Interleukin-4 Can Be a Key Positive Regulator of Inflammatory Arthritis

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Objective. **Development of arthritis in the K/BxN mouse model depends on the induction of high titers of antibodies against the enzyme glucose-6-phosphate isomerase (GPI), promoted by CD4+ T cells expressing a GPI-specific transgenic T cell receptor (TCR). This study was undertaken to determine whether this strong autoantibody response depends on T cell differentiation to the Th1 or Th2 phenotype.**

Methods. **The roles of Th cell–biasing cytokines were investigated by introducing the interleukin-4 (IL-4) and IL-12–specific subunit p35 (IL-12p35)– knockout mutations into the K/BxN model and evaluating the impact of these deficiencies on disease. The IL-4–expressing cell types in K/BxN mice were revealed by crossing in a knockin alteration, which resulted in green fluorescent protein expression controlled by endogenous IL-4 gene–regulatory elements. Transfer experiments permitted the identification of the IL-4– producing cell type required for arthritis, and quantitative reverse transcriptase–polymerase chain reaction allowed for determination of the cytokine profile of K/BxN T cells.**

Results. **While IL-12p35 appeared dispensable for the development of arthritis, IL-4 was crucial for full development of disease. The GPI-reactive TCR of standard K/BxN mice induced the transcriptional activation of the IL-4 locus in CD4+ T cells and eosinophils, and CD4+ T cells were the obligatory source of IL-4 for disease. However, the cytokine profile of K/BxN T cells revealed that K/BxN arthritis is not a "pure" Th2 disease.**

Conclusion. **The K/BxN model, although not a classic Th2 disease, depends critically on IL-4. The potential of IL-4 to promote inflammatory arthritis should be considered when proposing therapies for rheumatoid arthritis aimed at biasing T cells toward IL-4 production.**

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease of unclear etiology. Many cellular and molecular factors contribute to the pathogenesis, rendering proposed disease scenarios very complex. Cytokines play major roles in the development of RA (for review, see ref. 1), and significant progress has been made in identifying those that contribute to the effector phase of disease. Therapeutic strategies aimed at neutralizing proinflammatory effector cytokines such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and IL-6 have greatly improved the treatment of RA and other inflammatory arthritides (2–5). In terms of disease initiation, it is often considered that the autoreactive T cell response in RA is biased toward a Th1-type response (6), and exploration of therapeutic strategies that entail blockade of the Th1-associated cytokine interferon-γ (IFNγ) has begun (7). However, the evidence for such a classification is circumstantial, mainly based on the presence of inflammatory cells in the lesion. The actual roles of various Th1- and Th2-type cytokines in the induction of RA remain rather unclear. Understanding how these cytokines regulate disease may prove critical for designing effective therapies.

The functions of IL-4 impinge on many facets of

the immune response (for review, see ref. 8). IL-4 induces the expression of class II major histocompatibility complex (MHC) molecules on macrophages and dendritic cells (DCs). It also contributes to DC maturation and activation, and to B cell proliferation and activation. IL-4 is a well-documented mediator of Th2 cell commitment, and induces Ig class switching to the Th2-associated isotypes IgG1 and IgE. However, IL-4 can exhibit antiinflammatory effects, including suppression of macrophage functions such as IL-1 and TNFα production (9,10). IL-4 also has the ability to suppress synoviocyte proliferation (11). Although the positive contribution of IL-4 to Th2-biased responses is usually emphasized, it is the balance of the diverse functions of IL-4 that determines its overall effect on a particular immune response.

IL-12 is produced mainly by activated antigen-presenting cells (APCs), including DCs (for review, see ref. 12). Because it has an impact on many components of cell-mediated immunity, this cytokine is important for immune responses against intracellular pathogens, and is also known to influence a diversity of autoimmune diseases. IL-12 is considered to be at the apex of Th1 responses because it induces IFNγ in various cell types, including T cells and natural killer (NK) cells, and promotes the differentiation of naive CD4+ T cells into Th1 cells.

The role of IL-4 in RA has been studied using various mouse models, with often-divergent findings. For example, in the collagen-induced arthritis (CIA) model, some studies demonstrated IL-4 to be dispensable for disease, while others showed arthritis to be reduced in its absence (13–15). Experiments in the proteoglycan-induced arthritis model indicated that IL-4 has a protective role in that disease (16,17). The role of IL-12 has also been studied in the CIA model. Administration of the antibody against the p40 subunit of IL-12 resulted in decreased titers of anti–type II collagen antibody, and led to either the abrogation (18) or the decreased severity (19) of arthritis. However, since blockade of the p40 subunit would have affected both IL-12 and IL-23, these studies are difficult to interpret. More recently, Murphy and colleagues reported that mice genetically deficient in the IL-12–specific subunit p35 (IL-12p35) had more severe CIA (20).

The K/BxN mouse model of inflammatory arthritis affords a good opportunity to examine the role of IL-4 and IL-12 in an autoantibody-mediated model of RA. In this model, the initiation and effector phases of disease are readily distinguished, the autoantigen has been identified, and the autoreactive T cell population

can be conveniently monitored. The K/BxN mouse expresses the KRN T cell receptor (TCR) transgene and the NOD-derived class II MHC molecule A^{g7}. K/BxN mice spontaneously develop an arthritis with many similarities to human RA (21–23). The majority of T cells in K/BxN mice express the KRN TCR, which recognizes a peptide derived from the ubiquitous glucose-6-phosphate isomerase (GPI) enzyme presented by A^{g7} molecules (24). Thus, KRN T cells in a mouse that expresses A^{g7} molecules are constitutively activated by endogenous GPI/A^{g7} complexes. These T cells subsequently help B cells to produce anti-GPI antibodies, which induce the development of severe arthritis.

