Arthritis Provoked by Linked T and B Cell Recognition of a Glycolytic Enzyme

Isao Matsumoto,* Adrien Staub, Christophe Benoist,*† Diane Mathis*†

The hallmark of rheumatoid arthritis (RA) is specific destruction of the synovial joints. In a mouse line that spontaneously develops a disorder with many of the features of human RA, disease is initiated by T cell recognition of a ubiquitously expressed self-antigen; once initiated, pathology is driven almost entirely by immunoglobulins. In this study, the target of both the initiating T cells and pathogenic immunoglobulins was identified as glucose-6-phosphate isomerase, a glycolytic enzyme. Thus, some forms of RA or related arthritides may develop by a mechanism fundamentally different from the currently popular paradigm of a joint-specific T cell response.

The joint degradation characteristic of RA is thought to result from an autoimmune response, although the precise roles and targets of self-reactive B and T cells remain controversial (*1*–*4*). Given the disease's articular localization, it is tempting to infer that the response is elicited by an antigen (Ag) specifically expressed in the joints. However, there is currently no compelling evidence for or against this, so identification of a self-Ag that can spontaneously provoke arthritis would be an important advance.

The K/B×N T cell receptor (TCR) transgenic (tg) mouse line spontaneously develops a joint disorder with many of the clinical, histological, and immunological features of RA in humans (*5*, *6*). This line was generated by crossing the KRN/C57Bl/6 TCR tg strain with the NOD strain. By chance, the KRN TCR sees an unknown peptide bound to NOD-derived A^{g7} molecules on all major histocompatibility complex (MHC) class II–positive cells. Thus, in the F_1 animals, KRN T lymphocytes emerge into an environment of systemic self-reactivity. There is tolerance induction at multiple levels, but it is only partial. B lymphocytes are also critical for the K/B×N disease (*5*), engaging in a critical A^{g7}:TCR-mediated interaction with KRN T cells and ultimately acting by secreting arthritogenic immunoglobulins (Igs) (*7*). Injection of as little as 100 μl of serum from sick K/B×N mice into healthy animals, even lymphocyte-deficient ones, provokes arthritis within days. The arthritogenic activity in K/B×N serum resides in the immunoglobulin G (IgG) fraction and depends on particular Ig specificities, not rheumatoid factor [RF; antibody to IgG (anti-IgG)] or anti–collagen-II.

As a first step in identifying the Ag recognized by the arthritogenic Igs, we prepared different types of extracts (for example, NP-40 and GuHCl) from various mouse tissues and analyzed them by protein immunoblotting with K/B×N or control littermate serum, revealing bound Igs with an anti–mouse IgG reagent (*8*). With K/B×N serum, several bands showed up, but one band from the NP-40 extracts appeared dominant, corresponding to a protein of about 60 kD. When a large number of sera from K/B×N and control mice were screened, the band at 60 kD was always observed and always dominant with the former, but not with the latter; it was not specific to ankle extracts, also being present in spleen and kidney extracts (for example, Fig. 1A). The 60-kD protein was further highlighted in immunoprecipitation analyses: Precipitates of [^{35}S]methionine-labeled ankle extracts with K/B×N, but not control, sera yielded a single 60-kD protein (Fig. 1B).

To identify the 60-kD protein, we purified IgGs from a large pool of control or K/B×N sera and attached them to a matrix (*9*). Pooled NP-40 kidney extracts were passed over these matrices, the columns were washed extensively, and bound proteins were eluted. Upon electrophoresis and staining, only weak bands appeared with the control column eluate, but a dominant band showed up at 60 kD with the eluate from the K/B×N IgG column (Fig. 1C). This band was excised, the protein in the gel slice was digested by trypsin, and the resulting peptides were resolved by high-performance liquid chromatography (HPLC), allowing the isolation of several of them. Three peptides were sequenced by automatic Edman degradation (Fig. 1D), and the sequences were compared with public data banks with the BLAST program on the Swissprot database. All three peptide sequences resided within the glucose-6-phosphate isomerase (GPI; E.C.5.3.1.9) protein sequence. The molecular mass of GPI (62.636 kD) coincides well with that of the protein recognized by K/B×N serum (~60 kD), as estimated from the protein immunoblots.

