

## **Arthritogenic Monoclonal Antibodies from K/BxN Mice**

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### **Abstract**

Arthritis in the K/BxN mouse model is provoked by pathogenic antibodies (Abs) directed against a ubiquitously expressed protein, glucose-6-phosphate isomerase (GPI). To begin dissecting the repertoire of arthritogenic immunoglobulins (Igs) in the K/BxN model, and to provide a basis for comparison with RA patients we have generated anti-GPI monoclonal Abs (mAbs) from spontaneously activated B cells in the lymphoid organs of arthritic mice. B cell clones with anti-GPI specificities were present at extraordinarily high frequencies in the spleen, and less frequently in other lymphoid organs and in the synovial fluid. None of the anti-GPI mAbs induced arthritis when injected individually into healthy recipients, but most were effective when combined in pairs or larger pools. Arthritogenic combinations depended on mAbs of the IgG1 isotype, which bound to GPI with K<sub>d</sub> in the 10<sup>-9</sup> M range, with no indication of cooperative binding between complementing pairs. Pathogenicity was not associated with recognition of a particular epitope, but the ability to form mAb/GPI multimers by simultaneous recognition of different epitopes was clearly required, consistent with the known role of complement and FcRs in this model. Sequence analysis revealed structural similarities amongst the mAbs, indicating that a particular subset of B cells may evade tolerance in K/BxN mice, and that affinity maturation by somatic mutation likely takes place. These results confirm that GPI itself, rather than a cross-reactive molecule, is the target of pathogenic Igs.

**Key words:** autoimmunity • arthritis • autoantibodies • glucose-6-phosphate isomerase • mouse model

### **Introduction**

A wide spectrum of auto-Abs can be found in rheumatoid arthritis (RA) and related arthritides, not only the rheumatoid factor typical of RA patients, but also a variety of other reactivities. However, the true role of auto-Abs in the pathogenesis of these diseases has been a matter of substantial controversy (1). The best way to assign a disease-provoking function to a given auto-Ab is to transfer it into a healthy recipient and reproduce disease manifestations, as has been done for several autoimmune diseases, including autoimmune thrombocytopenic purpura (2) and pemphi-

gus vulgaris (3). A few experiments involving transfer of human sera from arthritic patients into normal individuals have been performed, but did not provoke pathology. It is possible that too few donors or recipients were sampled, that inappropriate experimental read-outs were used, particularly for transfers across species, or that transferred doses were too low. Nonetheless, these data contributed to a major shift to T cell-centric paradigms of RA pathogenesis (e.g., reference 4). In recent years, however, studies of multiple murine models of arthritis have demonstrated an important role for pathogenic Igs in triggering the effector phase of this disease (5–7). Understanding the mechanism of action of these murine arthritogenic Igs could yield new insights into the pathogenesis of human arthritis.

K/BxN TCR transgenic (tg) mice express a transgene-encoded TCR reactive to a self-peptide derived from the

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ubiquitously expressed glycolytic enzyme, glucose-6-phosphate isomerase, presented by the MHC class II molecule A<sup>g7</sup> (7–9). These animals spontaneously develop a very aggressive form of arthritis, beginning at 3 to 4 wk of age. As in humans, the disease is chronic, progressive, and symmetrical, and it exhibits all of the classical histological features: leukocyte invasion, synovitis, pannus formation, cartilage, and bone destruction. The articular manifestations are the result of arthritogenic Abs directed against GPI, which develop at high titers in K/BxN mice because of the preferential help that B cells expressing GPI-specific Igs receive from GPI-reactive T cells displaying the transgene-encoded TCR. Affinity-purified anti-GPI IgG from these mice can transfer disease to healthy recipients in the absence of any lymphocytes.

A great paradox in the K/BxN model is the exquisite specificity of the joint attack provoked by the auto-Abs, reactive against a ubiquitously expressed antigen. GPI is a cytoplasmic enzyme, essential for basic carbohydrate metabolism, and is normally sequestered in the cell cytoplasm, only being released in the circulation in minute amounts in conditions that involve cell damage or apoptosis (for refs, see reference 9). One possible resolution to the paradox is that the anti-GPI Abs are cross-reactive to a joint-specific antigen. It was therefore important to analyze the specificity of the anti-GPI response. We derived hybridomas from K/BxN mice, screening for anti-GPI reactivity by an ELISA; we report the molecular and biophysical properties of a panel of anti-GPI mAbs, in relation to their capacity to induce arthritis.

## Materials and Methods

**Mice.** KRN TCR tg mice have been described (8). They were maintained on the C57Bl/6 (B) background. Crossing these K/B animals with NOD/Lt (N) mice generated arthritic K/BxN offspring. Balb/c mice were 4 wk old when used as serum recipients.

