20

The K/BxN Mouse Model of Inflammatory Arthritis

Theory and Practice

Paul Monach, Kimie Hattori, Haochu Huang, Elzbieta Hyatt, Jody Morse, Linh Nguyen, Adriana Ortiz-Lopez, Hsin-Jung Wu, Diane Mathis, and Christophe Benoist

Abstract

Mice expressing the KRN T cell receptor transgene and the MHC class II molecule A^{g7} (K/BxN mice) develop severe inflammatory arthritis, and serum from these mice causes similar arthritis in a wide range of mouse strains, owing to pathogenic autoantibodies to glucose-6-phosphate isomerase (GPI). This model has been useful for the investigation of the development of autoimmunity (K/BxN transgenic mice) and particularly of the mechanisms by which anti-GPI autoantibodies induce joint-specific imflammation (serum transfer model). In this chaper, after a summary of findings from this model system, we describe detailed methods for the maintenance of a K/BxN colony, crossing of the relevant TCR and MHC genes to other strain backgrounds, evaluation of KRN transgenic T cells, measurement of anti-GPI antibodies, induction of arthritis by serum transfer, and clinical and histological evaluation of arthritis.

Key Words: Rheumatoid arthritis; glucose-6-phosphate isomerase; GPI; anti-GPI; serum transfer; autoantibodies; mouse model; KRN; methods.

1. Introduction

The K/BxN T cell receptor (TCR) transgenic mouse model of spontaneous inflammatory arthritis was described in 1996 *(1)*. Arthritis appeared serendipitously when the KRN transgene was crossed into mice carrying the $H\text{-}2^{g7}$ haplotype of the major histocompatibiity locus. Subsequent work has shown that autoimmunity is initiated by TCR recognition of a peptide derived from the glycolytic enzyme glucose-6-phosphate isomerase (GPI) in the context of the major histocompatibility complex (MHC) class II molecule $H2\text{-}A^{g7}$, resulting in production of high titers of autoantibodies to GPI *(2)*. These antibodies

From: *Methods in Molecular Medicine, vol. 136: Arthritis Research, Volume 2*
Edited by: A. P. Cope © Humana Press Inc., Totowa, NJ

are essential for the production of arthritis, and serum (or anti-GPI antibodies purified from serum, or monoclonal anti-GPI antibodies) of K/BxN mice induces arthritis in a wide range of mouse strains *(3,4)*. This final stage requires no input from the adaptive immune system, but involves several players of the innate immune system: lymphocytes and the cytokines they produce are necessary to generate the high titers of anti-GPI but become dispensible for the effector phase, which relies instead on responses and mediators from neutrophils, mast cells, or the vascular endothelium. Many genes required for or influencing the severity of arthritis in this model have been identified. Breeding of the KRN transgene and MHC restriction element to genetically-deficient backgrounds has allowed assessment of genetic effects on either the breakdown of tolerance to GPI or on subsequent effector mechanisms. More easily tractable has been genetic analysis isolated to the effector phase by use of the serum transfer model. By transfer of K/BxN serum into gene-deficient strains, arthritis induced by anti-GPI antibodies has been shown to require mast cells *(5)*, neutrophils *(6)*, the alternative pathway of complement activation *(7)*, the C5a receptor *(7)*, the low-affinity IgG receptor FcγRIII *(7,8)*, the cytokines interleukin (IL)-1 and tumor necrosis factor (TNF) *(9)*, the integrin LFA-1 *(10)*, and the MHC-like Fc receptor FcRn *(11)*. Mechanisms by which these factors interact to produce joint-specific inflammation are being actively investigated in several laboratories. The system has also allowed the relatively facile analysis of natural allelic variants of key genes, identifying several quantitative trait (QT) regions, and pinpointing variation in the C5 and IL-1β genes as influencing susceptibility to arthritis among inbred mouse strains *(12,13)*. Overall, the K/BxN serum transfer system very likely involves the same effector mechanisms as the model in which arthritis is induced by transfer of a cocktail of monoclonal antibodies (mAbs) directed against type-II collagen (cII) *(14–16)*. The K/BxN system does have the important advantage, however, of not requiring the complementary injection of LPS needed for activity of the anti-cII mAbs. Thus, effects of genetic mutations or pharmacological intervention on the effector phase can be evaluated directly, without interference from effects on the TLR signaling pathway.

A current working model of the pathogenesis of arthritis in this system is shown in **Fig. 1**.

In KRN transgenic mice also carrying the H-2^{g7} haplotype, arthritis appears very stereotypically around 30 ± 5 d of age. In some backgrounds or genetic variants where the disease is particularly aggressive, the first signs can be observed between 22 and 25 d of age; conversely, in genetic backgrounds that are less susceptible, the first signs may only be visible between 35 and 45 d of age. Arthritis in the K/BxN model is predominantly distal: ankles, wrists, associated tendon sheaths, and digits are always affected, the knees less so, whereas