To confirm that GPI is indeed the protein seen by K/B×N serum, we produced recombinant mouse GPI in *Escherichia coli* (*10*). The recombinant protein, a fusion product with glutathione S-transferase (GST), was purified on a glutathione affinity column, electrophoresed, blotted, and probed with sera from K/B×N or control mice. All K/B×N sera reacted strongly with the recombinant protein, whereas con-

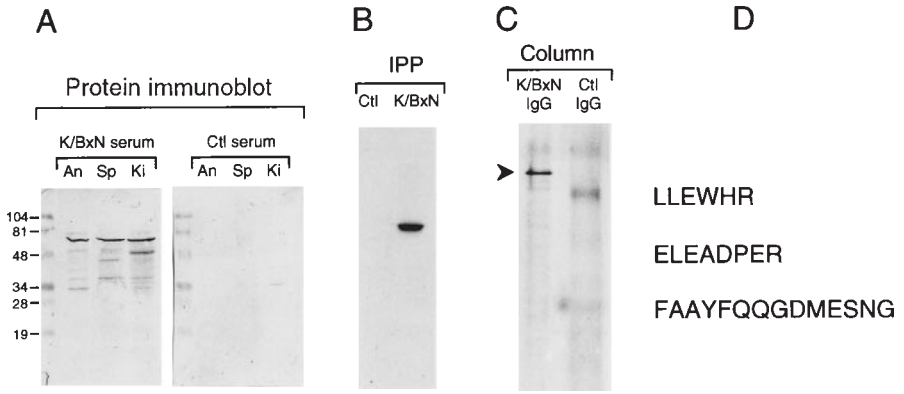

Fig. 1. Identification of GPI. (**A**) Protein immunoblot analysis of NP-40 extracts from ankle (An), spleen (Sp), or kidney (Ki) of Ig-deficient mice, with serum from a 35-day-old K/B×N mouse or normal littermates (*28*). Ctl, control. Numbers at left give molecular mass in kilodaltons. (**B**) Immunoprecipitation (IPP) with similar sera from [^{35}S]methionine-labeled ankle extracts (3 hours of metabolic labeling in vitro). (**C**) Immunopurification of kidney extracts on affinity matrices coupled with IgG from arthritic or control sera. (**D**) Peptides sequenced from the immunopurified 60-kD protein. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; W, Trp; and Y, Tyr.

Institut de Génétique et de Biologie Moléculaire et Cellulaire (CNRS/INSERM/ULP), BP 163, 67404 Illkirch, C.U. de Strasbourg, France.

*Present address: Joslin Diabetes Center, Harvard Medical School, One Joslin Place, Boston, MA 02215, USA.

†To whom correspondence should be addressed.

trol sera did not (Fig. 2A). Enzyme-linked immunosorbent assays (ELISA) with recombinant GPI/GST indicated that K/B×N sera also saw GPI (Fig. 2B). Reactive antibodies (Abs) were detected with K/B×N sera up to dilutions of $1/10^6$. [Sometimes, at far lesser dilutions (for example, 1/100), trace reactivity could also be found with control sera.] Thus, GPI is the 60-kD protein seen by serum Igs from arthritic K/B×N mice.

To address the question of whether anti-GPI Igs are really the pathogenic Abs, we made GST or GPI/GST affinity columns and passed a pool of sera from arthritic K/B×N mice sequentially over them (*11*). The bound proteins were eluted and injected into $RAG^{-/-}$ mice, as were portions of the starting material and the flow-through fraction (*12*). All arthritogenic activity resided in the fraction bound to the GPI/GST column; none appeared in the flow-through fraction, even though it contained a large portion of the Igs (Fig. 3). Therefore, antibodies to GPI are the pathogenic Igs contained in serum from arthritic K/B×N mice, and no other arthritogenic Abs can be detected. GPI-specific Abs were first detected in the blood at low concentrations around the third week after birth, corresponding to the onset of clinical arthritis, and steadily increased until 8 to 10 weeks (*13*).