**mAbs.** Hybridomas producing anti-GPI mAbs were generated using standard procedures. Single-cell suspensions were prepared from different organs of arthritic K/BxN mice of different ages and, without prior culture or stimulation, were fused to the nonsecreting myeloma cell line P3X63-AG8.653. The primary plates were screened for Ab production by ELISA against recombinant GPI. Positive wells were subcloned two or three times by limiting dilution and selection for GPI reactivity, and were expanded. mAbs were purified from supernatants on protein G Sepharose (Amersham Pharmacia Biotech), 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 eluted fractions were concentrated and switched to PBS buffer by centrifugation (Centricon-30; Amicon) and then titered by ELISA for anti-GPI activity. Heavy and light chain VDJ regions were sequenced from amplified mRNA (see online supplemental data).

ELISAs were performed as described (9). Briefly, 96-well flat-bottom ELISA plates (Maxisorb; Nunc) were coated with 5 μg/ml of recombinant GPI as a fusion protein with glutathione S-transferase (GST) (9) or 5 μg of GST in PBS at 4°C overnight. They were then blocked with bovine BSA, incubated with the supernatant, washed, and incubated with alkaline phosphatase-coupled Abs to mouse total IgG (or, when appropriate, with isotype-specific anti-IgG1, -IgG2a, -IgG2b, or -IgG3 reagents [Jackson ImmunoResearch Laboratories]).

**Arthritis Transfer.** Sera from paired lots of K/BxN mice or control BxN littermates (40 to 60 d of age) were pooled and injected intraperitoneally, in 150–250 μl total volume (adjusted with PBS if necessary). In most cases, recipients got two injections at a two-day interval. Purified mAbs were also injected at a 2 d interval. Arthritis was scored by clinical examination (clinical index as described [10], caliper measurement of ankle thickness [7, 11]).

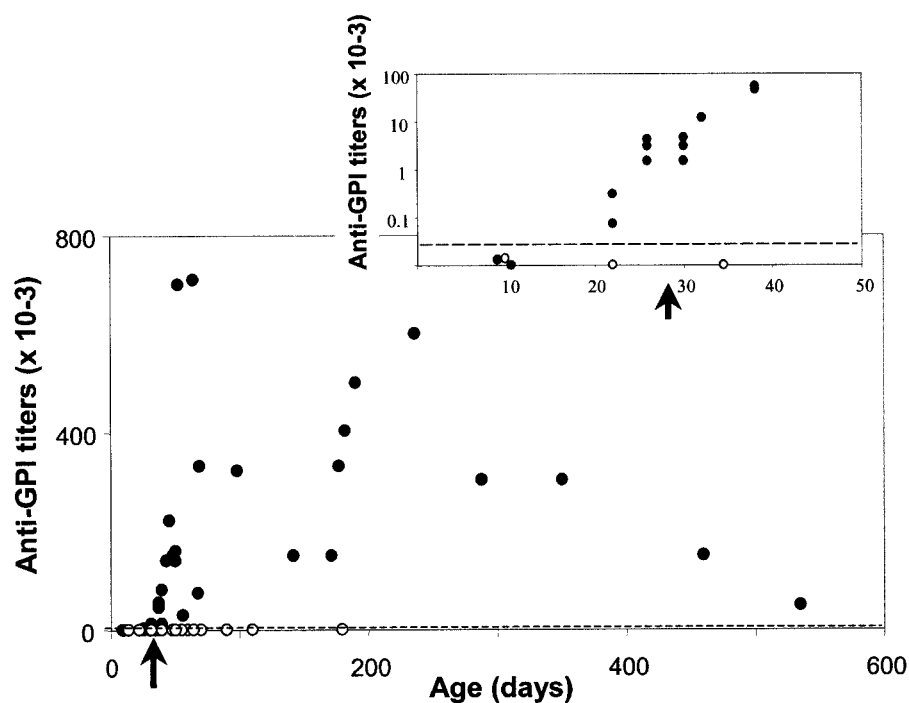
**Surface Plasmon Resonance Analyses.** Surface plasmon resonance (SPR) measurements were performed with a BIAcore 2000 (Biacore) as detailed in online supplemental data, with rabbit anti-mouse Fc or anti-GPI mAbs immobilized on the sensor chip via amino groups (12). Soluble rGPI (9) was injected in the fluid phase, and data were fitted to 1:1 Langmuir binding or to two-state conformational change models (BIA evaluation 3.0 software). For the two-Ab assays, the capturing anti-GPI mAb1 was directly immobilized on the sensor chip at high density (3 to 10 kRU). GPI was injected for 10 min, and the mAb-GPI complexes washed, leaving stable mAb1-GPI complexes. The second mAb2 was then injected, and its binding quantitated as the molar ratio (MR) of the reactant by the following relationship:  $(MR = [RU_{mAb}/RU_{Ag}] \times [MW_{Ag}/MW_{mAb}])$  (13).