In hybridoma fusion experiments, we found an amazingly high frequency of GPI-specific cells among antibody-producing B cells in arthritic K/BxN mice (25). Presumably, a unique set of factors comes into play during T cell–B cell collaboration in K/BxN mice, which promotes exuberant expansion of autoreactive B cells and autoantibody production. After the initiation phase of disease, which culminates in the production of pathogenic antibodies, neither T cells nor B cells are required for the subsequent effector phase. Accordingly, injection of serum or purified IgG from arthritic K/BxN mice into alymphoid recipients can transfer arthritis (26). Therefore, one can easily dissect out the effector phase of disease using the K/BxN serum-transfer model.

We evaluated the roles of IL-4 and IL-12 in K/BxN arthritis by crossing the corresponding knockout mutations into the model. Surprisingly, only IL-4 proved important. We then focused on the cellular and molecular underpinnings of this dependence, and concluded that the Th1/Th2 paradigm is inappropriately simplistic in this instance.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) and NOD/Lt (NOD) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). KRN TCR–transgenic mice have been described previously (21). They were maintained on the B6 background (K/B6). Crossing K/B6 animals with NOD mice generated arthritic K/BxN offspring. H-2^{g7} congenic mice on the B6 background (B6^{g7}) were created in our animal facility. Mice deficient in IL-4 (IL-4$^{-/-}$) (27), TCRα (C$\alpha^{-/-}$) (28), and IL-12p35 (29), all on the B6 background, were purchased from The Jackson Laboratory. Male 4get mice (knockin mutants in which the endogenous *Il4* gene has been replaced with an *Il4*/internal ribosome entry site/enhanced green fluorescent protein [EGFP] construct, resulting in the generation of a bicistronic transcript under the control of endogenous *Il4* gene–regulatory elements) on the BALB/c background (30) were bred with female K/BxN mice, and their offspring were intercrossed to

obtain $KRN^+4get^+H-2^{g7+}$ mice or those lacking 1 of those 3 elements. Experiments were conducted in compliance with federal and institutional guidelines and with the approval of the Institutional Animal Care and Use Committee at Harvard University (protocol no. 3024).

Serum transfer model and evaluation of arthritis. Serum-induced arthritis was transferred by intraperitoneal injection of 150 μl serum from 8-week-old K/BxN mice on days 0 and 2. Ankle thickness was measured with a caliper (J15 micrometer; Blet, Lyon, France). Each limb was scored on a scale of 0 (no observable swelling) to 3 (severe inflammation). The scores of the 4 limbs were added together to obtain the clinical index (maximum score 12 points). Histologic analysis of ankle specimens was performed as previously described (22).

Genotyping. Genotyping of the KRN transgene and I-A molecules was performed by flow cytometric analysis of peripheral blood using anti-TCR $V_\beta6$, anti-CD4, anti-A^{g7}, and anti-A^b antibodies. The presence of the 4get construct was determined by EGFP in the peripheral blood, as detected by flow cytometry. Genotyping of the IL-4$^{-/-}$ and C$\alpha^{-/-}$ mice was performed by polymerase chain reaction (PCR) with genomic DNA.

Proliferation assays. Responder splenocytes were sorted as described below and plated at 10^4 cells per well. B6^{g7} splenocytes were used as stimulator cells and were pretreated with 50 μg/ml mitomycin C and plated at 10^5 cells per well. Recombinant murine GPI–glutathione S-transferase (GST) was added to the cultures at various concentrations. After 3 days, cultures were pulsed overnight with 1 μCi/well of ^{3}H-labeled thymidine, harvested, and the counts per minute determined using a beta counter.

Enzyme-linked immunosorbent assay (ELISA). Titers of anti-GPI antibody were measured by ELISA. Recombinant mouse GPI was coated on ELISA plates at 5 μg/ml. Mouse sera were serially diluted (1:100 to 1:64,000). Subsequently, alkaline phosphatase (AP)–conjugated anti-mouse total IgG or biotinylated anti-mouse IgG1, IgG2a (cross-reactive with IgG2c), IgG2b, IgM, IgA, or IgE, followed by AP-conjugated streptavidin, were applied. After substrate addition, the reaction was determined using an ELISA reader. Arbitrary units were assigned based on the serum dilution factor at which the optical density first appeared above background.

Cell sorting. For cell transfers, CD4+ splenocytes were positively enriched with directly conjugated MACS beads (Miltenyi Biotec, Sunnyvale, CA). For proliferation assays, CD4+ T cells were enriched by negative sorting using MACS beads directly conjugated to antibodies against CD8, CD11b, and B220. To sort GFP+ and GFP− cells, as well as CD4+ T cells for cytokine messenger RNA (mRNA) real-time PCR analysis, a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO) was used.

Real-time reverse transcriptase–PCR (RT-PCR). RNA was isolated from sorted cells using TRIzol reagent and subjected to reverse transcription with oligo(dT) primer and SuperScript II polymerase (Invitrogen, San Diego, CA). Real-time RT-PCR was then performed (Mx3000P kit; Stratagene, La Jolla, CA). Gene-specific fluorogenic assays (TaqMan; Applied Biosystems, Foster City, CA) of IL-4, IFNγ, IL-5, IL-13, IL-10, and TNFα were performed. Nonspecific estimation of product accumulation by intercalator dye fluorescence (SYBR Green) was used to quantitate transforming growth factor $\beta1$ (TGF$\beta1$) transcripts (assay was verified by gel electrophoresis and melting curve analysis). Hypoxanthine guanine phosphoribosyltransferase was used as an internal standard.