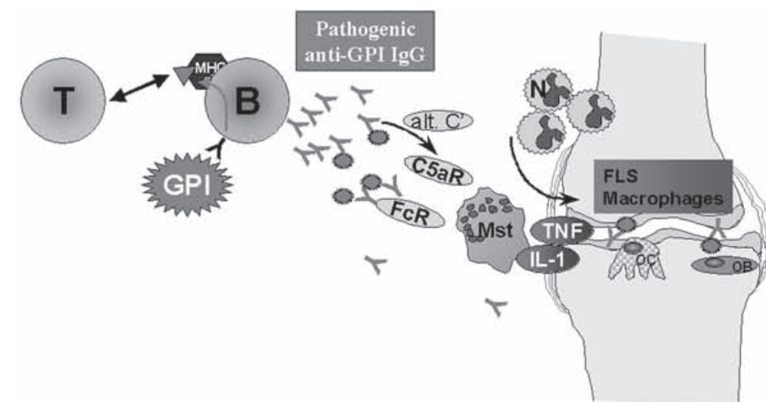

Fig. 1. Model of arthritis induction by anti-GPI antibodies. T-cell tolerance is broken, and T-cells reactive against a peptide derived from GPI, presented by the A^{g7} MHC molecule, provide potent help for those B-cells whose GPI-binding receptor make them particularly good antigen-presenting cells. These B-cells convert to plasma cells and produce high titers of high-affinity anti-GPI antibodies of the IgG1 isotype. These antibodies form immune complexes in the circulation and, more importantly, in the joint tissues, activating mast cells and complement (via the alternative pathway), leading to the influx of neutrophils. A chronic inflammatory state then arises, similar to that seen in human rheumatoid arthritis, that features marked expansion of macrophages and fibroblast-like synoviocytes (FLS) in the synovial tissue, production of TNF and IL-1, ongoing neutrophil recruitment into synovial fluid, erosion of cartilage, and erosion of bone via activation of osteoclasts (OC). Arthritis in the K/BxN model also features prominent growth of new bone around inflammatory lesions, mediated in part by osteoblasts (OB).

hips and shoulders normally show no involvement. Histological lesions of the spine are sporadic and limited to one or two levels. In addition, there is a degree of stochastic variability in the topography of the joints affected in any individual mouse, particularly when disease is induced by limited amounts of serum.

2. Breeding of KRN T Cell Receptor Transgenic Mice and K/BxN Arthritic Mice

The overall operation of a colony of K/BxN model involves two sets of breedings:

1. A core line in which the KRN transgene is maintained on an inbred background where the MHC haplotype is not $H2^{g7}$, and the mice are thus free of arthritis. Although fairly healthy, these mice are somewhat immunocompromised, because

the TCR transgene does limit their T-cell repertoire. Slightly delayed growth is not unusual. On such backgrounds (we have used either C57Bl/6 or B10.BR), animals are reasonably fertile. It is possible to generate animals carrying two copies of the transgene, indicating that the KRN transgene does not induce a homozygous lethal mutation, as do a number of transgenes. On the other hand, we found these animals to have significantly reduced breeding performance (Hergueux, J., unpublished work); thus, and although it would appear theoretically advantageous to use such homozygous animals in breedings that would produce 100% transgenic offspring, we have found that the colonies perform better in the long term when involving only heterozygous KRN mice, even at the cost of having to genotype breeders and of obtaining only 50% arthritic offspring.

2. To generate cohorts of arthritic animals, KRN TCR transgenic mice are crossed to mice carrying the stimulating MHC haplotype. We typically use NOD/Lt mice for this purpose. NOD females are good breeders with large litters (often >10 offspring), and the hybrid vigor of the BxNOD F1 combination is favorable to the offspring's health. In practice, a proven KRN male is rotated weekly between 2 or 3 cages, with 2 NOD females in each. These KRN males can be bred productively from 7 wk of age onwards, and usually remain fertile until 10 mo of age. The size of such a colony obviously depends on the experimental requirements for serum or arthritic experimental groups, but should probably involve 6 or more such rotated breeders (1 male replaced every month) for regular generation of mice and arthritogenic serum. NOD females do succumb to diabetes between 12 and 25 wk of age, and must be replaced when signs of diabetes are present (i.e., soiled fur, wet litter, and early weight loss). We have recently begun to use NOD. Eα16 mice *(17)* in such breedings: the Eα16 transgene encodes an MHC-II molecule that protects NOD mice from diabetes, but does not seem to affect the parameters of arthritis or anti-GPI titers in the progeny (Tran, V., unpublished work). It is also possible to breed KRN$^+$ females to NOD males, but litter sizes are smaller in this combination.

Over the course of 1 yr, it was noticed that the K/BxN serum produced in different animal facilities can have somewhat different titers. In particular, serum produced from one K/BxN colony housed in conditions of high health safety had a mean titer 5-fold lower than that produced in another colony at the same time (Morse, J., and Hattori, K., unpublished work). This difference was not the result of genetic drift, nor could it be attributed to any clear bacteriological agent. This has been a very rare occurrence, however, and most serum production colonies operated by various investigators generate K/BxN serum of high titer and arthritogenic activity.

3. Genotyping

The KRN transgene is integrated within 5 c*M* of the *Tyr* locus on Chr 7, from the original B6xSJL background on which the KRN transgene was generated *(1)*, most likely on the chromosome from the SJL parent which carries the

agouti allele at the *Tyr* locus. We have strived to keep the loci together over long periods: it allows a preselection of the animals to genotype, as most agouti-gray animals type positive (but not all, as some recombinants do appear, and coat color should *not* be used alone to identify KRN$^+$ mice). Genotyping can be done by flow cytometric analysis or by polymerase chain reaction (PCR).