**Online Supplemental Data Section.** Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20011941/DC1>

## Results and Discussion

**An Extraordinarily High Frequency of GPI-reactive B Cell Clones in K/BxN Mice.** As the self-reactive T cells of K/BxN mice constantly stimulate B cells capable of internalizing GPI and presenting peptides derived from it, we hypothesized that GPI-specific B cells would be highly represented among the activated, Ig-secreting population in these animals, and that they would be amenable to fusion without additional immunization. To establish the optimum age for hybridoma production, we titered anti-GPI Abs in the sera of a large panel of different-aged K/BxN mice. Anti-GPI specificities were detectable at the very onset of disease, and increased markedly to reach a plateau at around 60 d of age (Fig. 1). Hybridoma fusions were subsequently performed with cells from different lymphoid organs of young donors at the initiation of arthritis manifestations (day 29) and with older donors exhibiting well-established disease and higher Ab titers (50–60 d).

Many GPI-reactive hybridomas resulted from these fusions, the fraction of positive wells in the first screening progressing with age (Table I). This fraction reached impressive levels (100% in two different experiments) with splenocytes from animals older than day 50. The plating densities after fusion and during HAT selection were such that an average of 2 to 4 B cell clones grew in each well. Thus, while it is not possible to formally deduce clonal frequencies from these data, they do suggest that a major proportion (25%, if not more) of Ig-secreting B cells in the spleen of K/BxN mice produce anti-GPI Abs. These numbers are consistent with the very high anti-GPI titers in the serum. This impressive representation probably reflects the power of T-B cell cooperation in such animals (7), as those B cells able to pick up the very low concentrations of circulating GPI via high-affinity surface receptors will preferentially receive T cell help. The



**Figure 1.** Anti-GPI Abs at different ages. Serum from K/BxN mice (filled circles) or transgene-negative littermates (open circles) was tested by ELISA. Titters were determined as the serum dilution for anti-GPI reactivity fivefold above background. Each point represents an individual mouse. Inset: results from mice at early ages (note the different scale for the titers). Arrows: day of arthritis onset (day  $30 \pm 3$ ) in most K/BxN mice.

KRN TCR tg is unusual in this regard, as such an amplification of matching B cells has not been observed in other autoreactive TCR tg animals. This property may be a peculiarity of the clonotype-expressing T cells and their escape from complete clonal deletion (8), the manner in which GPI-reactive B cells are tolerated, or the biochemistry of GPI itself.

Interestingly, isotype usage also varied with age: anti-GPI Igs of the IgG2b and IgG2a isotypes were found with younger mice, but the proportion of IgG1 increased to become the exclusive isotype with older animals (Table I), in keeping with the dominance of IgG1 anti-GPI Abs in sera from K/BxN mice (8).

In contrast to spleen, much more modest frequencies of anti-GPI hybridomas resulted from fusions of lymph node or

bone marrow cells (Table I). Attempts were also made to fuse lymphocytes from the synovial fluid of arthritic joints, but few wells showed hybridoma growth, and these were not GPI-reactive (not shown), consistent with the low abundance of B cells in the K/BxN arthritic lesion. Thus, the spleen appears to be the main site of production of anti-GPI Abs in this model.

*Arthritogenic Activity of mAbs.* The pathogenic potential of these mAbs was then tested. In pilot studies, small-scale preparations of Igs were made from minimally expanded uncloned hybridomas; in other experiments, the hybridoma cells were transferred directly into irradiated mice. No arthritic recipients were ever observed, testing an aggregate of 40 GPI-reactive hybridomas. In parallel, a panel of hybridomas was selected and subcloned twice by limiting dilution. Secreted

**Table I.** Frequency of GPI-reactive Wells in the Initial Screens

Fusion	Age (d)	Organ	No. of wells screened	Percent GPI reactive <sup>a</sup>	Isotype among GPI-reactive (%)		
					IgG1	IgG2b	IgG2a
1	29	Spleen	175	8	nd	nd	nd
2	29	Spleen	137	12	70	23	6
9	35	Spleen	207	13	89	11	0
7	40	Spleen	264	42	100	0	0
6	50	Spleen	290	100	100	0	0
5	60	Spleen	128	100	100	0	0
7	40	Bone marrow	33	3	100	0	0
8	50	Bone marrow	300	7	100	0	0
8	50	Lymph node	65	20	100	0	0

<sup>a</sup>Proportion of positive wells. Fusion efficiencies were comparable in the various experiments, with an average of 2–4 hybridoma clones in each well.

IgG was purified and tested for arthritogenic activity by injection into naive Balb/c recipients, using regimens and doses that consistently gave disease with affinity-purified serum anti-GPI IgG. Balb/c was the recipient of choice because, of the common strains, it shows the highest susceptibility to K/BxN serum-induced arthritis (11). Again, none of the individual mAbs could induce either clinical symptoms of arthritis or histological manifestation of a joint disorder (Table II).

