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IDO2 Is a Critical Mediator of Autoantibody Production and Inflammatory Pathogenesis in a Mouse Model of Autoimmune Arthritis

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Rheumatoid arthritis and other autoimmune disorders are associated with altered activity of the immunomodulatory enzyme IDO. However, the precise contributions of IDO function to autoimmunity remain unclear. In this article, we examine the effect of two different IDO enzymes, IDO1 and IDO2, on the development of autoimmune arthritis in the KRN preclinical model of rheumatoid arthritis. We find that IDO2, not IDO1, is critical for arthritis development, providing direct evidence of separate in vivo functions for IDO1 and IDO2. Mice null for Ido2 display decreased joint inflammation relative to wild-type mice owing to a reduction in pathogenic autoantibodies and Ab-secreting cells. Notably, IDO2 appears to specifically mediate autoreactive responses, but not normal B cell responses, as total serum Ig levels are not altered and IDO2 knockout mice are able to mount productive Ab responses to model Ags in vitro and in vivo. Reciprocal adoptive transfer studies confirm that autoantibody production and arthritis are modulated by IDO2 expression in a cell type extrinsic to the T cell. Taken together, our results, provide important insights into IDO2 function by defining its pathogenic contributions to autoantibody-mediated autoimmunity. The Journal of Immunology, 2014, 192: 000–000.

Pathogenic drivers of autoimmunity remain a major focus of research aiming to reduce morbidity and mortality in patients who suffer from autoimmune disease. Therapeutic strategies to relieve or reprogram inflammation and deplete autoantibodies or B cell populations have been explored with variable clinical success (1–3). However, new strategies that target the underlying mechanisms driving autoimmune responses are still urgently needed. Rheumatoid arthritis (RA), a debilitating condition characterized by inflammation of the synovial joints and eventual degradation of cartilage and bone, represents one such autoimmune disease. Although increased knowledge has favorably improved options for therapeutic management, like other autoimmune diseases, RA remains in need of treatments that can target disease more specifically (3–5). Long-standing evidence for reduced tryptophan levels and increased tryptophan catabolites in the serum and urine of patients with autoimmune disorders has implicated the degradation of cartilage and bone, represents one such autoimmune disease. Although increased knowledge has favorably improved options for therapeutic management, like other autoimmune diseases, RA remains in need of treatments that can target disease more specifically (3–5). Long-standing evidence for reduced tryptophan levels and increased tryptophan catabolites in the serum and urine of patients with autoimmune disorders has implicated the tryptophan-catabolizing enzyme IDO in autoimmunity (6–11).

Indeed, dysregulation of IDO has been directly correlated with disease activity in the autoimmune disorders RA and systemic lupus erythematosus (12, 13).

IDO has been known to have immunomodulatory effects since the unexpected discovery that IDO was necessary for maternal tolerance to fetal tissue (14). Since then, it has been linked to immune modulation in a variety of diseases (15–17), although its function is best established as a critical mediator of tumor immune evasion (reviewed in Refs. 18, 19). In these contexts, IDO is considered immunosuppressive. In the context of autoimmunity, however, the function of IDO is less clear. Several studies have demonstrated that IDO has an immunosuppressive role in inducible models of autoimmunity, such as trinitrobenzene sulfonic acid–induced colitis, collagen-induced arthritis, and experimental autoimmune encephalomyelitis (20–22). Other models, including the KRN transgenic (Tg) [KRN (C57BL/6 × NOD)F1 (K/BxN) and KRN,g7] mouse model of RA (23) as well as models of inflammatory airway disease (24), allergy (25), and contact hypersensitivity (26), have provided evidence that IDO plays a positive role in inflammatory responses. These models may be more relevant to inflammatory autoimmune disease in humans, given correlations of elevated tryptophan degradation with disease activity in autoimmune patients (12, 13). The contrasting results seen in the different models of autoimmunity and inflammation may reflect mechanistic differences in the disease induction process and demonstrate that our understanding of the role of IDO in immune modulation is incomplete.