Given precedents that B cells present Ags to T cells particularly effectively when they can internalize them through their Ig receptors (*14*), we wondered whether KRN T cells might not see the same Ag as the arthritogenic Igs. Indeed, they do: Recombinant GPI, when offered by NOD antigen-presenting cells (APCs), could stimulate either the original KRN T cell hybridoma or KRN T cells from tg mice (*15*) (Fig. 4). Stimulation was efficient, comparable with that by $H\text{-}2^k$ APCs presenting bovine ribonuclease [the original specificity of the KRN TCR (*16*)], and was specific because non–TCR tg cells did not respond.

Thus, the molecular target of both self-reactive KRN T cells and the arthritogenic Igs in K/B×N serum is GPI, a critical enzyme of the glycolytic pathway, expressed in essentially all tissues from the earliest stages of embryogenesis until death, although with some quantitative variations (*17*–*19*). It normally resides in the cytoplasm, but soluble GPI has been detected in human (*20*) and murine (*13*) serum. This finding substantiates our earlier suggestion that a joint-specific disease can result from systemic self-reactivity (*5*). However, arthritis is not an inevitable consequence of widespread autoreactivity in TCR tg mice: KRN T cells also recognize an unknown self-peptide presented by a variant A^k complex, but when this class II molecule was expressed in KRN TCR tg mice, there were no signs of joint pathology (*21*). No doubt, a strict set of conditions must be met for arthritis to develop. The key to pathology is probably linked T/B cell stimulation by a self-protein that resides in the cytoplasm of most cells and also occurs at a very low concentration in the blood. All MHC class II–positive cells synthesize GPI, and certain of these, in particular dendritic cells, should be capable of stimulating T cells that can recognize it (*5*, *13*). However, most cells, including the bulk of B cells, will not present GPI very effectively because they are poor at displaying peptides derived from cytoplasmic proteins and are inept at taking up and displaying peptides derived from low-concentration, blood-borne proteins. Only those rare B cells expressing high-affinity GPI-specific Igs on their surface will be capable of extracting it from the blood and efficiently presenting derivative peptides (*14*). These cells will be activated and helped by their T cell cognates in a manner that is qualitatively different from any influence on the bulk of B cells, resulting in high-level production of a very narrow slice of the Ig repertoire.

A critical outstanding issue is how this systemic self-reactivity provokes arthritis in the absence of other autoimmune manifestations. It is possible that special features of GPI expression in synovial tissues are key—concentration, subcellular partitioning, and secretion—especially because joints are characterized by high metabolic activity. Or it is possible that the pathogenic Igs cross-react with another protein specifically expressed in joints. However, we did not find evidence of any such pathogenic cross reactivity: K/B×N serum did "light up" a few bands in GuHCl extracts of ankle, but not

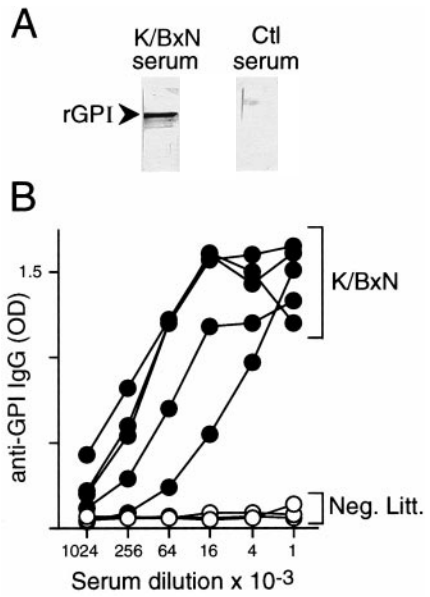

Fig. 2. GPI is recognized by Igs from arthritic K/B×N mice. Sera from arthritic or control mice were tested for reactivity to recombinant GPI/GST (rGPI) protein by (**A**) protein immunoblots (sera at 1/100 dilution) or (**B**) ELISA (each curve represents an individual mouse). Control GST protein gave no detectable signal in either assay.