RESULTS

IL-4 is crucial for the development of arthritis. To evaluate the contribution of cytokine biases to the K/BxN model of inflammatory arthritis, we generated mice that were deficient in either IL-4 or IL-12p35 and that expressed the KRN transgene on the B6.H-2^{g7} background (IL-4$^{-/-}$ K/B6^{g7} and IL-12p35$^{-/-}$ K/B6^{g7} mice). All IL-4–expressing control mice (IL-4$^{+/+}$ K/B6^{g7} and IL-4$^{+/-}$ K/B6^{g7}) developed severe arthritis. In contrast, as a group, the IL-4$^{-/-}$ K/B6^{g7} mice showed drastically reduced arthritis. The clinical indices and changes in ankle thickness of 5 representative mice are shown in Figure 1A, and the distribution of mice of each genotype according to age at disease onset and maximum ankle thickness is plotted in Figure 1B. Of the 21 IL-4$^{-/-}$ K/B6^{g7} mice that were examined, 7 mice (33%) did not develop arthritis, 9 (43%) showed slight to moderate disease, which subsided after several weeks, and the remaining 5 (24%) developed severe arthritis, comparable in intensity with that of IL-4–expressing littermates, although somewhat delayed in time of onset. Histologic examination of ankle joints from nonarthritic IL-4$^{-/-}$ K/B6^{g7} mice revealed no signs of inflammation, such as leukocyte infiltration or synovial cell hyperplasia (Figure 1C). In contrast, the joints from arthritic IL-4$^{-/-}$ K/B6^{g7} mice showed a degree of cell infiltration, synovial cell hyperplasia, pannus formation, and destruction of cartilage and bone comparable with findings in the joints of arthritic IL-4$^{+/-}$ K/B6^{g7} mice (Figure 1C).

We also evaluated the role of IL-12, a key initiator of Th1-biased responses, in K/BxN arthritis. IL-12 belongs to a family of covalently linked heterodimeric cytokines. Only the p35 chain of IL-12 is unique to IL-12; thus, in order to evaluate the role of this cytokine in our model, we generated IL-12p35–proficient and IL-12p35–deficient K/B6^{g7} mice. As shown in Figure 1D, there were no substantial differences in the development of arthritis between IL-12p35$^{-/-}$ K/B6^{g7} and IL-12p35$^{+/-}$ K/B6^{g7} mice, which showed that IL-12 is dispensable in this model.

IL-4 is not required during the effector phase of arthritis. Arthritis developed spontaneously in K/B6^{g7} mice, the culmination of distinct initiation and effector phases. The markedly reduced arthritis in IL-4$^{-/-}$
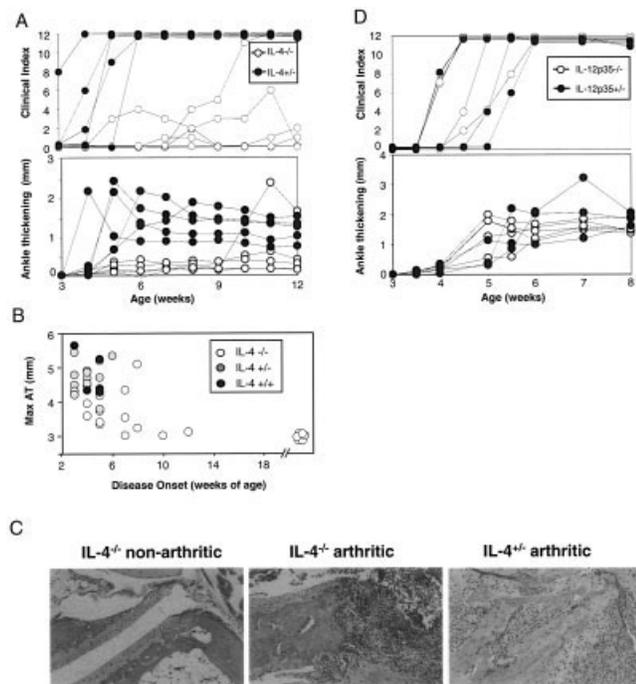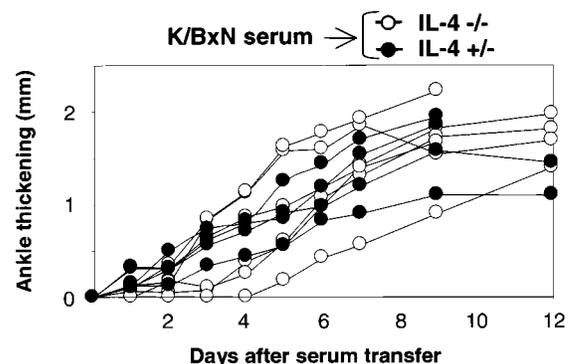
Figure 1. Role of interleukin-4 (IL-4) in K/BxN arthritis. **A,** Clinical index and change in ankle thickness (relative to baseline measurements) over time in IL-4$^{-/-}$ or IL-4$^{+/-}$ KRN-transgenic mice on the B6^{g7} background (K/B6^{g7}). Data from 5 representative mice from each group are shown. **B,** Age at disease onset and maximum ankle thickness (Max AT) in each IL-4$^{-/-}$, IL-4$^{+/-}$, or IL-4$^{+/+}$ K/B6^{g7} animal. **C,** Joint sections from arthritic and nonarthritic IL-4$^{-/-}$ K/B6^{g7} mice and from arthritic IL-4$^{+/-}$ K/B6^{g7} mice (hematoxylin and eosin stained; original magnification × 200). **D,** Clinical index and change in ankle thickness over time in IL-12p35$^{-/-}$ and IL-12p35$^{+/-}$ K/B6^{g7} mice.

K/B6^{g7} mice indicated that IL-4 is required for disease development, but did not distinguish its requirement during the initiation phase, effector phase, or both. The K/BxN serum-transfer model bypassed the initiation phase and focused on the effector phase subsequent to autoantibody accumulation. To determine whether IL-4 is required in the effector phase of the K/BxN disease, serum from arthritic mice was transferred into IL-4$^{-/-}$ or IL-4$^{+/+}$ animals. The IL-4$^{-/-}$ mice developed disease as severe as did their wild-type counterparts (Figure 2). Together with the severely impaired arthritis development observed in IL-4$^{-/-}$ K/B6^{g7} mice, these results indicated that IL-4 plays an essential role before, but not after, the generated pathogenic autoantibodies.