The KRN model is a spontaneous murine model of inflammatory autoimmune disease characterized by a rapid symmetrical onset of joint inflammation induced by the production of autoantibodies (27, 28). This model uses a TCR transgene, KRN, that when present in a genetic background expressing the I-A\(^{b}\) MHC class II molecule, leads to the development of joint-specific autoimmune disease. In this model, the autoreactive T and B cells both recognize the glycolytic enzyme glucose-6-phosphate isomerase (GPI) as an autoantigen,
and disease severity correlates with rising titers of anti-GPI Ig in the serum (28–30). The K/BxN model has many features in common with human RA, including pathological changes in the joints, cellular infiltrates, proinflammatory cytokines, and autoantibody production (27, 28). However, as with all animal models, some differences can be noted. In particular, the specificity of the autoantibodies produced in K/BxN mice is to GPI rather than to rheumatoid factor or citrullinated proteins, the autoantibodies present in the majority of human RA patients (31). As in human RA, arthritis in KRN mice is correlated with increased tryptophan catabolism, implicating the IDO pathway in the disease process (23).

Most previous studies of IDO and autoimmunity, including work demonstrating a reduced autoantibody response and an attenuated course of arthritis in the KRN-Tg mouse model of RA (23, 32), have used the compound D/L-1-methyl-triptophan (1MT) to inhibit IDO. Although widely considered an IDO inhibitor, 1MT, particularly the D-1MT stereoisomer, likely inhibits the IDO pathway rather than directly inhibiting the enzyme itself (33). The IDO pathway is complex, and the mechanistic underpinnings of immune modulation are only beginning to be established (19). Two closely related IDO genes, IDO1 and IDO2, appear to be inhibited by the different stereoisomers of 1MT (34) and may have different roles in immune regulation. The relationship of IDO1 versus IDO2 in the context of autoimmunity has yet to be established, and, indeed, the in vivo function of IDO2 is poorly understood in any context. To determine if IDO1, IDO2, or both are responsible for driving inflammation in the KRN model of RA, we follow arthritis induction in genetic knockout (ko) mouse mutants of IDO1 and IDO2.

In this study, we provide, to our knowledge, the first direct evidence of a specific pathogenic function for IDO2 in the establishment and development of autoimmune arthritis in the KRN-Tg preclinical model of RA. The severity of arthritis is significantly reduced in arthritic mice lacking IDO2, as measured by multiple parameters, including ankle inflammation and histological examination of the joints for immune cell infiltrates, synovial hyperplasia, pannus formation, and cartilage and bone erosion. The reduction in arthritis is mediated by a specific decrease in the production of autoantibody, but not total Ab, in IDO2-deficient mice. In contrast, IDO2 does not appear to affect normal B cell responses, as ko mice are able to make high-affinity, isotype-switched Abs in response to immunization with a model Ag, as well as maintain in vitro B cell proliferation and Ab production in response to polyclonal stimulation. The reduced autoantibody response is accompanied by a diminished CD4+ T cell response; however, reciprocal adoptive transfer studies demonstrate that IDO2 is necessary in the host, not the T cell itself, for robust arthritis development in this model. Together, these data associate the function of IDO2 with production of pathogenic Abs that generate an autoimmune phenotype. Thus, our results offer a possible explanation for the seemingly opposing roles of the IDO pathway in suppressing T cell responses in cancer, but promoting inflammatory responses in autoimmune disorders, by distinguishing a unique function for IDO2 as an important mediator of inflammatory autoimmunity.

