carry an additional receptor containing an endogenously encoded TCRα chain, TCRβ chain, or both. Since it has been suggested that such “dual-receptor” cells may be prone to autoimmunity (9,10), we wondered whether TCRs other than that encoded by the KRN transgenes are necessary for arthritis to appear in K/BxN mice.

First, we generated K/BxN mice that were incapable of rearranging endogenous TCR loci because of a null mutation at the RAG-1 locus (14). No disease was observed in these animals (results not shown). However, this result is not informative because B cells are absent from these animals, and we know that B cells are absolutely required for arthritis development in K/BxN mice (7).

To circumvent this difficulty, we developed a system that would allow us to monitor the behavior of monoclonal T cells expressing only the KRN TCR in the context of a normal B cell pool. This was achieved by adapting a previously described transfer system: transfer of bone marrow cells from a K/B donor into irradiated BxN hosts gives rise to arthritis in the recipients (16).

In the experiments illustrated in Figure 1 (schematized in part A), the bone marrow cell inoculate was either from K/B mice or from their RAG-deficient counterparts (K/B/R⁰); the recipients were Cα⁰/BxN animals, which lack conventional T cells due to a mutation inactivating the TCRα locus but which possess a healthy B cell compartment (15). That the T cells in the

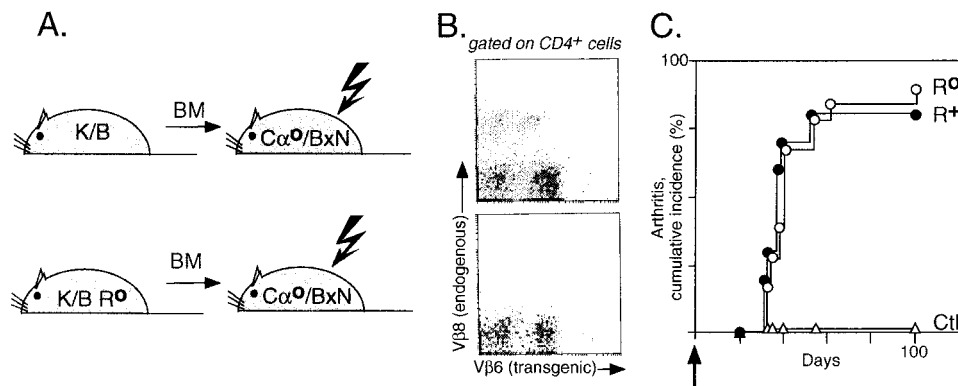


Figure 1. Induction of arthritis by the KRN T cell receptor (TCR) itself. **A**, Schematic of the bone marrow (BM) transfer experiments. BM from mice with the KRN transgene, either with wild-type recombination-activating gene (RAG) genes (K/B mice; **top**) or RAG deficient (K/B R^0 ; **bottom**), was injected intravenously into lethally irradiated (800 rads; **jagged arrow**) Ca^0/BxN recipients. The latter mice possess the H-2^{g7} haplotype at the MHC, which stimulates the KRN TCR, have a normal population of B lymphocytes, but are lacking T cells because of a knockout mutation in the TCR α locus. **B**, Phenotypic verification of the recipients 50 days after reconstitution. Gated $CD4^+$ lymph node cells were stained for transgene-encoded TCR V $\beta 6$ and for the V $\beta 8$ chain encoded by endogenous (nontransgenic) TCR genes. **Top**, Chimera reconstituted with K/B bone marrow; **bottom**, chimera reconstituted with K/B R^0 bone marrow. **C**, Cumulative incidence of arthritis in K/B bone marrow chimera (R^+) and K/B R^0 chimera (R^0). Controls (Ctl) were reconstituted with KRN-negative B bone marrow.

experimental animals were properly reconstituted was verified by flow cytometry.

In Ca^0/BxN mice in which the lymphocyte compartments had been reconstituted with bone marrow cells from K/B donors, a sizeable compartment of $CD4^+$ T cells was found in the spleen and lymph nodes 40 days after transfer. As expected from previous studies (7), a number of these cells expressed TCR β chains other than the V $\beta 6$ chain encoded by the KRN transgene, which was manifested as a large population of V $\beta 8^+$ cells (Figure 1B, top). Note that a high proportion of V $\beta 8^+$ cells also expressed the transgene-encoded V $\beta 6$ chain. In contrast, when the bone marrow came from RAG-deficient donors, only the transgene-encoded V $\beta 6$ chain could be detected (Figure 1B, bottom). The onset of arthritis, however, was very similar in the 2 types of chimeric mice, most becoming arthritic between 40 days and 60 days after transfer (Figure 1C).

These results indicate that the expression of other TCR chains is not required for arthritis to occur in K/BxN mice. Thus, the joint-specific autoimmunity can be attributed to the KRN receptor itself.