Activation of GPI-specific T cells is not impaired in the absence of IL-4. There is an obligatory series of events that precedes autoantibody production in K/BxN mice. First, potentially autoreactive KRN thymocytes

must escape central tolerance induction and must be exported from the thymus into the periphery. These T cells must be responsive to activation by APCs presenting the cognate GPI peptide. Following T cell activation, crosstalk between GPI-specific T cells and B cells occurs, resulting in the production of extremely high titers of anti-GPI autoantibodies. To elucidate the point along this pathway at which IL-4 affects disease initiation, we followed these events in IL-4$^{-/-}$ K/B6^{g7} mice.

The phenotype and activation status of peripheral T cells were evaluated by flow cytometric analyses of lymph node (Figure 3) and spleen (data not shown) cells from IL-4$^{-/-}$ K/B6^{g7} and IL-4$^{+/+}$ K/B6^{g7} mice at 7–9 weeks of age. Based on the expression of CD4 and the transgene-encoded TCR chain (V$_\beta$6), IL-4 deficiency seemed to have no effect on the selection or expansion of KRN T cells in the periphery (Figure 3A). In both IL-4 wild-type and mutant mice, the transgene-encoded V$_\beta$6 chain was expressed by the majority of CD4+ T cells, but with a telltale accumulation of cells with intermediate-to-low V$_\beta$6 levels, in response to the GPI/A^{g7} complex (21). The IL-4 deficiency did not affect the proportion of T cells displaying 2 TCR chains (V$_\beta$6 and V$_\beta$8) (Figure 3B), a feature previously reported for T cells in the K/BxN model (21). Based on expression of the very early activation marker CD69, no difference was seen in the activation of IL-4$^{-/-}$ and IL-4$^{+/+}$ K/B6^{g7} T cells (Figure 3C). The CD25+,CD69− populations, presumed to include regulatory T cells, were also similar in size in IL-4$^{-/-}$ and IL-4$^{+/+}$ K/B6^{g7} mice (Figure 3C), which showed that an expansion of this regulatory T cell population did not contribute to disease suppression in IL-4$^{-/-}$ K/B6^{g7} mice.

Figure 2. Interleukin-4 (IL-4) in the effector phase of arthritis. K/BxN serum (150 μl) was injected into IL-4$^{-/-}$ mice and control B6 mice on days 0 and 2. The change in ankle thickness (relative to baseline measurements) over time in 6 IL-4$^{-/-}$ mice and 4 control B6 mice was plotted. Results are representative of 3 independent experiments.
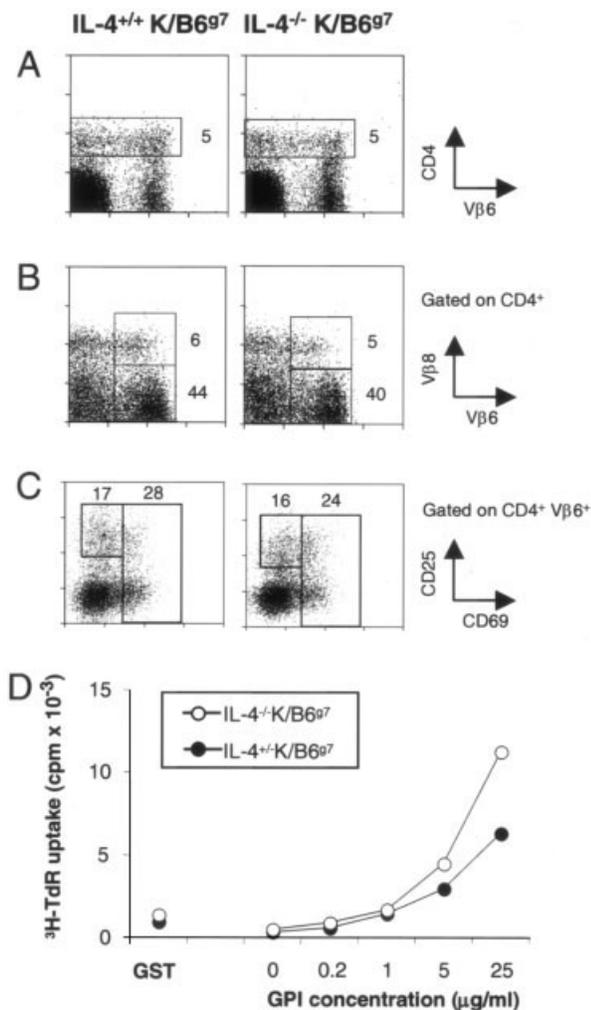
Figure 3. Comparison of T cell responses in interleukin-4–knockout (IL-4$^{-/-}$) K/B6^{g7} mice and IL-4$^{+/+}$ K/B6^{g7} mice. **A–C,** Lymph node cells from 7–9-week-old IL-4$^{+/+}$ K/B6^{g7} and IL-4$^{-/-}$ K/B6^{g7} mice were stained with antibodies against CD4, V$_\beta$6 T cell receptor (TCR) (transgenic), V$_\beta$8 TCR (endogenous), CD69, and CD25. Fluorescence-activated cell sorting profiles representative of 3 IL-4$^{+/+}$ K/B6^{g7} and 5 IL-4$^{-/-}$ K/B6^{g7} mice are shown. Percentages in the gated areas are shown in each panel. **D**, T cell proliferation assay. Sorted CD4+ cells from IL-4$^{-/-}$ K/B6^{g7} mice or IL-4$^{+/-}$ K/B6^{g7} mice were cultured with B6^{g7} splenocytes and various dosages of recombinant murine glucose-6-phosphate isomerase (GPI)–glutathione S-transferase (GST) protein or 25 μg/ml recombinant murine GST for 72 hours, with ^{3}H-thymidine (^{3}H-TdR) added during the last 16 hours.