For flow cytometry, KRN$^+$ mice are detected by staining blood cells for CD4 and Vβ6; in KRN$^+$ mice, most CD4$^+$ cells are Vβ6$^+$, compared with only about 10% of CD4$^+$ cells in KRN$^-$ mice. In practice, one recovers one or two drops of blood, from the lateral tail vein of an immobilized mouse, into a 1.5-mL tube containing 45 µL FACS wash buffer (PBS + 30 m*M* HEPES [pH 7.4], 3% heat-inactivated horse serum, and 0.1% sodium azide) and 5 µL heparin (from 5000 U/mL stock). Mix, then store on ice. Add 1 mL red blood cell (RBC) lysis buffer (10 µ*M* HEPES [pH 7.3], 0.15*M* NaCl, and 0.1 m*M* ethylene diamine tetraacetic acid [EDTA]), mix well. Leave at room temperature for 4 to 10 min. Centrifuge and discard supernatant. Repeat if the pellet appears red. Resuspend cells in FACS wash buffer containing diluted antibodies: 1:50 fluorescein isothiocyanate (FITC)-conjugated anti-mouse Vβ6 TCR (Pharmingen; cat. no. 01364C) and 1:25 PE-conjugated anti-mouse CD4 (Caltag; cat. no, RM2504-3). Leave on ice for 30 min and then wash once with FACS wash buffer. Resuspend in 100 to 200 µL of 1% paraformaldehyde in phosphate buffered saline (PBS) for flow cytometry.

For PCR typing, 1 µL of DNA (derived from a 100 µL proteinase K digestion of a 2-mm piece of tail: 50 µg/mL proteinase K added fresh from 400X stock to buffer of 10 m*M* Tris [pH 8.3], 50 m*M* KCl, 0.01 mg/mL gelatin, 0.045% NP-40, and 0.045% Tween-20, incubated at 50°C overnight) is amplified in a 25 µL reaction, using 0.5 U Taq polymerase, 3 m*M* Mg, 1*M* betaine, and otherwise standard conditions for Taq amplification. One of two sets of oligos can be used: TCR α primer sequences giving a 146-bp product (forward 5'-AGG TCC ACA GCT CCT TCT GA-3'; reverse 5'-GTA TTG GAA GGG GCC AGA G-3'), or TCR β primers giving a 227-bp product (forward 5'-GGG CAA AAA CTG ACC TTG AA-3'; reverse 5'-GAG CCT GGT TGT TTG TGG AT-3'). We typically use only the β primers. PCR conditions are the same with either primer set: 95°C for 5 min; then 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min; and finally 72°C for 5 min. Products are detected using 1% agarose gels impregnated with ethidium bromide.

4. Clinical Evaluation of Arthritis

Ankle thickness is measured with a caliper (this laboratory uses the Kafer dial thickness gauge with flat anvils) (Long Island Indicator; cat. no. 21-790-1) placed across the ankle joint at the widest point (the malleoli) (*see* **Fig. 2A**). Measuring should be done quickly. Once both sides of the caliper touch the

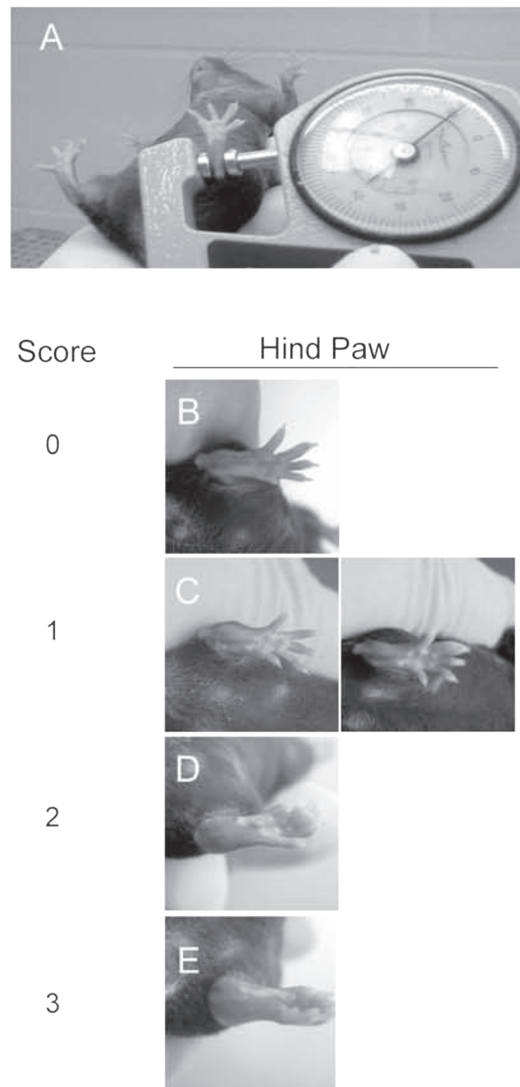

Fig. 2. Evaluation of arthritis severity. **(A)** Demonstration of technique for measuring ankle thickness using a precision caliper. **(B–E)** Examples of hind paws assessed as having clinical severity **(B)** 0 (nonarthritic), **(C)**1 , **(D)**2 , or **(E)** 3 (maximum). Note that clinical severity of 1 can represent either mild swelling of the ankle and surrounding tendon sheaths, or focal swelling of one or more digits.