In contrast, pools of the mAbs did provoke arthritis. The pool of all nine mAbs induced a severe and robust disease (Table II), characterized by a rapid onset and joint swelling largely equivalent to that observed with polyclonal preparations of anti-GPI IgG. The histological alterations were comparable with those described previously, including inflammatory infiltration of the synovium and articular cavity, synovial hyperplasia, pannus formation, and cartilage erosion (data not shown). As is typical of K/BxN arthritis, spontaneous or induced by serum transfer, there was a strong distal predominance. The pool induced the same disease manifestations in lymphoid-deficient RAG<sup>o/o</sup> mice (not shown). Robust disease could also be conferred by a subset of seven Abs, all of the IgG1 isotype, or by smaller pools of only four, three, or even two mAbs. Yet, as the number of Abs was reduced, the disease became less reproducible and less aggressive (Table II).

To test which mAbs were most effective at complementing each other, we injected all 36 possible pairwise combinations into Balb/c recipients (Fig. 2 A). Only 10 of these were able to provoke clinical symptoms of disease, which in some cases reached the maximum score of 4 and showed the typical histological alterations described above. However, whereas the injection of serum Ig or larger pools caused aggressive disease in nearly 100% of the recipients, such a high incidence was never achieved with the mAb pairs; rather, when arthritis could be induced, it showed partial penetrance. With the marked exception of the two

IgG2b mAbs, all individual Abs within the pool participated to at least some extent in arthritogenic pairs.

All of the arthritogenic combinations involved IgG1 Abs, confirming that anti-GPI Igs of this isotype are capable, on their own, of inducing arthritis. Mouse IgG1 has a very poor capacity for interacting with C1q, the initiator of the classical pathway of complement activation. Thus, Abs of this isotype would seem, at first, to be unlikely candidates for the initiating agent in a model totally dependent on complement activation (11). However, we have recently found that it is the alternative, not the classical, pathway that is involved in arthritis induction by anti-GPI Abs (10). In the alternative pathway, Igs enhance C3 activation by binding C3b in a covalent fashion, thus preventing its inactivation by factor H. Mouse IgG1 is quite active in this regard (14). Multimerized IgG1 is also efficient at activating FcγRIII, the dominant player in the second arm of the K/BxN effector phase (10). Thus, the identification of IgG1 as the main and only required isotype for pathogenesis is fully consistent with what we know so far about the effector mechanisms in K/BxN arthritis. As IgG1 is an isotype that is tightly associated with Th2-biased help and IL-4 activities, these two elements may be elements of K/BxN pathogenesis. The implication is that, if this model proves relevant to human arthritis, one should view with great circumspection proposed therapeutic protocols involving Th2-promoting regimens.

**Kinetics and Affinity of the Anti-GPI mAbs.** To generate clues about why some mAb pairs, but apparently not others, could complement for arthritis induction, we measured the binding kinetics and GPI affinity of the nine mAbs via Biacore analysis.

First, experimental conditions were optimized to limit the complexity of the analyzed interaction. To eliminate mass transport as a limiting factor, we captured small quantities of mAbs (100 RU) on anti-Fc surfaces. Before kinetic runs, each

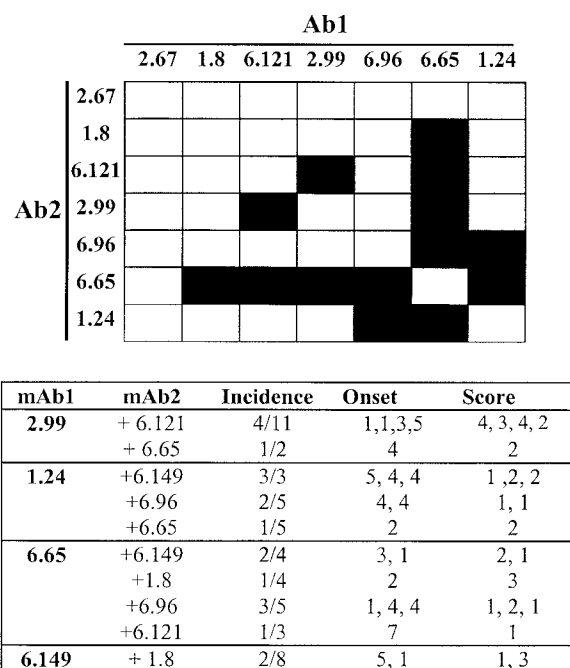
**Table II.** *Anti-GPI mAbs Can Induce Arthritis when Combined, but Not Individually*