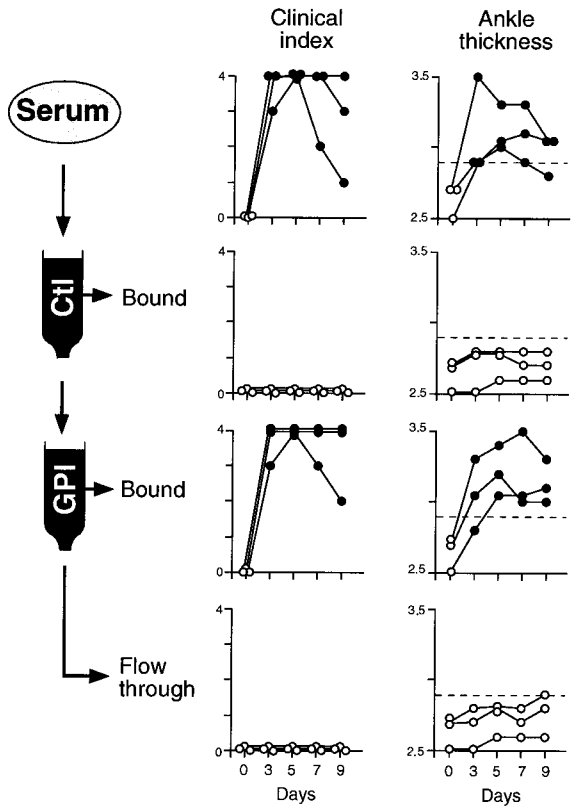

Fig. 3. GPI can adsorb arthritogenic Igs. Whole serum from K/B×N mice was passed sequentially over GST- or GPI/GST-coupled affinity columns, and portions of the fractions were tested by injection into $RAG^{-/-}$ mice. Arthritis was read out as clinical index (*12*) or ankle thickness (millimeters).

other tissue, but these specificities partitioned in the nonarthritogenic flow-through fraction when K/B×N sera were passed over GPI affinity columns (*13*). We favor the notion that some unusual physiological feature of joints is responsible for focusing the autoimmune destruction, perhaps related to their being rich in proteoglycans, devoid of a basement membrane, hypoxic, and subject to reduced blood flow. Some aspect of the following pathway might thereby be promoted: Immune complexes, formed inside or outside the joint, activate the complement cascade and engage Fc receptors (*13*), setting off a chain of destructive events, including recruitment and activation of inflammatory cells and synoviocytes, overproduction of growth factors and cytokines, and synthesis of degradative enzymes. A likely possibility is enhancement of the production or retention of immune complexes, but effects on downstream events, for example, the recruitment of inflammatory cells, are also possible. The development of arthritis in lymphocyte-deficient mice systemically overproducing tumor necrosis factor (TNF)–α (*22*) may reflect convergence somewhere along such a pathway.

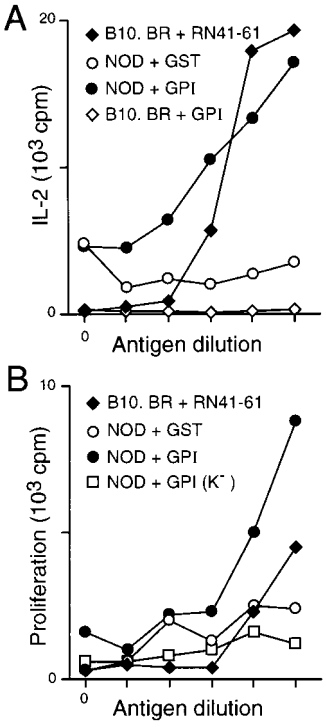

Fig. 4. (**A** and **B**) GPI is recognized by KRN T cells. The KRN T cell hybridoma or primary T cells from KRN TCR tg mice (on the neutral C57Bl/6 background) were stimulated with APCs from NOD or B10.BR mice and varying doses of Ag (fivefold serial dilutions; 41 to 61 peptide from bovine ribonuclease, maximum concentration, 10 μg/ml; recombinant GPI/GST or GST control protein, maximum concentration, 1 mg/ml). K^-, response of lymph node cells from nontransgenic control littermates. The bovine ribonuclease peptide presented by A^k represents the original specificity of the KRN receptor (*16*).