Materials and Methods

Mice

KRN TCR Tg (27), IDO1-deficient (IDO1 ko) (35), and IDO2 ko (26) mice on a C57BL/6 background have been described. Arthritic mice were generated by breeding KRN Tg C57BL/6 mice expressing the I-A\(^{\text{K}}\) MHC class II molecule (KRN.g7). This process was repeated to generate arthritic mice lacking IDO1 or IDO2 (IDO1 ko KRN.g7 or IDO2 ko KRN.g7). KRN.g7 mice develop arthritis with kinetics similar to that in the original K/BxN mice (23). C57BL/6 IDO2 wild-type (wt) and ko mice lacking the TCR a-chain (Co) and carrying a single copy of the I-A\(^{\text{K}}\) allele (Co ko B6.g7b and Co ko IDO2 ko B6.g7b) were generated as recipient mice for adoptive transfer of T cells. T cell donor mice were KRN TCR Tg (KRN B6) or IDO2 ko KRN TCR Tg (IDO2 ko KRN B6), both carrying two copies of the I-A\(^{\text{K}}\) allele. All mice were bred and housed under specific pathogen–free conditions in the animal facility at the Lankenau Institute for Medical Research. Studies were performed in accordance with National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care guidelines with approval from the Lankenau Institute for Medical Research Institutional Animal Care and Use Committee.

Administration of 1MT

Mice were given 400 mg/kg/dose (100 μl total volume) of D/L-1MT (Sigma-Aldrich) diluted in Methocel/Tween (0.5% Tween 80, 0.5% methylcellulose [v/v in water]) twice daily by oral gavage starting at weaning (3 wk of age).

Arthritis incidence

The two rear ankles of wt, IDO1, and IDO2 ko KRN.g7 mice were measured starting at weaning (3 wk of age). Measurement of ankle thickness was made above the footpad axially across the ankle joint, using a Fowler Metric Pocket Thickness Gauge. Ankle thickness was rounded off to the nearest 0.05 mm. Data are represented as the change (Δ) in ankle thickness compared with that measured at 3 wk of age. At the termination of the experiment, ankles were fixed in 10% buffered formalin for 48 h, decalcified in 14% EDTA for 2 wk, embedded in paraffin, sectioned, and stained with H&E. Histology sections were imaged using a Zeiss Axioplan microscope with a Zeiss Plan-Apochromat ×100/0.32 objective and Zeiss AxioCam HRC camera using AxioVision 4.7.1 software. The images were then processed using Adobe Photoshop CS2 software.

IDO1 and IDO2 RNA expression

Liver and spleen tissues from 6- to 8-wk-old KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice were harvested and passed through a 70-μm nylon strainer to generate a single-cell suspension. RNA was extracted with TRIzol (Invitrogen) and first-strand cDNA synthesized using oligo-dT primer (Promega GoScript). IDo1 and Ido2 expression were measured by real time PCR using SYBR Green for detection (Sigma SYBR Green JumpStart Taq Ready Mix). Expression of target genes was determined relative to GAPDH and calculated as 2^(-ΔΔCt), as primers had similar efficiencies. IDO1, IDO2, and GAPDH primers are as previously described (26).

Kynurenine assay

Serum was collected from 6-wk-old KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice. Serum was diluted in water (1/4 v/v), deproteinated, and analyzed by HPLC coupled to electrospray ionization tandem mass spectrometry (liquid chromatography/mass spectrometry/mass spectrometry) analysis, as previously described (36). Quantitation of kynurenine was based on analysis of the major daughter ion.

ELISPOT assay

Cells from the joint draining lymph nodes (dLNs) (axillary, brachial, and popliteal LNs) from 6-wk-old KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice were plated at 4 × 10^6 cells per well and diluted serially 1:4 in MultiScreen-HA mixed cellulose ester membrane plates (Millipore) coated with GPI-his (10 μg/ml). The cells were incubated on the Ag-coated plates for 4 h at 37°C. The Ig secreted by the plated cells was detected by alkaline phosphatase–conjugated goat anti-mouse total Ig secondary Ab (Southern Biotechnology Associates) and visualized using NBT/BCIP substrate (NBT/5-bromo-4-chloro-3-indolyl phosphate; Sigma-Aldrich).