Injection	Dose	Max CI	Onset (d)	Δ Ankle thickness (mm) <sup>a</sup>
Individual mAbs 2.56 <sup>a</sup> , 2.67 <sup>a</sup> , 1.8, 6.121, 2.99, 6.96, 6.65, 1.24, 6.149	2 × 1 mg	0, 0	–	–
Pools				
All nine	(2 × 222 μg/ea)	4, 4, 4, 3	3, 1, 2, 2	0.32, 0.73, 0.86, 0.73
All IgG1s (all but 2.56 and 2.67)	(2 × 286 μg/ea)	3, 3	4, 3	1.01, 0.8
1.8 + 6.121 + 6.149 + 6.96	(2 × 500 μg/ea)	4, 0, 2, 2, 1	3, –, 3, 3, 4	0.8, –, 1.01, 1.1, 0.9
1.8 + 6.121 + 6.149 + 6.65	(2 × 500 μg/ea)	3, 4	1, 3	1.11, 0.93
2.56 + 2.67 + 6.149	(2 × 660 μg/ea)	1, 0, 0, 4, 1, 0	24, –, –, 2, 6, –	
1.8 + 6.65 + 6.149	(2 × 660 μg/ea)	4, 4, 2.5, 4, 4	1, 1, 1, 2, 2	1.19, 0.96, 0.51, 0.99, 0.99
6.121 + 6.65 + 6.149	(2 × 660 μg/ea)	3, 4, 2.5	1, 2, 2	0.81, 0.98, 0.58
6.65 + 6.149	(2 × 1 mg/ea)	2, 0, 1, 0	3, –, 1, –	

Abs purified by protein-G chromatography were injected into 4-wk-old Balb/c recipients either individually or as pools. Arthritis was scored as clinical index and by measurement of ankle thickening (represented as the difference between thickness at day 7 and day 0).

<sup>a</sup>IgG2b (all others IgG1).

## A Pathogenicity



## B Concomitant binding (MR)

		Ab1						
		2.67	1.8	6.121	2.99	6.96	6.65	1.24
Ab2	2.67	-0.03	0.06	-0.01	0.2	0.16	0.31	0.09
	1.8	0.07	0.03	0.3	0.46	0.4	0.42	0.19
	6.121	-0.01	0.10	0	0.22	0.13	0.37	0.1
	2.99	0.43	0.48	0.45	0.01	0.03	0.69	0.57
	6.96	0.40	0.38	0.40	0	0	0.45	0.51
	6.65	0.36	0.28	0.46	0.38	0.36	0.02	0.26
	1.24	0.55	0.32	0.19	-0.10	0.40	0.73	0

**Figure 2.** Complementing pairs of anti-GPI mAbs. (A) Pairs of mAbs were tested by injection into healthy mice. Black squares indicate pathogenicity (clinical score >1, incidence >20%), white squares no clinical or histological lesions (at least two mice tested for each pair). Illustrative day of onset and maximum arthritis scores are shown below. (B) Anti-GPI mAbs were tested as pairwise combinations in Biacore experiments, where Ab1 was first bound to the chip, GPI was added, and the binding of the second Ab tested for reactivity to the preformed Ab1-GPI complex. Data are expressed as molar ratio, representing the relative increase in signal generated by binding of the second antibody (0, no binding; 1, theoretical maximum binding). Black squares represent an absence of binding over background, gray squares detectable binding of Ab2.

mAb surface was tested by injecting the lowest concentration of GPI, at variable flow rates. Flow rates had no effect on binding rates, indicating that mass transport limitations were not an issue (15). The nine mAbs were analyzed by measuring the binding of soluble GPI injected in the fluid phase (Table III; online supplemental data). In general, all mAbs had a high affinity for GPI, with a  $K_d$  of  $5 \times 10^{-8}$  M or better. Simple 1:1 Langmuir binding models fit readily the curves of two sets of mAbs: one group ("low  $k_{off}$ " mAbs), which includes mAbs

2.56, 6.149, 1.8, and 6.96, formed complexes with GPI with a half-life of  $\sim 1/2$  h. A second group ("very low  $k_{off}$ " mAbs), consisting of 6.65 and 1.24, formed complexes more stable by an order of magnitude. A third set included mAbs 2.67, 6.121, and 2.99, whose interaction with GPI was more complex, a two-state reaction involving a conformational change that stabilizes the complex ( $A + B \leftrightarrow AB \leftrightarrow AB^*$ ) giving the best fit with the experimental data.

In general, arthritogenic pairs tended to involve the mAbs with the slowest off-rates (6.65, 1.24), but this was not an absolute. Complementation did not necessarily require combination of mAbs with different affinities or dissociation rates.

As a minimum of two mAbs was needed to provoke disease, an immediate question was whether simultaneous binding of these arthritogenic pairs to GPI was required to initiate the events culminating in joint inflammation, or whether complementing but uncoordinated binding of different molecules was involved. The former would be necessary should complementation function by inducing cross-linking of GPI into multivalent complexes. Therefore, dual interactions were examined. Saturating amounts of capture mAb (mAb1) were first immobilized, and stable complexes were formed by injecting GPI. A second mAb (mAb2) was then injected and its interaction with the mAb1-GPI complex evaluated; the signal increment due to the binding of mAb2 was expressed as molar ratio (MR) values (Fig. 2 B).