KRN TCR recognition of the A^{g7}-peptide complex to engender arthritis. We wondered whether the tissue tropism of the K/BxN disease might somehow be inherent to the KRN TCR, independent of the particular antigen that stimulates the T cell expressing it. To

address this question, we made use of 2 additional ligands known to activate T cells displaying the KRN receptor. The first was the endogenous superantigen mls-1^a. This molecule, encoded in the 3' open-reading frame of the *mtv7* endogenous retrovirus, strongly stimulates broad populations of T cells by binding to TCRs that utilize particular V β regions (20). Among these is V $\beta 6$, the variable segment expressed by the KRN TCR, and so the mls-1^a molecule can activate KRN T cells.

The second ligand was a mutant class II MHC molecule, $A\alpha^{k69}A\beta^k$. This is a closely related variant of the normal $A\alpha^kA\beta^k$ heterodimer, differing by a single amino acid replacement at position 69 of the $A\alpha$ chain. In studies on alanine-scan mutants of the $A\alpha^k$ molecule, we found that this variant triggers the KRN TCR in vitro in the absence of added antigen (13). In addition, it can stimulate KRN T cells when expressed on antigen-presenting cells (APC) of transgenic mice (Mangialaio S: unpublished observations).

We generated mice expressing one or the other of these stimulatory ligands together with the KRN receptor, and sought to determine whether they would provoke arthritis, as does the A^{g7} molecule in K/BxN animals. The mls-1^a molecule was introduced simply by crossing K/B mice with DBA/2 animals, which carry the *mtv7* locus. The T cell compartment of the transgenic progeny was analyzed by flow cytometry (Figure 2A).

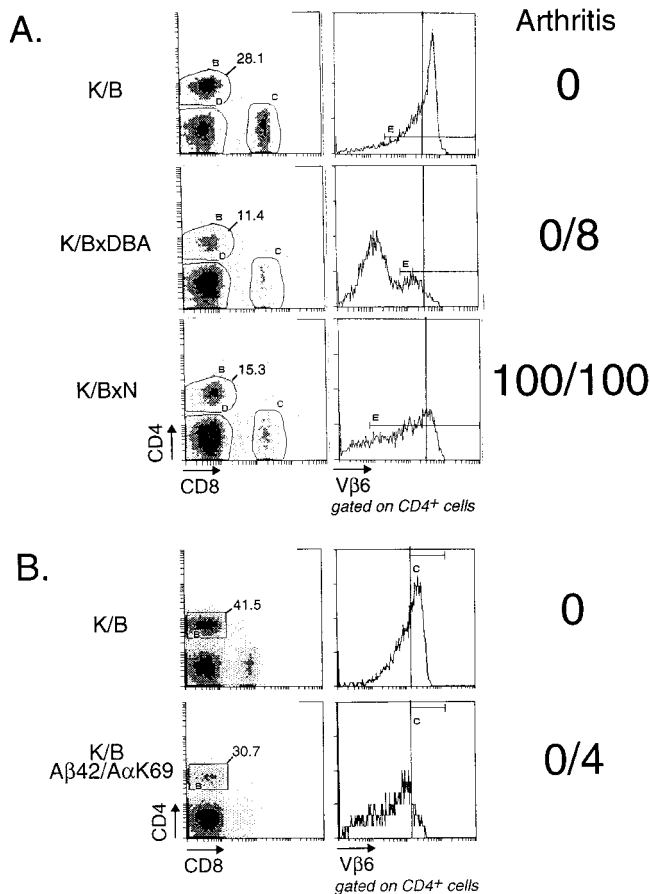


Figure 2. Demonstration that the KRN T cell receptor (TCR) requires a particular target to be arthritogenic. The KRN transgene was crossed onto several different genetic backgrounds (see Table 1). Different experimental groups are shown. **A**, The TCR trigger was the *mls-1^a* superantigen from the DBA/2 strain (K/BxDBA), compared with the usual *A^{g7}* molecule in the K/BxN mouse and with the neutral background of the K/B mouse. **B**, The TCR trigger was the mutant *Aα^{k69}Aβ^k* class II MHC molecule (K/B-Aβ42Aαk69 mouse), compared with the neutral B background (K/B mouse). Splenocytes (A) or lymph node cells (B) were stained with anti-CD4 and anti-CD8 monoclonal antibodies (left panels). Values are the percentages of CD4+ cells. Gated CD4+ cells were analyzed for transgene-encoded Vβ6 (right panels). Vertical lines show the cells expressing normal levels of Vβ6; note the loss of these cells, to different extents, on the triggering backgrounds. The numbers of arthritic mice in these crosses are shown to the right, with followup to 40 days minimum for the K/B-Aβ42Aαk69 mice and to 60 days for the K/BxDBA mice. Small letters and horizontal lines with bars represent gates.