ankle, the meter should be read immediately. The swelling of ankles is caused by inflammatory fluid and soft tissue. Failure to read the meter immediately results in squeezing of this pliable tissue, therefore under-estimating the ankle thickness. When uncertain about the first measurement, let the mice rest for 5 min before taking the measurement again so that the swelling returns to the original size. With these precautions, the measurement of ankle swelling is a surprisingly sensitive indicator: in the hands of an experienced investigator, an increase of 0.1 mm from one day to the next is an unmistakable sign of incipient arthritis. For optimal precision, it is preferable to measure both hind limbs. For the serum transfer model, the ankle thickness is measured before serum injection, daily or at least every other day during the first week, and every other day afterward until the disease severity plateaus or starts to dwindle (around 12–15 d). Because of variation of ankle width even among unmanipulated animals of similar age, it is suggested to use ankle thickening (the difference between ankle thickness at day 0 and at later time points) instead of ankle thickness to analyze data. For the spontaneous arthritis that develops in the transgenic mice, measurements are first taken at 22 d of age (i.e., before the onset in essentially all cases), then every 2 to 3 d thereafter, typically until 40 d of age. To obtain a "clinical index," each limb is also scored on a scale of 0–3, where "0 = indicates no observable swelling; 1 = indicates one involved digit or mild swelling of the foot and ankle, but where the foot maintains its original V shape; 2 = indicates that the long edges of foot are parallel with each other, with disappearance of the normal V shape; and 3 = indicates inversion of the V shape by expansion of the ankle and hindfoot to greater than the width of the forefoot (*see* **Fig. 2**). The four limb scores are added together to give the clinical index (maximum 12 points).

5. Induction of Arthritis by Serum Transfer

The induction of arthritis by transfer of arthritogenic immunoglobulins provides a robust tool to analyze the impact of genetic variations or therapeutic interventions on the disease process. With serum transfer, the onset of arthritis is very robust and follows a reproducible course in genetically identical animals, although there is some inter-individual variability. We have reported *(4)* that arthritis can be induced by transfer of a cocktail of monoclonal antibodies (mAbs), which in theory would provide a good replacement for serum from arthritic K/BxN mice. Unfortunately, the disease induced by mAbs did not prove, in the long run, to be as robust as that induced by serum transfer; in addition, we have prepared some large batches of anti-GPI mAbs which were very poor at inducing arthritis, even though they had respectable anti-GPI titers. The cause of these problems (e.g., contamination from tissue culture, and possible drift in the post-translational modifications of the hybridomas over

time) has not been sorted out, but we have not adopted monoclonal transfer in routine use, and do not recommend it.

Arthritis is induced by intraperitoneal injection of serum from 8-wk-old K/BxN mice on days 0 and 2, 150 µL/injection (intravenous [iv] injection can also be used, particularly when wishing to analyze very early aspects of the response, but intraperitoneal [ip] injection is perfectly adequate in general practice). It is advised to use at least 5-wk-old males or 6-wk-old females as the recipients. Complement is critical for this model, and mice younger than these ages usually are relatively deficient in serum complement factors, which results in decreased incidence and severity of arthritis. For B6 mice, the first signs of arthritis and ankle thickening are detectable after 2 to 3 d, and maximum severity of arthritis usually occurs between days 8 and 10 after serum transfer. Male mice tend to reach the plateau of severity faster, but both males and females reach the same level of ankle thickening. There are different sensitivities to serum transferred arthritis among various inbred strains of mice, which translate as different times of onset and/or extent of joint swelling. The sensitivities of some common strains are listed in **Table 1**. At the end of a serum transfer experiment, it is advised to collect serum from each individual mouse and examine its serum anti-GPI titer, particularly in the event that some mice do not develop severe arthritis. This allows one to confirm that all mice received accurate ip injection of serum (misinjection into the bladder can occur), and also can be helpful in ensuring that the mechanism for arthritis-resistance in a given strain is not caused by a shortened half-life of the anti-GPI antibodies.

6. Crossing KRN and H-2^{g7} to Other Genetic Nackgrounds

The spontaneous K/BxN transgenic model can be used to study the effects of other genes on the immunological or effector phases of disease. This is usually done by crossing the transgene to mice deficient for a particular gene X of interest ($X^{-/-}$). Because the KRN TCR transgene and the MHC class II H-2^{g7} allele are both required for arthritis, and because breeding performance of arthritic animals is quite poor, the breeding strategy we have used is to breed a gene-deficient line independently with KRN TCR transgenic mice (on the H-2^b B6 background) and with B6 H-2^{g7} congenic mice. This first cross generates $KRN^+X^{+/-}$ and H-$2^{g7/b}X^{+/-}$ animals, respectively. Intercrossing these mice can be performed at this stage, but with only a very low yield of the desired mice (1/16), so we usually resort to an additional round of "gene enrichment" to produce more favorable breeders. Typically, and depending on the viability/fertility of $X^{-/-}$ mice, H-$2^{g7/b}X^{+/-}$ mice are further intercrossed to generate H-$2^{g7/g7}X^{+/-}$ or H-$2^{g7/g7}X^{-/-}$ mice, and/or $KRN^+X^{+/-}$ mice are intercrossed to generate $KRN^+X^{-/-}$ animals (this second round of "preliminary crossing" thus