Finally, how do these results relate to human arthridites? Whether or not RA patients' arthritis is initiated by a joint-specific Ag has been debated for years, without a satisfying resolution. The view that it is has been espoused by many ever since injection of collagen-II into rodents was found to provoke arthritis (*23*). However, there is no compelling evidence that collagen-II or any other joint-specific Ag is a central player in RA—indeed, attempts to modulate disease by inducing tolerance with a joint-specific Ag administered orally (by means of a bystander effect) have been disappointing (*24*). Whether the identification of GPI as the Ag that initiates arthritis in K/B×N mice bears on this issue obviously depends on how relevant this model is to human RA. As has been discussed at length (*5*, *6*), it does show marked similarities but is certainly not a perfect mimic. It remains possible that the K/B×N model is most relevant to a related form of arthritis (for example, psoriatic arthritis) or a subset of RA (for example, juvenile RA, which often shares a lack of RF and a similar slightly atypical pattern of joint involvement). We suggest that a scenario similar to that proposed for the K/B×N model might unfold in at least some arthritis patients. GPI could be the inciting Ag, but equally plausible is that other self-Ags with similar properties are responsible. Certain aspects of this scenario are reminiscent of disease mechanisms proposed in the 1970s (*25*, *26*), never refuted but eventually disregarded because a paradigm focusing on a joint-specific T cell response became the dominant one. This route to autoimmune disease may not be restricted to arthritis as Igs recognizing ubiquitously expressed proteins have been observed in other organ-specific autoimmune diseases, although their pathological relevance remains to be established (*27*).