When compared with parent K/B mice (Figure 2A, top), the K/BxDBA mice (Figure 2A, middle) showed a clear effect of the *mls-1^a* superantigen: there were reduced CD4+ cells, with a strongly shifted pattern of the transgene-encoded Vβ6 chain; and very few cells still displayed the normal high level of Vβ6. The reduc-

tion in Vβ6 expression was more extensive than that seen in K/BxN mice (Figure 2A, bottom), which is consistent with the fact that *mls-1^a* is a very potent activator of the KRN TCR, more so than *A^{g7}*. Thus, *mls-1^a* induces a stronger negative selection than the *A^{g7}*-peptide ligand. No arthritis was observed in any of the K/BxDBA mice, however (see also ref. 7). In fact, this strong negative selection was dominant: in second-generation progeny carrying both the *A^{g7}* and *mls-1^a* molecules, disease was prevented (results not shown).

The second cross introduced transgenes expressing the *Aβ^k* chain (from the Aβ42 transgene [12]) and the *Aα^{k69}* chain (from the K69 transgene). In the triple-positive mice (i.e., those carrying the KRN, Aβ42, and K69 transgenes), we again noted a clear deletion phenotype, with a reduction in the proportion of CD4+ T cells, and a diminished level of expression of the transgene-encoded Vβ6 chain (Figure 2B). There was a significant residual level of Vβ6 expression, though, which was comparable with that of K/BxN mice. Yet, no arthritic manifestations were detected in these mice either.

The inescapable conclusion from these experiments is that the KRN TCR is not heedlessly arthritogenic. It must recognize a specific *A^{g7}*-peptide target. A related conclusion is that not all alloreactive TCR/MHC-peptide combinations provoke arthritis. Carrasco-Marín et al (21) have suggested that the *A^{g7}* molecule is peculiar in its poor binding of peptides, potentially leading to inefficient inactivation of autoreactive T cells. It is not clear whether this property plays a role in the present model or whether any target of autoreactive T cells, as long as it presented the proper self peptide in a corresponding affinity/avidity window, could generate arthritis. The coincidence is suggestive, though.

No joint specificity to target antigen of the KRN TCR. As mentioned above, the KRN TCR reacts to *A^{g7}* molecules presenting self peptides; the relevant peptides are found in lymphoid tissues, since splenic APC stimulate KRN T cells and thymic stromal cells delete them (7). This is paradoxical, given the restricted nature of the disease, which only affects joints. One possibility is that the antigen, although not joint specific, is more abundant in the joint than in other tissues. We tested this hypothesis by measuring the in vivo potency of APC from different lymphoid organs. Several studies have demonstrated that APC in lymph nodes draining the organ in which an antigen is specifically expressed are uniquely able to prime the corresponding T cells (22,23). Thus, we considered that if the peptides recognized by