Table 1
K/BxN Serum-Ttransferred Arthritis in Diverse Inbred Strains

Strain	Max. clinical index	Day of onset[a]	Max. ankle thickening (mm)	No. of mice examined	Histological evaluation	
					Inflammation	Cartilage destruction
Balb/c	4.0	1.2 (1–2)	1.5	11	(++)	(++)
C57Bl/10	3.9	1.3 (1–3)	1.4	4	(++)	(+)
PL	3.8	2.0 (1–4)	1.4	8	(++)	(++)
C57Bl/6	3.5	2.9 (1–6)	1.5	19	(++)	(+)
DBA/1	3.5	2.8 (2–5)	1.4	4	(+)	(+)
CBA	3.5	3.5 (2–5)	1.1	4	(++)	(+)
MRL/Mp	2.9	3.8 (2–4)	0.9	4	(++)	(+++)
NZW	2.9	4.8 (1–14)	0.7	4	(++)	(+++)
C3H/He	2.2	6.4 (3–12)	1	5	(+/–)	(+/–)
SJL	2.2	12.4 (2–28)	0.6	12	(+)	(+/–)
129/Sv	1.6	3.5 (3–4)	0.3	4	(+/–)	(+/–)
DBA/2	ND	ND	0	4	(–)	(–)
FvB/N	ND	ND	0	4	(–)	(–)
NZB	ND	ND	0	4	(–)	(–)
NOD	ND	ND	0	4	(–)	(–)

[a] Mean (range).

Note: Different strains of mice were transferred with 7.5 µL/g body weight of serum from K/BxN mice on days 0 and 2. Two mice were killed at day 7 for histology, and the others followed until days 12–28. The "day of onset" is the first day of overt disease on one limb. Reprinted with permission from **ref. *12***.

enriches the frequency of the H-2^{g7} and X^- chromosomes in the final crosses). Finally, $KRN^+X^{+/-}$ mice are crossed to H-$2^{g7/g7}X^{+/-}$ (or H-$2^{g7/g7}X^{-/-}$) to generate experimental mice (KRN^+H-$2^{g7/b}X^{-/-}$) and control littermates (KRN^+H-$2^{g7/b}X^{+/+}$ or KRN^+H-$2^{g7/b}X^{+/-}$). We have also used a similar strategy for crossing to mouse strains deficient for two genes. Screening for the KRN transgene and MHC haplotype in these breeding schemes is most readily done by flow cytometry of peripheral blood. KRN^+ mice are detected as above. H-$2^{g7/b}$ and H-$2^{g7/g7}$ can be distinguished by staining with monoclonal antibodies that distinguish A^{g7} from A^b (typically mAbs 10.2.16 and Y-3P, respectively).

7. Analysis of KRN Transgenic T-Cells

In order to evaluate T-cell tolerance in this model, key markers can be examined in thymocytes. Perturbations in thymocyte negative selection or positive selection can be manifested as changes in the subpopulations delineated

by CD4 and CD8 expression. Selection of T-cells bearing the KRN TCR can be evaluated by the expression of the transgene-encoded TCR-β chain (Vβ6) vs other variable regions from endogenous TCR loci (e.g., Vβ8), in the CD4 single positive T-cell subset. In addition, thymocyte development can be evaluated based on the expression of the maturation markers CD44 and CD5 in various populations: $CD4^-$ $CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), $CD4^+CD8^-$ single positive (SP) and $CD4^-CD8^+$ SP populations. Combinations of labeled antibodies that we have found to be effective for such analysis, using a Cytomation MoFlo instrument, include: (1) CD8α-FITC, Vβ6-PE, Vβ8-Bio/SA-PE-Cy7, CD3-APC, and CD4-PE-TR; and (2) CD8α-FITC, CD4-PE, and CD5(or CD44)-APC. Some of the populations in K/BxN mice are not as well defined as they are in non-TCR transgenic mice. Thus, we find it useful to stain cells from non-TCR transgenic mice in parallel with cells from experimental mice, and to set some of the gating (e.g., $V\beta6^+$, $V\beta8^+$, $CD44^{high}$) based on the populations in non-TCR transgenic mice. Thymi from K/BxN mice display an altered CD4/CD8 profile compared with non-TCR transgenic mice, the most prominent differences being a decrease in the percentage of CD4 SP thymocytes and an increase in the percentage of DN thymocytes. Thymocyte subsets in K/BxN mice are approx 20% DN, 75% DP, 3% CD4 SP, and 2% CD8 SP. In the CD4 SP subset, the majority (approx 60–70%) of cells express the transgenic Vβ6 TCR, and notably, several percent of cells express both transgenic and endogenous TCRs ($V\beta6^{int}$ $V\beta8^+$). Levels of Vβ6 and CD3 expression are very tightly correlated. In nontransgenic mice, levels of CD5 increase as thymocyte differentiation progresses to the SP stage. In contrast, CD5 expression is already high on DN thymocytes in K/BxN mice, perhaps resulting from early expression of and signaling through the transgenic TCR. In order to evaluate T-cell activation in peripheral lymphoid organs, we examine spleen and lymph node suspensions for expansion of CD4 T-cells, expression of transgenic and endogenous TCR chains, and expression of CD44, CD62L, CD25, and CD69. We also evaluate the regulatory T-cell subset as defined by $CD4^+$ $CD69^-$ $CD25^+$ T-cells. Effective combinations of labeled antibodies include (1) CD4-PE-TR, Vβ6-PE, B220-Bio/SA-PE-Cy7, CD69-FITC, and CD25-APC; and (2) CD4-PE-TR, Vβ6-PE, B220-Bio/SA-PE-Cy7, CD62L-FITC, and CD44-APC. For the markers we have examined, similar results have been obtained from spleen and lymph node cells. In addition, we have not detected significant differences between cells from lymph nodes draining inflamed joints (e.g., axillary) vs nondraining lymph nodes (e.g., cervical). The distribution of Vβ6, Vβ8, and CD3 on $CD4^+$ T-cells in the periphery is similar to that in CD4 SP thymocytes. One difference is that the percentage of T-cells expressing both transgenic and endogenous TCRs is slightly higher in the periphery than in the thymus. $CD4^+$ and $CD8^+$ populations remain at fairly low