To further assess T cell activation in the absence of IL-4, we tested the proliferative capacity of IL-4$^{-/-}$ KRN T cells in response to GPI/A^{g7} in vitro. Splenocytes enriched in CD4+ cells from IL-4$^{-/-}$ K/B6^{g7} and IL-4$^{+/-}$ K/B6^{g7} mice were cultured with B6^{g7} splenocytes and various concentrations of recombinant GPI protein

(GPI–GST fusion protein), and proliferation was assayed by incorporation of ^{3}H-labeled thymidine. T cells from the 2 types of mice proliferated equally well in response to GPI stimulation (Figure 3D). In summary, T cell selection and activation were not detectably impaired in IL-4$^{-/-}$ K/B6^{g7} mice.

Reduction of anti-GPI antibody titers in IL-4$^{-/-}$ K/B6^{g7} mice. We then evaluated the next step in progression to arthritis, collaboration of T cells and B cells. To this end, titers of anti-GPI antibody were determined in sera from IL-4$^{-/-}$, IL-4$^{+/-}$, and IL-4$^{+/+}$ K/B6^{g7} littermates at 8 weeks of age (Figures 4A and B). The former exhibited markedly lower titers of total anti-GPI IgG (all IgG isotypes). Even one of the most severely arthritic IL-4$^{-/-}$ K/B6^{g7} mice had only one-fortieth of the anti-GPI IgG found in IL-4–expressing animals. In this particular mouse, antibody titers did not increase significantly, even at 11 weeks of age, when the arthritis index reached its maximal score (data not shown). A plot of the maximum ankle thickness of each mouse against its anti-GPI IgG titer at 8 weeks of age revealed that the reduced titer of pathogenic autoantibodies conferred by the IL-4 deficiency largely accounted for the reduced severity of joint inflammation.
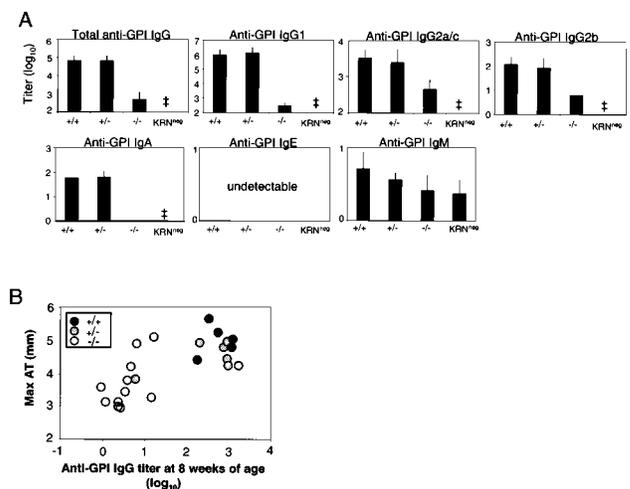
Figure 4. Mediation of anti–glucose-6-phosphate isomerase (anti-GPI) antibody production by interleukin-4 (IL-4). **A,** Anti-GPI antibody titers of total IgG and the isotypes IgG1, IgG2a/c (the B6 form of the hallmark Th1 isotype IgG2a), and IgG2b, as well as anti-GPI IgA, IgE, and IgM were measured by enzyme-linked immunosorbent assay in the sera of IL-4$^{+/+}$, IL-4$^{+/-}$, or IL-4$^{-/-}$ K/B6^{g7} mice and KRN-negative littermates at 8 weeks of age. Mice (5–10 from each group) were studied; mean and SD titers are shown. ‡ = undetectable. **B,** Titer of total anti-GPI IgG at 8 weeks of age plotted against the maximum ankle thickness (Max AT) in each IL-4$^{+/+}$, IL-4$^{+/-}$, or IL-4$^{-/-}$ K/B6^{g7} mouse.

The specific IgG isotypes of the anti-GPI IgG in IL-4 wild-type or mutant animals were also quantitated (Figure 4A). Consistent with the role of IL-4 in promoting antibody switching to IgG1, titers of anti-GPI IgG1 were much lower in IL-4$^{-/-}$ K/B6^{g7} mice than in the control animals. Titers of anti-GPI IgG2c (the B6 form of the hallmark Th1 isotype IgG2a [31]), IgG2b, and IgA were also reduced in IL-4$^{-/-}$ K/B6^{g7} mice, although not nearly to the same extent as IgG1. Anti-GPI IgE was undetectable in both types of mice. Titers of anti-GPI IgM in IL-4$^{+/+}$ and IL-4$^{-/-}$ K/B6^{g7} mice were at similarly low levels, but not very different from those of nontransgenic controls. These data demonstrated that IL-4 contributes to the initiation phase of arthritis by promoting the production of isotype-switched anti-GPI autoantibodies, and, in particular, is crucial for generating high levels of anti-GPI IgG1.

IL-4 expression in CD4+ T cells, eosinophils, and mast cells. To further understand how IL-4 affects K/BxN arthritis, we sought to identify the cell types that produce this cytokine in the K/BxN model. It has been reported that IL-4 can be synthesized not only by CD4+ T cells but also by other cell types, including γ/δ T cells, NKT cells, mast cells, and eosinophils (8). To identify the IL-4–producing cell types in K/BxN animals, we exploited the 4get mouse (30), a knockin mutant in which the endogenous *Il4* gene has been replaced with an *Il4*/IRES/EGFP construct, resulting in the generation of a bicistronic transcript under the control of endogenous *Il4* gene–regulatory elements. IL-4 production and activity in 4get mice remain intact, and cells that express IL-4 also express EGFP. The 4get mutation was crossed with KRN and B6^{g7} mice.