Some mAb pairs were mutually incompatible (MR of 0; for example 2.67/6.121, or 2.99/6.96), most likely due to overlapping epitopes. On the other hand, many of the mAb pairs were characterized by a significant MR, indicating that they could bind simultaneously to GPI. Most of these interactions were symmetrical, as would have been predicted, although this was not always the case. In some instances, it was important which Ab bound to GPI first: for example, 1.24 could bind to the 2.67-GPI complex, but the opposite did not happen. The epitope map resolved the mAbs into four groups. According to the  $M_r$  criterion, mAbs 2.67, 1.8, and 6.121 could be grouped together, as they bound to the same cluster of epitopes, showing a similar, although slightly different, pattern of recognition. The second group corresponded to mAbs 2.99 and 6.96, while mAbs 6.65 and 1.24 were distinct. From competitive ELISA results (not shown), 6.149 would belong to the same group as 1.24. Comparison of these cobinding patterns with the pathogenicity of individual pairs (Fig. 2 B) clearly showed that corecognition of the same GPI molecule was required for arthritogenesis: none of the cross-blocking pairs induced disease.

As illustrated above, the more individual Abs injected, the more robust the disease. The data rule out several potential explanations for this phenomenon. First, it does not reflect a need for effector functions mediated by Abs of different isotypes, as IgG1 mAbs alone sufficed. Nor does it reflect epitope unmasking and cooperative binding — rather, if anything, the opposite. On the other hand, these pathogenic mAbs had to bind GPI simultaneously, and the improvement in disease induction with increasing mAb number implies that large multimeric complexes must be formed. This notion of large Ab/Ag complexes is consistent

**Table III.** Kinetics Constants of Anti-GPI mAbs–GPI Interactions

Kinetics parameters		Low k-off mAbs				Very low k-off mAbs		Biphasic dissociation mAbs		
		2.56	6.149	1.8 <sup>a</sup>	6.96	1.24 <sup>a</sup>	6.65	2.99	6.121	2.67 <sup>a</sup>
$k_{\text{off}}$	$1 \times 10^{-4} (\text{s}^{-1})$	$3.3 \pm 0.01$	$6 \pm 0.1$	$3.6 \pm 1.4$	$4.7 \pm 0.01$	$0.2 \pm 0.1$	$0.5 \pm 0.01$	$65 \pm 0.7$	$40 \pm 0.3$	$100 \pm 4$
	$2 \times 10^{-4} (\text{s}^{-1})$	–	–	–	–	–	–	$5 \pm 0.03$	$2 \pm 0.03$	$2.9 \pm 0.4$
$t_{1/2}$	1 min	35	19	32	24	640	240	2	3	1
	2 min	–	–	–	–	–	–	23	60	40
$k_{\text{on}}$	$1 \times 10^{-4} (\text{M}^{-1}\text{s}^{-1})$	$5.0 \pm 0.01$	$11.6 \pm 0.06$	$42 \pm 0.9$	$78 \pm 0.1$	$14.6 \pm 0.2$	$9.2 \pm 0.01$	$54 \pm 0.2$	$78 \pm 0.2$	$180 \pm 10$
	$2 \times 10^{-4} (\text{M}^{-1}\text{s}^{-1})$	–	–	–	–	–	–	$37 \pm 0.2$	$28 \pm 0.2$	$56 \pm 18$
$k_{\text{d}}$	$\times 10^{-9} (\text{M})^{\text{b}}$	6.6	5	$0.8 \pm 0.3$	0.6	$0.12 \pm 0.07$	0.5	1.6	0.37	$1.8 \pm 0.03$
	Pathogenicity <sup>c</sup>	0	3	2	2	2	6	2	2	0

Kinetic data were obtained by capturing mAbs by a first anti-IgGFc (RAM) immobilized on the dextran and using GPI as soluble analyte. Association ( $k_{\text{on}}$ ) and dissociation ( $k_{\text{off}}$ ) rate constants were obtained by numerical integration combined with global fitting using five different GPI concentrations (9.5 nM to 152 nM). Data were fitted to a simple 1:1 bimolecular interaction model or a two state reaction model for mAbs 2.67, 6.121, and 2.99. The quality of the fits was assessed by the random distribution of the residuals and  $\chi^2$  values usually below 3.0.

<sup>a</sup>For mAbs 2.67, 1.8, and 1.24, the kinetic constants are the mean  $\pm$  SD of data obtained on two different sensor chips.

<sup>b</sup>The  $K_{\text{d}}$  are obtained from the ratio  $k_{\text{off}}/k_{\text{on}}$  and for mAbs 2.67, 6.121, and 2.99 from  $(k_{\text{off1}} \times k_{\text{off2}})/k_{\text{on1}} \times k_{\text{on2}}$ .