proportions in the periphery (approx 3-7% $CD4^+$, 1–2% $CD8^+$). $CD4^+$ T-cells display clear evidence of activation, with the early activation marker CD69 upregulated on approx 20% of cells, and the activated/memory cell marker CD44 upregulated on approx 60% of cells. Generally, characteristics of the peripheral T-cell compartment are consistent between 2 and 10 wk of age, with some populations expanding over time (e.g., $CD4^+$ T-cells, dual TCR expressors, and $CD44^{high}$ cells). In both thymus and periphery, the frequency of $CD4^+$ cells in adult K/BxN mice is lower than what is usually found with mice expressing MHC class II-restricted TCR transgenes. Before thymic tolerance of KRN T-cells is broken between 2 and 3 wk of age in K/BxN mice, the number of $CD4^+$ cells is very low. GPI-specific T-cells can also be followed by staining with an MHC tetramer (A^{g7} MHC molecule presenting a GPI-derived peptide) ***(18)***.

8. Screening K/BxN Serum for Anti-GPI Antibodies

Levels of anti-GPI antibodies in individual or pooled K/BxN sera can be measured by enzyme linked immunosorbent assay (ELISA). Microtiter plates are coated with the recombinant fusion protein mouse GPI-GST (*see* below), 5 μg/mL in phosphate buffered saline (PBS), at 4°C overnight or longer. Plates are then blocked with 1% bovine serum albumin (BSA) in PBS (with 2 changes to effect washing as well) for 30 min at room temperature, then with serum dilutions in PBS for 1 h. The linear range of anti-GPI activity in K/BxN serum is typically between dilutions of 1:3,000 and 1:1,000,000. After incubation with serum, plates are washed 3 times with PBS, then incubated with alkaline phosphatase-conjugated $F(ab')_2$ fragments of goat-anti-mouse IgG (Jackson ImmunoResearch), typically diluted 1:2000 in PBS, again for 1 h at room temperature. After 3 more washes with PBS, phosphatase substrate is added. We use substrate pellets (Sigma), dissolved in 8% diethanolamine, 2.4 m*M* MgC12 (pH 9.8); plates are read at 405 nm with the linear range typically apparent after 5 to 10 min. This ELISA assay can also be adapted to confirm transfer of anti-GPI antibodies, or measure their half-life, after transfer of K/BxN serum into other mice. The starting serum dilution should then be lower (i.e., 1:100 or 1:300). To produce recombinant GPI, mouse GPI cDNA was cloned into the pGEX-4T-3 vector (Amersham/Pharmacia), which creates an N-terminal GST fusion protein. The manufacturer's standard protocol for induction and one-step purification of the fusion protein (Amersham/Pharmacia) has produced good purity, excellent yields, and no significant problems with solubility. Briefly, a 10 mL overnight culture is diluted into 1 L of 2X YT medium and grown at 20 to 30°C until the A_{600} is between 0.5 and 2. Then, IPTG is added to a final concentration of 0.1 m*M*, and incubation is continued for an additional 4 h. The culture is centrifuged, the supernatant removed, and the pellet of cells

by CD4 and CD8 expression. Selection of T-cells bearing the KRN TCR can be evaluated by the expression of the transgene-encoded TCR-β chain (Vβ6) vs other variable regions from endogenous TCR loci (e.g., Vβ8), in the CD4 single positive T-cell subset. In addition, thymocyte development can be evaluated based on the expression of the maturation markers CD44 and CD5 in various populations: $CD4^-$ $CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), $CD4^+CD8^-$ single positive (SP) and $CD4^-CD8^+$ SP populations. Combinations of labeled antibodies that we have found to be effective for such analysis, using a Cytomation MoFlo instrument, include: (1) CD8α-FITC, Vβ6-PE, Vβ8-Bio/SA-PE-Cy7, CD3-APC, and CD4-PE-TR; and (2) CD8α-FITC, CD4-PE, and CD5(or CD44)-APC. Some of the populations in K/BxN mice are not as well defined as they are in non-TCR transgenic mice. Thus, we find it useful to stain cells from non-TCR transgenic mice in parallel with cells from experimental mice, and to set some of the gating (e.g., $V\beta6^+$, $V\beta8^+$, $CD44^{high}$) based on the populations in non-TCR transgenic mice. Thymi from K/BxN mice display an altered CD4/CD8 profile compared with non-TCR transgenic mice, the most prominent differences being a decrease in the percentage of CD4 SP thymocytes and an increase in the percentage of DN thymocytes. Thymocyte subsets in K/BxN mice are approx 20% DN, 75% DP, 3% CD4 SP, and 2% CD8 SP. In the CD4 SP subset, the majority (approx 60–70%) of cells express the transgenic Vβ6 TCR, and notably, several percent of cells express both transgenic and endogenous TCRs ($V\beta6^{int}$ $V\beta8^+$). Levels of Vβ6 and CD3 expression are very tightly correlated. In nontransgenic mice, levels of CD5 increase as thymocyte differentiation progresses to the SP stage. In contrast, CD5 expression is already high on DN thymocytes in K/BxN mice, perhaps resulting from early expression of and signaling through the transgenic TCR. In order to evaluate T-cell activation in peripheral lymphoid organs, we examine spleen and lymph node suspensions for expansion of CD4 T-cells, expression of transgenic and endogenous TCR chains, and expression of CD44, CD62L, CD25, and CD69. We also evaluate the regulatory T-cell subset as defined by $CD4^+$ $CD69^-$ $CD25^+$ T-cells. Effective combinations of labeled antibodies include (1) CD4-PE-TR, Vβ6-PE, B220-Bio/SA-PE-Cy7, CD69-FITC, and CD25-APC; and (2) CD4-PE-TR, Vβ6-PE, B220-Bio/SA-PE-Cy7, CD62L-FITC, and CD44-APC. For the markers we have examined, similar results have been obtained from spleen and lymph node cells. In addition, we have not detected significant differences between cells from lymph nodes draining inflamed joints (e.g., axillary) vs nondraining lymph nodes (e.g., cervical). The distribution of Vβ6, Vβ8, and CD3 on $CD4^+$ T-cells in the periphery is similar to that in CD4 SP thymocytes. One difference is that the percentage of T-cells expressing both transgenic and endogenous TCRs is slightly higher in the periphery than in the thymus. $CD4^+$ and $CD8^+$ populations remain at fairly low