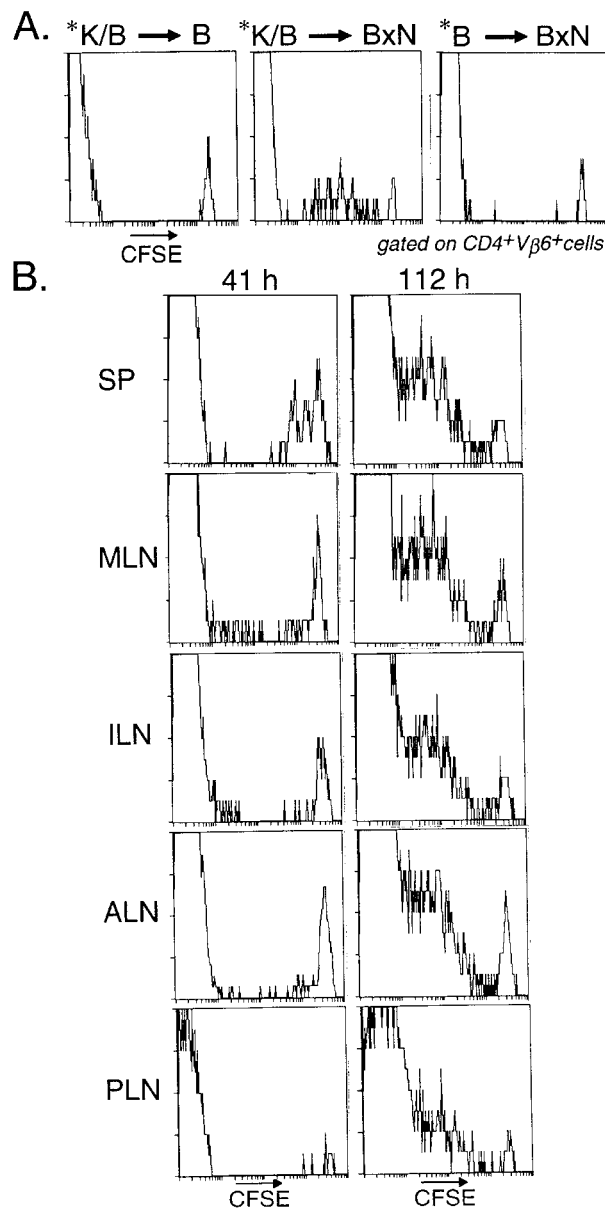


Figure 3. Demonstration that proliferation of KRN T lymphocytes is essentially equivalent in all lymphoid organs. **A**, Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled splenocytes from transgenic K/B mice were transferred intravenously into control B recipients (**left**) or to BxN recipients (**middle**); labeled nontransgenic (control) splenocytes were transferred to BxN recipients (**right**). After 72 hours, the intensity of remaining CFSE staining in gated CD4+V β 6+ donor cells was analyzed by flow cytometry (a reduction in staining intensity indicating cell proliferation). Results are representative of at least 3 independent experiments. **B**, CFSE-labeled K/B splenocytes were transferred to BxN recipients. Proliferation of CD4+V β 6+ cells in spleen (SP) and in the mesenteric (MLN), inguinal (ILN), axillary (ALN), and popliteal (PLN) lymph nodes was assessed as in **A** at 41 hours (**left**) and 112 hours (**right**) after transfer. Results are representative of at least 5 independent experiments.

the KRN TCR were preferentially expressed in the joints, there should be more stimulatory capacity in lymph nodes draining the joints than in the spleen or in visceral nodes.

The test system entailed infusing BxN or negative control B mice with antigen-inexperienced K/B splenocytes that had been labeled in vitro with CFSE. Cells labeled with this cytoplasmic dye can be readily traced in recipients, and each round of cell division is manifested by a halving of the CFSE fluorescence intensity. This is illustrated in Figure 3A for KRN T cells: transferred K/B cells proliferated and lost label in the spleen of BxN recipients (Figure 3A, middle). They did not do so when the host was a control B mouse that did not express the target molecule (Figure 3A, left), nor was there proliferation when control donor cells from a transgene-negative B mouse were introduced into BxN hosts (Figure 3A, right).

We next analyzed the kinetics of KRN T cell proliferation in several lymphoid organs. As shown in Figure 3B, proliferation in the spleen was only beginning at 41 hours after transfer and was, if anything, less advanced in the limb-draining nodes (axillary, popliteal). Extensive proliferation was seen at 112 hours and was essentially equivalent in all organs.

By this assay, then, antigen recognized by the KRN TCR in A^{g7}-positive mice does not appear to exhibit joint specificity. It is important to recall that KRN T cells probably do not preferentially home to the joint itself, because they are rather rare in the arthritic lesion (7).

DISCUSSION

The three novel conclusions from this study are 1) that the KRN T cell receptor is the sole TCR responsible for arthritis development in K/BxN mice; 2) that the KRN TCR must recognize a particular A^{g7}-peptide(s) ligand to engender arthritis; and 3) that this ligand is broadly distributed, apparently equivalently, throughout the body. These conclusions represent critical details in the scenario of disease pathogenesis in K/BxN mice, especially at the stage of disease initiation.

This set of data solidifies our contention that an organ-specific disease can result from systemic self-reactivity (7). The processes responsible for focusing autoimmune destruction to the joints in K/BxN mice still remain to be identified. Immunoglobulins are somehow involved, since we have recently demonstrated that transfer of small amounts of serum Ig from arthritic K/BxN mice into healthy recipients induces arthritis in

days, even in the complete absence of lymphocytes (16). However, this does not necessarily mean that the Ig are joint-specific; indeed, we have very recently established that the arthritogenic specificities are directed at a ubiquitously expressed enzyme (Matsumoto I, Benoist C, Mathis D: unpublished observations). Focusing inflammation/destruction in the joint may occur even further downstream in the pathogenic process, perhaps reflecting unusual physiologic features, such as a lack of a basement membrane or a diminished blood flow (1).

Yet, not all systemic self-reactivity, even through the KRN receptor, results in autoimmune disease. KRN TCR recognition of neither A^d:mIs-1^a nor of A α ^{k69}A β ^k provoked arthritis. It may be that the ligand has to be recognized within a particular window of affinity or avidity to be disease-provoking. Alternatively, or in addition, the timing of ligand expression during development or the subcellular compartment in which it resides (e.g., secreted versus membrane-bound) could be critical factors.

What does this scenario have to do with human RA? We cannot answer this question in the absence of more conclusive information on the pathway(s) by which arthritis unfolds in RA patients. However, it is important to keep in mind that there is no compelling evidence for a joint-specific antigen in the human disease (for review, see refs. 1 and 4–6) and that disease mechanisms highlighting physiologic features of the joint have been proposed (24,25).

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