Figure 5 illustrates expression of the GFP reporter by a range of cell types in the resulting animals, compared with control littermates that were missing either of the 2 genetic elements (H-2^{g7} or KRN). KRN$^+$4get$^+$H-2^{g7} mice showed GFP expression in CD4+ T cells and CD11b+ cells. Such expression required both the KRN transgene and its A^{g7} target: the corresponding GFP+ populations were absent in control mice missing either element. The expression of 4get by both splenic and lymph node CD4+ T cells (Figure 5B) is consistent with the idea that KRN T cell activation occurred in both locations (32).

For CD11b+ cells, the distinction was more obvious in the lymph nodes than in the spleen because of a higher background expression in the spleen. To identify the IL-4–producing CD11b+ cell type revealed by flow cytometric analysis, we sorted CD11b+,GFP+ and CD11b+,GFP− cells and Giemsa stained them (Figure
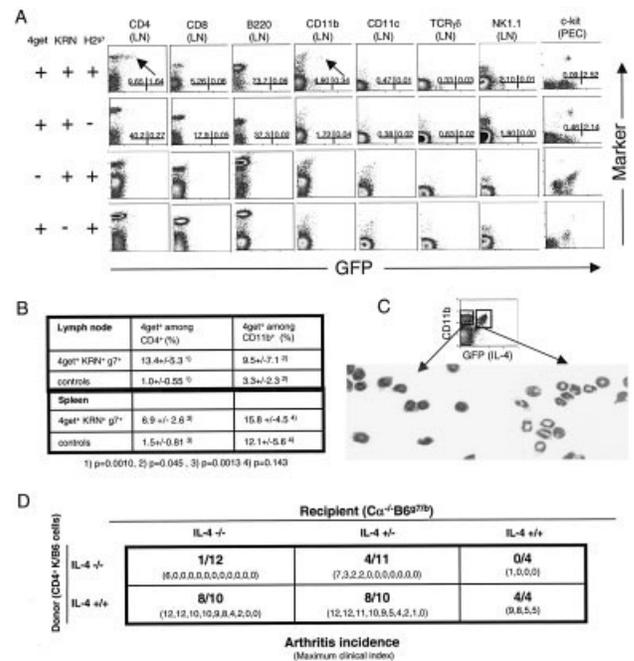
Figure 5. Interleukin-4 (IL-4) expression in various cell types. **A,** Lymph node (LN) cells and peritoneal exudate cells (PEC) were isolated from mice of the indicated genotypes and were stained and examined by flow cytometric analysis. The percentages of the upper left and right quadrant populations are shown. Profiles representative of 5 KRN$^+$4get$^+$H-2^{g7+} mice, 4 KRN$^+$4get$^+$H-2^{g7-} mice, 2 KRN$^+$4get$^-$H-2^{g7+} mice, and 2 KRN$^-$4get$^+$H-2^{g7+} mice are shown. GFP = green fluorescent protein. **B,** Percentage of GFP+ cells in CD4+ or CD11b+ cell populations. The mean ± SD percentages of GFP+ lymph node or spleen cells in the CD4+ or CD11b+ populations from 5 KRN$^+$4get$^+$H-2^{g7+} (arthritic) and 4 KRN$^+$4get$^+$H-2^{g7-} (nonarthritic [control]) mice are shown. Statistical significance of the difference between the arthritic and control groups was determined by *t*-test. **C,** GFP+,CD11b+ cells and GFP−,CD11b+ cells from KRN$^+$4get$^+$H-2^{g7+} spleens were sorted and then Giemsa stained. **D,** CD4+ T cells from IL-4$^{-/-}$ K/B6 or IL-4$^{+/+}$ K/B6 spleens were purified using MACS beads and 1×10^4 cells were transferred into C$\alpha^{-/-}$B6$^{g7/b}$ mice of IL-4$^{-/-}$, IL-4$^{+/-}$, or IL-4$^{+/+}$ genotypes. Recipient animals were monitored for 14 days. The incidence of arthritis and the maximum clinical index in each recipient animal (shown in parentheses), from 3 independent experiments, are shown. These results indicate that IL-4–producing T cells are essential for arthritis.

5C). GFP+,CD11b+ cells were exclusively eosinophils, whereas GFP−,CD11b+ cells were mostly neutrophils and monocyte/macrophages (Figure 5C). GFP expression was also observed in peritoneal mast cells (c-Kit+), but this expression was independent of the presence or activation of KRN T cells. With the caveat that GFP and IL-4 are not under the same translational control, these data indicated that T cell activation by interaction of the KRN TCR with GPI/A^{g7} results, directly or indirectly, in

the induction of IL-4 in at least 2 cell types: CD4+ T cells and eosinophils.

Importance of CD4+ cells to IL-4 production. To determine whether the source of IL-4 required for arthritis development was T cells, non–T cells, or both, we performed crisscross T cell transfer experiments. CD4+ T cells from the spleens of IL-4$^{-/-}$ or IL-4$^{+/+}$ KRN-transgenic mice (H-2$^{b/b}$) were sorted, and 10^4 cells were transferred into T cell–deficient animals (C$\alpha^{-/-}$B6$^{g7/b}$) of the IL-4$^{-/-}$, IL-4$^{+/-}$, or IL-4$^{+/+}$ genotype (Figure 5D). As expected, CD4+ T cells from IL-4–expressing K/B6 mice induced arthritis efficiently after transfer into T cell–deficient animals. The incidence and severity of arthritis did not depend on the IL-4 status of the recipients, which indicated that synthesis of this cytokine by non–T cells is not essential for arthritis induction. In contrast, CD4+ T cells from IL-4$^{-/-}$ K/B6 mice induced only mild arthritis in a small number of recipients, and no disease in most of them. Again, disease incidence and severity did not differ significantly according to the recipient's IL-4 genotype. The IL-4 deficiency in CD4+ T cells reproduced the effect of the full mutation in IL-4$^{-/-}$ K/B6^{g7} mice. Thus, CD4+ T cells are the source of IL-4 that is critical for the development of arthritis.