proportions in the periphery (approx 3-7% $CD4^+$, 1–2% $CD8^+$). $CD4^+$ T-cells display clear evidence of activation, with the early activation marker CD69 upregulated on approx 20% of cells, and the activated/memory cell marker CD44 upregulated on approx 60% of cells. Generally, characteristics of the peripheral T-cell compartment are consistent between 2 and 10 wk of age, with some populations expanding over time (e.g., $CD4^+$ T-cells, dual TCR expressors, and $CD44^{high}$ cells). In both thymus and periphery, the frequency of $CD4^+$ cells in adult K/BxN mice is lower than what is usually found with mice expressing MHC class II-restricted TCR transgenes. Before thymic tolerance of KRN T-cells is broken between 2 and 3 wk of age in K/BxN mice, the number of $CD4^+$ cells is very low. GPI-specific T-cells can also be followed by staining with an MHC tetramer (A^{g7} MHC molecule presenting a GPI-derived peptide) ***(18)***.

8. Screening K/BxN Serum for Anti-GPI Antibodies

Levels of anti-GPI antibodies in individual or pooled K/BxN sera can be measured by enzyme linked immunosorbent assay (ELISA). Microtiter plates are coated with the recombinant fusion protein mouse GPI-GST (*see* below), 5 µg/mL in phosphate buffered saline (PBS), at 4°C overnight or longer. Plates are then blocked with 1% bovine serum albumin (BSA) in PBS (with 2 changes to effect washing as well) for 30 min at room temperature, then with serum dilutions in PBS for 1 h. The linear range of anti-GPI activity in K/BxN serum is typically between dilutions of 1:3,000 and 1:1,000,000. After incubation with serum, plates are washed 3 times with PBS, then incubated with alkaline phosphatase-conjugated $F(ab')_2$ fragments of goat-anti-mouse IgG (Jackson ImmunoResearch), typically diluted 1:2000 in PBS, again for 1 h at room temperature. After 3 more washes with PBS, phosphatase substrate is added. We use substrate pellets (Sigma), dissolved in 8% diethanolamine, 2.4 m*M* MgC12 (pH 9.8); plates are read at 405 nm with the linear range typically apparent after 5 to 10 min. This ELISA assay can also be adapted to confirm transfer of anti-GPI antibodies, or measure their half-life, after transfer of K/BxN serum into other mice. The starting serum dilution should then be lower (i.e., 1:100 or 1:300). To produce recombinant GPI, mouse GPI cDNA was cloned into the pGEX-4T-3 vector (Amersham/Pharmacia), which creates an N-terminal GST fusion protein. The manufacturer's standard protocol for induction and one-step purification of the fusion protein (Amersham/Pharmacia) has produced good purity, excellent yields, and no significant problems with solubility. Briefly, a 10 mL overnight culture is diluted into 1 L of 2X YT medium and grown at 20 to 30°C until the A_{600} is between 0.5 and 2. Then, IPTG is added to a final concentration of 0.1 m*M*, and incubation is continued for an additional 4 h. The culture is centrifuged, the supernatant removed, and the pellet of cells

placed on ice, then resuspended in 50 mL cold PBS. This suspension is sonicated in short bursts until partial clearing is apparent, then Triton X-100 added to a final concentration of 1%, and the suspension incubated on ice for 30 min. Then, the suspension is centrifuged at 12,000*g* for 10 min and the supernatant applied to 2 mL of a 50% slurry of glutathione sepharose 4B (Amersham/Pharmacia) preequilibrated with PBS. The lysate/bead mixture is incubated for 30 min at room temperature with rotation, then the beads recovered and washed 3 times with PBS (10 mL/wash) by low-speed centrifugation (500*g*, 5 min). After the third wash, 1 mL of elution buffer (0.154 g reduced glutathione/50 mL of 50 m*M* Tris-HCl [pH 8.0]) is added to the 1 mL of packed beads, incubated for 10 min at room temperature, and the supernatant recovered after low-speed centrifugation. Elution is repeated twice and the three elutions pooled, dialyzed against PBS, and stored at –20°C after determination of concentration by ultraviolet (UV) absorption at 280 nm (assuming approximate A_{280} of 1.4 for a 1 mg/mL solution).