ELISA

To measure serum autoantibody titers, serum samples from 6-wk-old KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice were plated at an initial dilution of 1:100 and diluted serially 1:5 in Immulon II plates coated with GPI-his (10 μg/ml). Recombinant GPI-his protein was generated and purified as described previously (30). Donkey anti-mouse total Ig-HRP (Jackson ImmunoResearch) was used as a secondary Ab. Ab was detected using ABTS substrate (Fisher). The serum titer was defined as the reciprocal of the last dilution that gave an OD > 3× background. To measure serum total Ig levels, serum samples were plated at an initial dilution of 1:100, as above, and then serially diluted 1:10 in Immulon II plates coated with anti-mouse IgG (H + L) (Jackson ImmunoResearch). Donkey anti-mouse total Ig-HRP (Jackson ImmunoResearch) was used as a secondary Ab. Concentration was determined by comparison with a standard curve of IgG1 (BD Pharmingen).
Serum transfer

Serum from 8-wk-old untreated K/BxN mice was harvested, pooled, and filter sterilized. A total of 150 μl was transferred i.p. into wt or IDO2 ko C57BL/6 recipient mice (8–10 wk old), and arthritis progression followed as described above.

(4-Hydroxy-3-nitrophenyl)acetetyl keyhole limpet hemocyanin immunization

C57BL/6 or IDO2 ko C57BL/6 mice were immunized i.p. with 100 μg (4-hydroxy-3-nitrophenyl)acetetyl keyhole limpet hemocyanin (NP-KLH) (Biosearch Technologies) precipitated in alum, as described previously (37). Mice were boosted 10 wk after initial immunization with 100 μg NP-KLH precipitated in alum. Mice were bled 8 d following the primary and secondary immunizations, and NP titers were measured by anti-NP ELISA.

Anti-NP ELISA

Serum samples were plated at an initial dilution of 1:100 and diluted serially 1:4 on Immulon II plates coated with NP3-BSA (Biosearch Technologies). The serum titer was defined as the reciprocal of the last dilution that gave an OD > 3× background. Goat anti-mouse IgM-HRP or IgG-HRP (Southern Biotechnology Associates) were used as secondary Abs. Ab was detected using ABTS substrate (Fisher).

Proliferation

A total of 1 × 10⁶ splenic B cells, pooled from each of two C57BL/6 or two IDO2 ko C57BL/6 mice, were isolated by negative selection with CD43 MACS beads (Miltenyi Biotec), labeled with 5 μM CFSE for 10 min, then cultured with media alone, 25 μg/ml LPS, or 2 μg/ml anti-CD40 + 50 ng/ml IL-4. After 72 h, cells were analyzed for CFSE staining by flow cytometry (BD FACSCanto II). The percentage of proliferated cells was determined in comparison with the unstimulated control, using FlowJo software (TreeStar). In addition, total Ig production was measured from supernatants of proliferating cultures at 72 h by ELISA, as described above.

Analysis of Th subsets

Joint dLN cells from 6-wk-old KRN.g7 and IDO2 ko KRN.g7 mice were stained and cultured for CD4+ T cells (BioLegend clone GK1.5) and the following markers to distinguish Th subsets: bcl6 [T follicular helper cell (Thf)], BD Pharmingen clone K112g1), Foxp3 (regulatory T cells, BioLegend clone 150D), gata3 (Th2, eBioscience clone TWAJ), rorγ (Th17, eBioscience clone AFKJS-9), T-bet (Th1, eBioscience clone 4B10). The samples were acquired on a BD FACSCanto II flow cytometer using FACS Diva Software (BD Bioscience) and analyzed using FlowJo Software (TreeStar).

Cytokine secretion

Cells from the joint dLNs of 6-wk-old KRN.g7 and IDO2 ko KRN.g7 mice were harvested and cultured in either media alone or PMA (50 ng/ml) + ionomycin (500 ng/ml) for 24 h. The supernatants were then harvested and analyzed for the levels of IL-4, IL-6, IL-10, IL-17, RANTES, TNF-α, IFN-γ, and MCP-1 by cytometric bead array (BD Biosciences). The samples were stained according to the manufacturer’s instructions and analyzed on a BD FACSCanto II flow cytometer using FACS Diva software. Cytokine concentrations were calculated by comparing with standard curves, using FACS array analysis software (BD Biosciences).