K/BxN arthritis is not a typical Th2 disease. The development of K/BxN arthritis exhibits some of the features characteristic of a Th2-biased response. IL-4 is critical for the development of K/BxN arthritis and profoundly affects the titer of IgG1 isotype autoantibodies. However, the bias in K/BxN mice may not be that of a typical Th2 response, since K/BxN mice also produce other anti-GPI IgG isotypes. Another notable feature of K/BxN arthritis is the extraordinarily high frequency of GPI-specific B cells and autoantibodies, which suggests a unique quality or quantity of T cell help. To determine whether the K/BxN model represents a pure Th2 disease, and to gain insight into the cytokine bias associated with superefficient T cell help, we performed quantitative RT-PCR to assay the expression of a panel of cytokines in CD4+ T cells.

Consistent with the role of IL-4 in promoting arthritis and with the data on the 4get system, we found that, from 2 weeks on, IL-4 mRNA was markedly up-regulated in K/BxN CD4+ T cells in lymph nodes, ~100-fold relative to CD4+ T cells from background-matched nontransgenic BxN littermates (Figure 6A). IFNγ mRNA transcripts were also up-regulated in these cells, but only by ~10-fold (Figure 6A). In contrast to the up-regulation of IL-4, other Th2-associated cytokines, IL-13 and TGFβ, were not up-regulated in CD4+
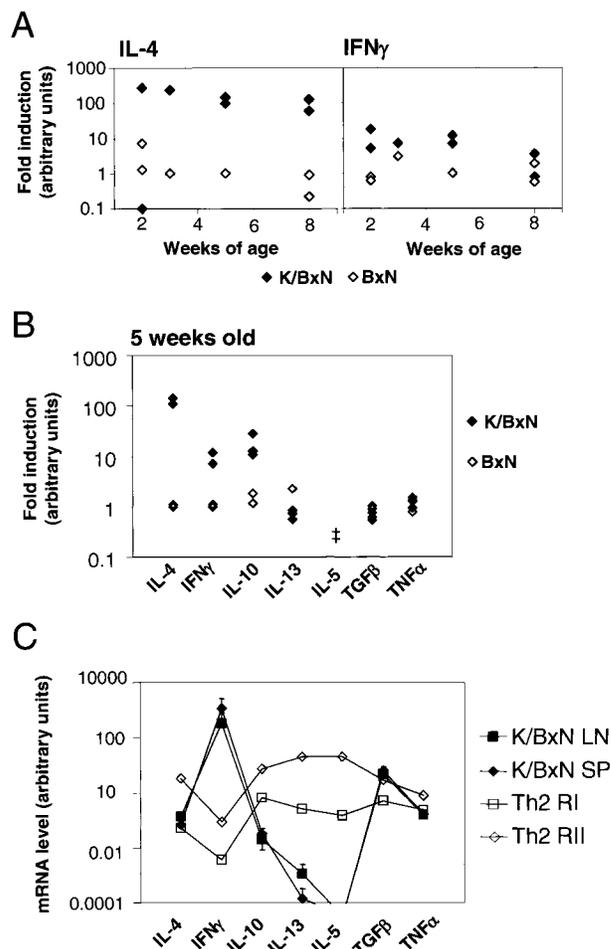
Figure 6. Cytokine profile of K/BxN T cells. **A** and **B,** Quantitative real-time reverse transcriptase–polymerase chain reaction was performed using mRNA from CD4+,CD69− T cells isolated from lymph nodes of K/BxN and BxN mice of the indicated ages. Hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA was used as an internal standard. The mRNA levels are expressed as the fold induction over the average level of each particular cytokine in 5-week-old BxN lymph node CD4+ T cells. Each symbol represents 1 sample from pooled samples from 3–5 mice. IL-4 = interleukin-4; IFNγ = interferon-γ; TGFβ = transforming growth factor β; TNFα = tumor necrosis factor α; ‡ = undetectable. **C,** The levels of each cytokine mRNA in the K/BxN cells (5-week-old mice) from lymph nodes (LN) or spleens (SP) were plotted against the levels in conventional Th2-polarized cells (stimulated once [RI] or twice [RII]). HPRT mRNA levels were used as internal standards. Note that the levels of one cytokine cannot be compared with the levels of another cytokine. Each symbol for Th2-polarized cells represents an individual replicate, whereas each symbol for K/BxN cells represents the mean expression from 2–3 replicates (each replicate from 3–5 pooled samples).

T cells from 5-week-old K/BxN mice (Figure 6B). We were unable to detect the Th2-associated cytokine IL-5 in these cells, while IL-10, which has been associated

with regulatory T cells as well as with Th2 cells, was induced. TNFα (predominantly Th1-associated) was not up-regulated in CD4+ cells from K/BxN mice. It was revealing to compare the levels of this panel of cytokine transcripts in K/BxN CD4+ T cells with those in "standard" Th2-biased cells, BALB/c CD4+ T cells (a kind gift from Drs. V. Dardalhon and V. Kuchroo), stimulated in vitro under Th2-biasing conditions (Figure 6C). There were some striking differences. First, K/BxN T cells expressed much higher amounts of IFNγ mRNA than did the conventional Th2 cells. In addition, the former expressed much lower amounts of several Th2-associated cytokines (including IL-10, IL-13, and IL-5) than did the latter. Thus, although our in vivo data clearly demonstrated the importance of T cell–derived IL-4 in K/BxN arthritis, this model does not represent a "textbook" Th2 disease.