9. Histology of Ankles

Ankle joints can be prepared for either paraffin or frozen sectioning. In both cases, we typically remove the skin, then cut above the toes and 5 to 10 mm above the ankle joint in order to isolate the ankle and midfoot joints. For paraffin sectioning, after fixation in 4% paraformaldehyde/PBS overnight at 4°C, the bones must be decalcified. This can be done rapidly with formic acid or nitric acid, over 48 h with Kristensen's solution (a mixture of 20% 8*N* formic acid and 80% 1*N* Sodium Formate), or over 2 wk with 0.375*M* EDTA (pH 7.5) at 4°C (4 mL/ankle, changed 2 times/wk). It is wise to use the latter, gentler method if there is any chance of using the tissue for immunohistochemistry to detect targets of unknown stability in acid. Decalcified ankles are then processed for paraffin sectioning and subsequent staining by standard protocols. Frozen sections may be required to detect certain targets by immunostaining (in our experience, C3 and GPI have not been detectable on paraformaldehyde-fixed paraffin sections, but IgG is readily detected). Sectioning of undecalcified bone requires special techniques to preserve tissue structure. Starting with ankles frozen in standard OCT medium, we use commercially-available (Instrumedics) equipment and reagents to obtain sections on pieces of tape, then transfer the sections to slides ("4x" from the Instrumedics line) precoated with a UV-activatable adhesive *(5,7)*. Immunofluorescence staining of frozen sections is then done by a standard protocol: fixation for 5 min in cold acetone, rehydration with PBS, blocking with PBS containing 4% BSA and 0.1% Tween-20, washing and dilution of primary and secondary antibodies in PBS containing 0.5% BSA and 0.1% Tween 20.

References

1. Kouskoff V., Korganow, A. -S., Duchatelle, V., Degott, C., Benoist, C., and Mathis, D. (1996) Organ-specific disease provoked by systemic autoimmunity. *Cell* **87,** 811–822.
2. Matsumoto, I., Staub, A., Benoist, C., and Mathis, D. (1999) Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science* **286,**1732–1735.
3. Korganow, A. S., Ji, H., Mangialaio, S., et al. (1999) From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity* **10,** 451–461.
4. Maccioni, M., Zeder-Lutz, G., Huang, H., et al. (2002) Arthritogenic monoclonal antibodies from K/BxN mice. *J. Exp. Med.* **195,** 1071–1077.
5. Lee, D. M., Friend, D. S., Gurish, M. F., Benoist, C., Mathis, D., and Brenner, M. B. (2002). Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* **297,** 1689–1692.
6. Wipke, B.T. and Allen, P.M. (2001) Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J. Immunol.* **167,** 1601–1608.
7. Ji, H., Ohmura, K., Mahmood, U., et al (2002) Arthritis critically dependent on innate immune system players. *Immunity* **16,** 157–168.
8. Corr, M. and Crain, B. (2002) The role of FcγR signaling in the K/B x N serum transfer model of arthritis. *J. Immunol.* **169,** 6604–6609.
9. Ji, H., Pettit, A., Ohmura, K., et al. (2002) Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. *J. Exp. Med.* **196,** 77–85.
10. Watts, G. M., Beurskens, F. J., Martin-Padura, I., et al (2005). Manifestations of inflammatory arthritis are critically dependent on LFA-1. *J. Immunol.* **174,** 3668–3675.
11. Akilesh, S., Petkova, S., Sproule, T. J., Shaffer, D. J., Christianson, G. J., and Roopenian, D. (2004) The MHC class I-like Fc receptor promotes humorally mediated autoimmune disease. *J. Clin. Invest.* **113,** 1328–1333.
12. Ji, H., Gauguier, D., Ohmura, K., et al.. (2001) Genetic influences on the end-stage effector phase of arthritis. *J. Exp. Med.* **194,** 321–330.
13. Ohmura, K., Johnsen, A., Ortiz-Lopez, A., et al. (2005) Variation in IL-1β gene expression is a major determinant of genetic differences in arthritis aggressivity in mice. *Proc. Natl. Acad. Sci. USA* **102,** 12,489–12,494.
14. Watson, W. C., Brown, P. S., Pitcock, J. A., and Townes, A. S. (1987) Passive transfer studies with type II collagen antibody in B10.D2/old and new line and C57Bl/6 normal and beige (Chediak-Higashi) strains: evidence of important roles for C5 and multiple inflammatory cell types in the development of erosive arthritis. *Arthritis Rheum.* **30,** 460–465.
15. Kagari, T., Doi, H., and Shimozato, T. (2002) The importance of IL-1 beta and TNF-alpha, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. *J. Immunol.* **169,** 1459–1466.
16. Kagari,T., Tanaka, D., Doi, H., and Shimozato, T. (2003) Essential role of Fc gamma receptors in anti-type II collagen antibody-induced arthritis. *J. Immunol.* **170,** 4318–4324.

17. Le Meur, M., Gerlinger, P., Benoist, C., and Mathis,D. (1985) Correcting an immune-response deficiency by creating E alpha gene transgenic mice. *Nature* **316,** 38–42.
18. Stratmann, T., Martin-Orozco, N., Mallet-Designe, V., et al. (2003) Susceptible MHC alleles, not background genes, select an autoimmune T cell reactivity. *J. Clin. Invest.* **112,** 902–914.