Intracellular IL-21

Cells from the joint dLNs of 6-wk-old KRN.g7 and IDO2 ko KRN.g7 mice were harvested and cultured for 4 h with 50 ng/ml PMA, 500 ng/ml ionomycin, and 3 μg/ml brefeldin A. After 4 h, cells were harvested, surface stained for CD4 and CD8 (eBioscience), fixed and permeabilized (IC Fixation and Permeabilization Buffer, eBioscience), and then stained for intracellular IL-21 or isotype control. The samples were acquired on a BD FACSCanto II flow cytometer using FACS Diva software and analyzed with FlowJo software.

Adaptive transfers

CD4+ T cells from KRN TCR Tg (KRN B6) or IDO2 ko KRN TCR Tg (IDO2 ko KRN B6) mice were purified by positive selection with MACS beads (Miltenyi Biotec). Following purification, 3.5 × 10⁷ CD4+ T cells were adoptively transferred i.v. into Cx ko B6.7 or Cx ko IDO2 ko B6.7 hosts. Arthritis was measured as described above. Mice were sacrificed after 2 wk, and anti-GPI titers were measured by ELISA.

Statistical analysis

Statistical significance was determined using an unpaired Student t test or the Mann–Whitney nonparametric test and Instat Software (GraphPad).

Results

Deletion of IDO2, but not IDO1, alleviates arthritis

To determine whether IDO1, IDO2, or both are necessary for the development of a robust arthritic response, we crossed the null alleles of IDO1 or IDO2 into the KRN model on a pure C57BL/6 background (KRN.g7) and monitored the development of joint inflammation (Fig. 1A). IDO1 ko KRN.g7 mice developed arthritis in a pattern statistically indistinguishable from that of their KRN.g7 counterparts. IDO2 ko KRN.g7 mice, however, had a significantly delayed onset of arthritis and overall reduction in ankle swelling compared with KRN.g7 or IDO1 ko KRN.g7 mice. Histological examination of ankles from arthritic mice confirms the reduction in arthritis, as measured by a decrease in immune cell infiltrates, synovial hyperplasia, pannus formation, and cartilage and bone erosion in IDO2 ko arthritic mice, revealing that IDO2, not IDO1, is critical for the development of arthritis (Fig. 1B).

Because IDO1 and IDO2 are located adjacent to each other on chromosome 8, genetic deletion of one of the IDO genes could cause a change in expression of the other (34, 38). Indeed, alternative splice variants of IDO2 are found in macrophages isolated from IDO1 ko mice (26). Using quantitative real time PCR, we find a 2-fold decrease in IDO2 message in the liver, but not spleen, of IDO1 ko KRN.g7 mice (Fig. 2A). In contrast, IDO2 ko KRN.g7 mice express normal levels of IDO1 in both the spleen and the liver, confirming that the decreased arthritis in IDO2 ko mice is solely due to the lack of IDO2 and not to an additional change in expression of IDO1 (Fig. 2B).

Reduction in arthritis in IDO2 ko mice is not due to reduced kynurenine production

The IDO1 enzyme is the first, rate-limiting step in the catabolism of tryptophan to kynurenine. IDO2 can also catalyze tryptophan to kynurenine, but with lower efficiency (34, 39, 40), and its overall function is less well established. Increased kynurenine has been associated with disease in autoimmune patients (see Refs. 6, 7, 11) and mice (23), suggesting an association between the IDO pathway and autoimmunity. To determine whether increased kynurenine production is correlated with arthritis development in KRN.g7 mice, kynurenine levels were compared in IDO1 ko and IDO2 ko KRN.g7 mice. Although IDO2 ko KRN.g7 mice exhibit an attenuated course of arthritis, they do not show decreased kynurenine levels, whereas IDO1 ko KRN.g7 mice develop robust arthritis despite reduced kynurenine levels (Fig. 2C). These data confirm that IDO1 is active in IDO2 ko KRN.g7 mice and show that the production of kynurenine by the IDO pathway is not responsible for arthritis.