<sup>c</sup>Number of pathogenic pairwise combinations in which the antibody participates.

with recent observations demonstrating the requirement for two major inflammatory effector arms in K/BxN arthritis: complement activation and FcR signaling (10). Complement activation is enhanced by Ig deposition and immune complexes, and the cross-linking of multiple FcRs by immobilized Igs arrays is most effective at initiating a functional response (16). As discussed elsewhere (17), multimerization may correspond either to deposition of preformed complexes that can be found circulating in the serum of K/BxN mice, or to multimeric stabilization of arrayed GPI on joint surfaces. Whichever the explanation (or both?), the data presented here indicate that multimerization of GPI/anti-GPI complexes is an essential component of K/BxN pathology.

Dual-binding assays showed that mAb pairs that could not bind simultaneously were ineffective at inducing arthritis. However, there was no explanation for other pairs that were capable of simultaneous binding yet were ineffective at inducing arthritis. These pairs might induce an infrequent disease that would not have been detected with the limited groups involved. They might also fall just below the threshold for productive activation — none of the mAb pairs was very efficient in any case. Or, more interestingly, the relative molecular angles adopted by these mAb1–GPI–mAb2 complexes might preclude assembly into larger lattices. These negative results must be treated with a certain degree of caution, however, as these combinations were not tested extensively.

The requirement for more than one mAb for disease induction has also been described in another mouse model of arthritis: a minimum combination of three anti-collagen II mAbs was needed to provoke collagen-induced arthritis (CIA; reference 6), suggesting that similar effector mechanisms might be involved in the two models. This is consistent with the fact that genetic factors that influence arthritis

in the K/BxN and CIA models appear quite similar (18, 19). However, there are some interesting differences in the effects of mAbs in the two cases. First, there is obviously far more collagen-II than GPI in the joint, and so one might have expected anti-collagen to be more effective than anti-GPI at eliciting inflammation. But the opposite was true, as the doses of Ig required for the latter were markedly lower than the 10 mg of anti-collagen mAbs routinely used. Second, the dominant and most efficient isotype of anti-GPI mAbs was IgG1, while the best results were obtained with a cocktail of IgG2a and IgG2b anti-collagen mAbs (6), in keeping with earlier observations that IgG2 subclasses correlated with CIA pathology (20).

*Ig Gene Usage.*  $V_{\text{H}}$  and  $V_{\text{L}}$  nucleotide sequences were determined for the nine anti-GPI mAbs by PCR amplification with degenerate primer, followed by sequencing of the PCR products (results presented in the online supplemental data). As the mAbs were derived from the spleens of several different mice, most should have been clonally independent. The sequences confirmed this, none of the heavy or light chain sequences of the nine mAbs being identical to another. On the other hand, there are distinct similarities between the heavy chains: six are encoded by genes derived from the  $V_{\text{H}}1$  family (aka J558), particularly intriguing as the  $V_{\text{H}}\text{s}$  used in 1.8, 6.121, 2.56, and 2.67 all stem from subgroup 20; in addition, 6.149 and 6.96 are both encoded by the  $V_{\text{H}}14$  gene (aka SM7).  $V_{\text{K}}$  gene usage shows no such restrictions, since 5 different  $V_{\text{K}}$  gene families encode the light chains of 8 of the anti-GPI mAbs in the pool.

The  $V_{\text{H}}$  and  $V_{\text{L}}$  sequences were compared with the germ-line genes. A large number of mutations is observed (from 3 to 36, median 18), the majority leading to amino acid replacements (online supplemental data). It is not possible to

conclude with certainty that any single mutation arose from somatic mutation, because the germline sequences could have arisen from either the B6 or NOD Ig loci of the donor K/BxN mice. On the other hand, that none of the sequences is identical or even closely related to any of the database germline sequences is certainly indicative of a high overall degree of somatic mutation. Perhaps the clearest example entails mAbs 6.149 and 6.96: they originated from the same mouse, and their heavy chain genes are clearly related, as the CDR3 joining regions are identical; yet, they show four nucleotide differences. This finding suggests a common IgH rearrangement event and later divergence by somatic mutation. Interestingly, sequences of anti-GPI Fabs from human RA patients also indicated somatic mutation (21).

**Conclusion.** These findings finalize the identification of GPI as the true target of the arthritogenic Igs in K/BxN mice. First, the fact that arthritis could be induced by the injection of pooled mAbs grown in vitro rules out the possibility that any serum-derived contaminant, cofractionated during the affinity purification of anti-GPI IgG (9), causes the joint lesions. Second, earlier results did not definitively eliminate the possibility that the anti-GPI Igs cross-reacted with another, joint-specific, molecule, although no such molecule could be detected (9). However, the fact that seven different anti-GPI mAbs reactive against at least four distinct epitopes could participate in arthritis induction renders such an explanation highly unlikely — it is difficult to conceive of how there could be cross-reactivity between this entire quartet and elements of another molecular species. Instead, multimeric crosslinking by high-affinity antibodies seems at the root of K/BxN arthritis.