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6. The major similarities are that both disorders are chronic, progressive, and symmetrical; have distal to proximal gradient of severity; are MHC allele-specific; display synovitis, pannus formation, and cartilage and bone destruction; display cytokine (TNF-α and interleukin-6) inbalances; and are TNF-α–dependent. The major differences are as follows: overall severity, distal interphalangial joint involvement, occasional mild spinal inflammation, and RF negativity (*5*, *13*).
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8. Minced tissues from Ig-deficient mice were extracted in 50 mM tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40 for 30 min on ice. Protein immunoblots were performed and probed with sera (1/30 dilution) or purified IgG; bound Ig was revealed with alkaline phosphatase-conjugated anti–mouse IgG. For immunoprecipitations, ankle tissue was minced, washed in methionine-free medium and 10% bovine serum albumin, and labeled with 250 μCi of [^{35}S]methionine in medium for 3 hours at 37°C. An NP-40 extract was prepared as above, precleared with normal mouse serum and protein A–Sepharose (Pharmacia), and precipitated again with K/B×N serum and protein A beads. These beads were washed three times, and the bound proteins were eluted in SDS sample buffer, separated by electrophoresis, and revealed by autoradiography.
9. NP-40 kidney extract (150 mg total protein) was passed over affinity columns containing 15 mg of protein G–purified IgG from K/B×N or control sera. After extensive washing, the bound proteins were eluted with 0.1 M glycine (pH 2.8), concentrated on Centricon, and separated by SDS–polyacrylamide gel electrophoresis. Coomassie-stained bands were cut out, freeze-dried, and digested with 2 μg of trypsin in 50 μl of 100 mM $(NH_4)_2CO_3$ (pH 8.0). The resulting peptides were separated on a C-18 reversed-phase HPLC column, and several were sequenced by automated Edman degradation (ABI-477A), or their molecular mass was measured by electrospray mass spectrometry (LCT, Micromass).
10. Murine GPI cDNA was amplified by reverse transcriptase polymerase chain reaction from total kidney RNA, with the addition of restriction enzyme sites for in-frame cloning into the pGEX-4T-3 vector (primers GGAGAATTCTATGGCTGCGCTCACCCGGAA and TTCCTCGAGTCCCAACAGCCTTGTAGTCC, where underlining indicates the cloning sites positions 53 and 1834 of M14220, respectively). Recombinant GST fusion protein was prepared from soluble *E. coli* extracts and purified by affinity chromatography on glutathione-Sepharose columns (as per manufacturer's instructions). The purified GPI/GST fusion protein was used as such in assays, with the GST moiety prepared in parallel as control. Serum reactivity was tested on protein immunoblots as above, with 2 μg of recombinant GPI/GST per lane, and detection with alkaline phosphatase-conjugated anti-IgG or by ELISA with plates coated with recombinant GPI/GST or GST control proteins (5 μg/ml) and bound Ab detected by alkaline phosphatase-conjugated anti–mouse IgG (Jackson ImmunoResearch).
11. Recombinant GPI-GST or control GST proteins (*10*) were coupled to Sepharose (2.5 mg, 0.5-ml bed volume); 300 μl of serum diluted in 5 ml of phosphate-buffered saline (PBS) was applied sequentially. After extensive washing, bound Ig was eluted at pH 2.8, and the fractions were concentrated and exchanged to PBS (Centricon) before injection.
12. For serum-transfer experiments, RAG-deficient mice on the C57Bl/6x129 background were injected at a 2-day interval with serum or fractions from affinity purifications, and arthritis was scored by clinical examination (clinical index defined as follows: 0, normal; 1, doubt; 2, two paws affected; 3, three paws affected; 4, all limbs affected) and by caliper measurement of ankle thickness (*5*, *7*).
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28. Arthritic K/B×N serum donors were bred by crossing asymptomatic KRN TCR tg mice on the C57Bl/6 or B10.BR background with NOD/Lt animals (*5*, *7*). The arthritic progeny were bled between 45 and 70 days of age for pools of arthritic serum. Control serum came from non-tg littermates.
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Proapoptotic Bcl-2 Relative Bim Required for Certain Apoptotic Responses, Leukocyte Homeostasis, and to Preclude Autoimmunity

Philippe Bouillet, Donald Metcalf, David C. S. Huang, David M. Tarlinton, Tom W. H. Kay, Frank Köntgen, Jerry M. Adams,*† Andreas Strasser*†

Apoptosis can be triggered by members of the Bcl-2 protein family, such as Bim, that share only the BH3 domain with this family. Gene targeting in mice revealed important physiological roles for Bim. Lymphoid and myeloid cells accumulated, T cell development was perturbed, and most older mice accumulated plasma cells and succumbed to autoimmune kidney disease. Lymphocytes were refractory to apoptotic stimuli such as cytokine deprivation, calcium ion flux, and microtubule perturbation but not to others. Thus, Bim is required for hematopoietic homeostasis and as a barrier to autoimmunity. Moreover, particular death stimuli appear to activate apoptosis through distinct BH3-only proteins.

Apoptosis is essential for normal development, tissue homeostasis, and immune function; its altered regulation can trigger cancer, autoimmunity, and degenerative disorders (*1*). Dismantling of the cell is carried out by cysteine proteases (caspases) (*2*), but initiation of many apoptotic responses is regulated by members of the Bcl-2 protein family (*3*). They appear to govern the activity of adapter proteins such as Apaf-1 required to activate apical caspases such as caspase-9 (*3*). Some members, like Bcl-2 itself, promote cell survival, but two other subfamilies instead promote cell death. Those such as Bax exhibit considerable sequence homology with Bcl-2, possessing three of the four Bcl-2 homology (BH) domains, but the others, which include EGL-1 of *Caenorhabditis elegans* and at least six mammalian proteins (Bad, Bik, Blk, Hrk, Bid, Bim), share only the short (9- to 16-residue) BH3 domain with the Bcl-2 family (*3*). This domain allows them to bind to the prosurvival Bcl-2-like molecules and neutralize their function. Several BH3-only proteins are present in healthy cells but are maintained, by different mechanisms, in a latent form until unleashed by cytotoxic signals (*4*–*6*). For example, after engagement of death receptors such as Fas/APO-1 (CD95), activated caspase-8 cleaves Bid, generating a more proapoptotic form (*4*). On the other hand, the potent proapoptotic molecule Bim (*7*), the subject of this study, is expressed in many cell types but normally sequestered to the microtubular dynein motor complex by interaction with dynein light-chain LC8 (*6*). Apoptotic stimuli provoke release of Bim and LC8, allowing Bim to associate with Bcl-2-like proteins.