The IDO pathway inhibitor 1MT does not affect arthritis in IDO1 or IDO2 ko mice

Our previous work showed that the IDO pathway inhibitor 1MT significantly attenuated arthritis in KRN.g7 and the related K/BxN mouse model of RA (23). To determine whether the effect of 1MT resulted from inhibition of IDO1, IDO2, or both, 1MT was administered to wt KRN.g7 and IDO1 and IDO2 ko KRN.g7 mice beginning at 21 d of age. As previously reported, 1MT alleviated arthritis in KRN.g7 mice (Fig. 2D). 1MT did not further reduce arthritis in the already attenuated IDO2 ko KRN.g7 mice, demonstrating that IDO2 is necessary for the function of 1MT (Fig. 2D). Unexpectedly, 1MT also did not reduce arthritis in IDO1 ko KRN.g7 mice, suggesting that IDO1 is likewise required for 1MT to exert...
its antiarthritic effect (Fig. 2D). Previous work in a transplantable tumor model has also demonstrated a dependence of 1MT on IDO1, despite lack of tumor inhibition in IDO1 ko mice (41). These data suggest that, although both IDO1 and IDO2 are involved in the pathway inhibited by 1MT, only IDO2 is required for the development of robust arthritis.

**IDO2 deletion reduces autoantibody, but not total Ab, production**

Autoantibodies to the glycolytic enzyme GPI are the major effector molecules in arthritis development and progression in the KRN model of RA (28, 29, 42). To ascertain whether reducing autoantibody levels is the mechanism by which the arthritic response is attenuated in IDO2 ko KRN.g7 mice, levels of GPI-specific autoantibodies in the serum and numbers of anti-GPI autoantibody secreting cells (ASCs) in the joint dLN were measured. In correlation with reduced arthritis severity, titers of anti-GPI Ig were significantly lower in the IDO2 ko KRN.g7, but not IDO1 ko KRN.g7, mice relative to the KRN.g7 controls (Fig. 3A). Likewise, the number of anti-GPI ASCs was reduced in the IDO2 ko compared with IDO1 ko and wt KRN.g7 mice (Fig. 3B). To determine whether the reduced Ab response is specific to GPI-reactive Ig, or reflected an overall reduction in Ab titers, total serum Ig levels were measured by ELISA. No significant differences in total Ig were seen between

**FIGURE 1.** Deletion of IDO2, but not IDO1, alleviates arthritis. (A) Rear ankles were measured as an indication of arthritis and represented as the mean change in ankle thickness ± SEM from n = 14 KRN.g7, n = 10 IDO1 ko KRN.g7, and n = 12 IDO2 ko KRN.g7 mice, pooled from three independent litters per genotype. (B) Metatarsal joint from KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice at 42 d of age stained with H&E. Representative sections from a total of n = 14 KRN.g7, n = 10 IDO1 ko KRN.g7, and n = 12 IDO2 ko KRN.g7 mice. Scale bar, 100 μm. *p < 0.05

**FIGURE 2.** IDO expression, kynurenine production, and the effect of the IDO inhibitor 1MT in IDO1 ko and IDO2 ko KRN.g7 mice. (A) IDO2 and (B) IDO1 mRNA expression was measured by real-time PCR. Data show the median and interquartile range of the fold change in IDO expression relative to KRN.g7 control mice. (A) KRN.g7: n = 13 (spleen and liver), IDO1 ko KRN.g7: n = 8 (spleen and liver); (B) KRN.g7: n = 11 (spleen and liver), IDO2 ko KRN.g7: n = 7 (spleen), n = 6 (liver). (C) Serum kynurenine levels were measured by mass spectroscopy. Data show mean ± SEM, n = 8 mice per group, pooled from three independent litters of each genotype. (D) KRN.g7, IDO2 ko KRN.g7, and IDO1 ko KRN.g7 mice were treated with 400 mg/kg D/L 1-MT or carrier alone, beginning at 21 d of age. Data show mean change in ankle thickness ± SEM from n = 10 1MT-treated and n = 7 carrier-treated KRN.g7, n = 5 1MT-treated and n = 5 carrier-treated IDO1 ko KRN.g7 and IDO2 ko KRN.g7 mice, pooled from two independent experiments. *p < 0.05.
IDO2-deficient mice develop arthritis in response to transferred arthritogenic autoantibodies