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## References

- Zvaifler, N.J. 1973. The immunopathology of joint inflammation in rheumatoid arthritis. *Adv. Immunol.* 265:265–336.
- Harrington, W.J., V. Minnich, J.W. Hollingsworth, and C.V. Moore. 1951. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J. Lab. Clin. Med.* 115:636–645.
- Ding, X., L.A. Diaz, J.A. Fairley, G.J. Giudice, and Z. Liu. 1999. The anti-desmoglein 1 autoantibodies in pemphigus vulgaris sera are pathogenic. *J. Invest. Dermatol.* 112:739–743.
- Panayi, G.S., J.S. Lanchbury, and G.H. Kingsley. 1992. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum.* 35:729–735.
- Stuart, J.M., and F.J. Dixon. 1983. Serum transfer of col-

- lagen-induced arthritis in mice. *J. Exp. Med.* 158:378–392.
- Terato, K., K.A. Hasty, R.A. Reife, M.A. Cremer, A.H. Kang, and J.M. Stuart. 1992. Induction of arthritis with monoclonal antibodies to collagen. *J. Immunol.* 148:2103–2108.
- Korganow, A.-S., H. Ji, S. Mangialaio, V. Duchatelle, R. Pelandra, T. Martin, C. Degott, H. Kikutani, K. Rajewsky, J.-L. Pasquali, C. Benoist, and D. Mathis. 1999. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity.* 10:451–461.
- Kouskoff, V., A.-S. Korganow, V. Duchatelle, C. Degott, C. Benoist, and D. Mathis. 1996. Organ-specific disease provoked by systemic autoreactivity. *Cell.* 87:811–822.
- Matsumoto, I., A. Staub, C. Benoist, and D. Mathis. 1999. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science.* 286:1732–1735.
- Ji, H., K. Ohmura, U. Mahmood, D.M. Lee, F.M.A. Hofhuis, S.A. Boackle, V.M. Holers, M. Walport, C. Gerard, A. Ezekowitz, et al. 2002. Arthritis critically dependent on innate immune system players. *Immunity.* 16:157–168.
- Ji, H., D. Gauguier, K. Ohmura, A. Gonzalez, V. Duchatelle, P. Danoy, H.J. Garchon, C. Degott, M. Lathrop, C. Benoist, and D. Mathis. 2001. Genetic influences on the end-stage effector phase of arthritis. *J. Exp. Med.* 194:321–330.
- Lofas, S., and B. Johnsson. 1990. A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands. *J. Chem. Soc. Chem. Commun.* 21:1526–1528.
- Scalice, E.R., D.J. Sharkey, and J.L. Daiss. 1994. Monoclonal antibodies prepared against the DNA polymerase from *Thermus aquaticus* are potent inhibitors of enzyme activity. *J. Immunol. Methods* 172:147–163.
- Klaus, G.G., M.B. Pepys, K. Kitajima, and B.A. Askonas. 1979. Activation of mouse complement by different classes of mouse antibody. *Immunology.* 38:687–695.
- Myszka, D.G. 1999. Improving biosensor analysis. *J. Mol. Recognit.* 12:279–284.
- Ravetch, J.V., and S. Bolland. 2001. IgG Fc receptors. *Annu. Rev. Immunol.* 19:275–290.
- Matsumoto, I., M. Maccioni, D.M. Lee, M. Maurice, B. Simons, M. Brenner, D. Mathis, and C. Benoist. 2002. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. *Nat. Immunol.* 3:360–365.
- Adler, A.J., D.W. Marsh, G.S. Yochum, J.L. Guzzo, A. Nigam, W.G. Nelson, and D.M. Pardoll. 1998. CD4<sup>+</sup> T cell tolerance to parenchymal self antigens requires presentation by bone marrow-derived antigen-presenting cells. *J. Exp. Med.* 187:1555–1564.
- Johansson, A.C., M. Sundler, P. Kjellen, M. Johannesson, A. Cook, A.K. Lindqvist, B. Nakken, A.I. Oldstad, R. Jonsson, M. Alarcon-Riquelme, and R. Holmdahl. 2001. Genetic control of collagen-induced arthritis in a cross with NOD and C57BL/10 mice is dependent on gene regions encoding complement factor 5 and FcγRIIb and is not associated loci controlling diabetes. *Eur. J. Immunol.* 31:1847–1856.
- Watson, W.C., and A.S. Townes. 1985. Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci. *J. Exp. Med.* 162:1878–1891.
- Schaller, M., D.R. Burton, and H.J. Ditzel. 2001. Autoantibodies to GPI in rheumatoid arthritis: linkage between animal model and human disease. *Nat. Immunol.* 2:746–753.