Little has been established about the physiological roles of the mammalian BH3-only proteins, but the finding that EGL-1 of *C. elegans* is required to initiate all its developmental cell death (*8*) argues that they may be critical triggers of apoptosis. For example, Bid appears to be important in the death of hepatocytes after Fas engagement (*9*). To identify the biological roles of Bim, we disrupted its gene in the mouse (*10*). The targeting vector replaces the exon encoding BH3, which is essential for proapoptotic function (*7*), with a *neo* cassette flanked by LoxP sites (Fig. 1A). Chimeric mice generated from two independent targeted embryonic stem (ES) cell clones (263 and 266) were crossed with C57BL/6 mice to produce animals heterozygous for the *bim* mutation, and crosses with Cre-expressing deleter mice (*11*) yielded a progeny line lacking the *neo* cassette (266 Del). ES cells and mice were genotyped by Southern blot and polymerase chain reaction (PCR) analysis (Fig. 1B). When $bim^{-/-}$ mice were generated, as expected their spleen cells contained no full-length Bim protein (Fig. 1C). A truncated BH3-less polypeptide anticipated from the mutant allele appears to be unstable, because it represented <5% of wild-type (WT) Bim. The mutant allele behaves as a null mutation, because the truncated polypeptide, even when highly expressed in transfected cells, does not affect apoptosis (*12*).

Bim appears to have an important, albeit unidentified, role in embryonic development. Although interbreeding of $bim^{+/-}$ mice produced healthy and fertile $bim^{-/-}$ offspring, their number was less than half that of +/+ progeny (Fig. 1D). The marked deficit in mutant progeny ($P < 0.001$) is not attributable to adventitious mutations introduced during ES cell manipulation, because it appeared in both independent *bim* mutant strains and persisted after deletion of the *neo* cassette (Fig. 1D). A preliminary analysis indicated that $bim^{-/-}$ fetuses die before embryonic day 10 (E10). However, the penetrance of the embryonic lethality appears to be strongly affected by genetic background (*13*), as found with mice lacking certain other cell death regulators (*14*). Hence, we are placing the mutation on inbred 129 and C57BL/6 backgrounds to permit studies of the basis for the embryonic deaths.

Bim is expressed in many hematopoietic cell types (*7*), and its loss markedly affected homeostasis in that compartment (Table 1). Although the number of red cells was normal, blood leukocytes were elevated severalfold because of two- to fourfold increases ($P < 0.05$) in B cells, mature T cells ($CD4^+8^-$ and $CD4^-8^+$), granulocytes, and monocytes (Table 1). Surprisingly, although megakaryocyte numbers were normal, platelets were half the normal amount (Table 1). This substantial drop may indicate that their shedding from megakaryocytes depends on Bim-dependent mechanisms akin to apoptosis. Like the blood, the spleen and lymph nodes of young adult $bim^{-/-}$ mice contained two to three times as many leukocytes as WT littermates, mostly because of elevated B and T cells (Table 1). The frequency of hematopoietic progenitors in bone marrow was normal, however, and most of the leukocytes were small, noncycling cells. Hence, their accumulation probably reflects extended cell survival rather than excessive proliferation.

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia.

*These authors share senior authorship.
†To whom correspondence should be addressed. E-mail: adams@wehi.edu.au and strasser@wehi.edu.au