DISCUSSION

Using mice deficient in IL-4 or IL-12p35, we have shown that the initiation of K/BxN arthritis is dependent on IL-4 produced by CD4+ T cells, and that the Th1 pathway, to the extent that it is controlled by IL-12, seems dispensable. The lack of IL-4 does not appear to affect the activation of autoreactive T cells but rather impinges on the collaboration of T cells and B cells, greatly diminishing the titer of anti-GPI antibodies, particularly those of the IgG1 isotype. Despite this pivotal role of IL-4 in disease, the cytokine profile of K/BxN T cells indicates that this arthritis model does not reflect a conventional Th2 response, at least not exclusively.

When arthritis development was monitored in IL-4$^{-/-}$ K/B6^{g7} mice, we noted that they exhibited various courses of disease: no arthritis, transient mild arthritis, or severe arthritis (Figure 1). One possible explanation for this variability is that IL-13, whose functions overlap with those of IL-4 (33), partially compensates for the absence of IL-4 in some mice. However, in preliminary experiments that compared IL-13 mRNA expression in CD4+ T cells from severely arthritic and nonarthritic IL-4$^{-/-}$ K/B6^{g7} mice, IL-13 transcription was actually lower in cells from the diseased mice (data not shown). Although these findings do not support the notion of compensation by IL-13, it is possible that other cytokines may exert this function, to a degree that varies among individual mice. But even IL-4$^{-/-}$ K/B6^{g7} mice with the most robust arthritis had greatly reduced titers of anti-GPI antibody. The highest titer measured was <10% of the average titer in IL-4$^{+/+}$

K/B6^{g7} mouse sera (Figure 4B). This observation suggests that the IL-4 deficiency decreases antibody titers to levels at which threshold effects come into play. Since pathogenesis in the K/BxN model clearly depends on feed-forward amplification loops (34), one can readily accept a scenario in which small changes in initial conditions have a strong impact on the ultimate outcome when anti-GPI titers are limiting.

The CD4+ T helper cell response is classically categorized into Th1 and Th2 responses, at least in mice (35). Th1 cells produce IL-2 and IFNγ, support IgG2a antibody production, and contribute mainly to cellular immunity phenomena. Th2 cells produce cytokines such as IL-4, IL-5, and IL-13, support IgG1 and IgE antibody production, and underlie humoral immunity. The Th1/Th2 dichotomy has provided a comfortable framework in which to compare types of immune responses. Unfortunately, this classification has also fostered the oversimplification that autoimmune diseases are either Th1 type or Th2 type, and RA is often categorized with type 1 diabetes mellitus and multiple sclerosis as a Th1-mediated disease (6). However, these assumptions have been challenged because disease mechanisms are clearly not that simple (36,37). For example, although experimental allergic encephalomyelitis is generally considered a Th1 disease, the Th1 cytokine IFNγ can suppress it (38,39). In the CIA model, IFNγ or IFNγ receptor–deficient mice showed accelerated arthritis (36). Clearly, the application of a simple Th1/Th2 paradigm to autoimmune diseases can mislead the effort to unravel disease mechanisms.

The K/BxN disease appears to exhibit elements of both conventional Th1 and conventional Th2 responses. Arthritogenic autoantibodies in K/BxN serum are extremely skewed to the Th2-associated IgG1 isotype (21) (Figure 4A), which is primarily induced by IL-4 (40). Furthermore, IL-4 mRNA is highly induced in K/BxN animals and is key to the initiation of disease. However, the cytokine profile indicated that this was not a typical Th2 response, with an absence of the Th2-associated cytokines IL-5 and IL-13. Furthermore, several hallmarks of a Th1-biased response were detected: up-regulation of IFNγ mRNA in K/BxN T cells, and high titers of the Th1-associated isotype IgG2c. In addition to the wide spectrum of IgG isotype autoantibodies in K/BxN mice, these animals are also distinguished by an astonishingly high frequency of GPI-specific B cells and high titers of anti-GPI autoantibodies. Thus, it is tempting to speculate that this unusual blend of "Th1-ness" and "Th2-ness" may be

partly responsible for these notable features of the K/BxN B cell response.

Although the relative importance of individual Th1- and Th2-associated cytokines in the K/BxN model is not yet known, the present study establishes a requirement for IL-4. This cytokine has been reported to be of variable consequence in other arthritis models. Two of 3 studies on CIA models showed that arthritis was reduced in IL-4–deficient mice (13–15). In the proteoglycan-induced arthritis model, mice lacking IL-4 developed more severe arthritis (17), and treatment with IL-4 dampened disease (16), demonstrating a clear antiinflammatory effect. Recently, it was reported that IL-4 deficiency does not influence the onset or severity of disease in the SKG arthritis model (41). Since the details of disease onset and progression clearly differ in many ways among these different models (42), it may not be surprising that the contribution of IL-4 also varies. Factors such as the genetic background of the mice and the degree to which pathogenic autoantibodies contribute to the disease process likely influence the role of IL-4 in each case.

The crucial question, then, is whether IL-4 is a promoter of RA in humans. It is plausible that this cytokine exacerbates human RA because it is a potent B cell activator, and there is by now ample evidence for an important contribution of B cells to this autoimmune disease. In particular, B cell depletion by anti-CD20 antibodies has been effective in the treatment of RA patients (43). Although they are clearly not a general feature of RA, anti-GPI antibodies have been detected in a proportion of patients (44–46). In addition, in human RA, IgG-type rheumatoid factor antibodies (anti-IgG Fc and anti-IgG Fab), both of which are detected in ~70% of RA sera, are often dominated by the IgG4 isotype (47,48), which is induced by IL-4 in humans. Although these examples do not provide direct evidence that IL-4 contributes to human RA, they do suggest that a role for IL-4 should be strongly considered. At the very least, attempts to treat RA patients with IL-4 (as has been done in a clinical trial for psoriasis [49]) should be weighed with great caution. If, in at least a fraction of patients, RA results from antibody-mediated mechanisms similar to those of the anticollagen and anti-GPI models, boosting IL-4 would be the last thing one would want to do.

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