The reduction in autoantibody titers could explain the attenuated arthritic response in IDO2 ko KRN.g7 mice; however, IDO2 could also be involved in effector responses downstream of autoantibody production. To find out whether the defect in IDO2 ko mice is in the production of autoantibodies and/or the physiological response to those autoantibodies, we made use of the serum transfer model of arthritis (Fig. 4). In this model, the initiation phase of the arthritic response, which includes the steps leading to the production of autoantibodies, can be experimentally separated from downstream effectors through the passive transfer of serum from arthritic K/BxN mice into nonarthritic mice (28). In this study, IDO2 ko or wt C57BL/6 mice were administered K/BxN serum and observed for the development of arthritis. Within 2 d of serum transfer, wt mice developed joint inflammation that peaked ∼1 wk later. IDO2 ko mice developed arthritis with an identical rate and severity, indicating that the downstream effectors in IDO2 mice are capable of mounting an arthritic response when supplied with arthritogenic autoantibody (Fig. 4). Therefore, the defect in IDO2 ko mice is upstream of the production of autoantibody.

IDO2 deletion does not affect total B cell responses

The reduced autoantibody levels in IDO2 ko KRN.g7 mice suggest that IDO2 plays a role in the pathway leading to autoantibody responses. To determine if IDO2 is critical for B cell responses in general, we measured the ability of IDO2 ko B cells to produce Ab in response to stimulation in vitro and in vivo. B cells were purified from IDO2 ko or wt C57BL/6 mice and stimulated in vitro with anti-CD40 + IL-4 to mimic T cell help or the TLR ligand LPS as a polyclonal stimulus. IDO2 ko B cells proliferated (Fig. 5A) and secreted Ig (Fig. 5B) in response to both stimuli at levels indistinguishable from those of wt B cells. Next, IDO2 ko and wt C57BL/6 mice were immunized with the model Ag NP-KLH and measured in both CD4+ and CD8+ T cell populations (Fig. 6C). Both IL-4 and IL-17 were found to be increased in IDO2 ko KRN.g7 lymphocytes, respectively. This finding suggests that T cell differentiation is reduced or impaired in IDO2 ko mice. To determine whether this decrease in differentiated Th cells might have a functional effect, concentrations of key inflammatory and B cell–related cytokines were measured from in vitro culture of KRN.g7 and IDO2 ko KRN.g7 dLN cells. No significant differences were found in the inflammatory cytokines IL-10, IL-17, IFN-γ, TNF-α, MCP-1, or RANTES. Significant decreases were found in IL-4 and IL-6, both of which can influence B cell proliferation, differentiation, and Ab production (Fig. 6B). IL-21, which can direct B cell Ab responses along with IL-4 and IL-6, was not detectable in the supernatants of in vitro cultures. However, intracellular IL-21 was found to be decreased in both CD4+ and CD8+ T cell populations (Fig. 6C). Both IL-4 and IL-21 are required for arthritis development in the KRN transgene model (44, 45). Although the role of IL-6 is less...
It is undoubtedly relevant to human disease, given the success of IL-6 inhibitors in the treatment of RA (Ref. 47, reviewed in Ref. 48). Overall, the data in our study suggest that the reduced autoantibody production and subsequent arthritis in IDO2 ko KRN.g7 mice is due to a diminished Th cell response.

**Mechanism for decreased arthritic response in IDO2 ko mice is not intrinsic to T cells**

The reduced Th response in IDO2 ko mice could be attributable to a T cell–intrinsic role for IDO2 or a T cell–extrinsic role that IDO2 plays in another cell type, which then influences Th cell function, autoantibody production, and arthritis induction. To distinguish...
between a T cell–intrinsic and T cell–extrinsic role for IDO2, we made use of the KRN T cell adoptive transfer model of arthritis, in which adoptive transfer of KRN T cells into wt C57BL/6 mice induces autoimmune arthritis with defined kinetics (49, 50). KRN T cells from IDO2 ko or wt C57BL/6 mice were adoptively transferred into IDO2 ko or wt T cell–deficient hosts (Cx67b or Cx62 ko IDO2 ko B6.g7b) (Fig. 7A). Both wt and IDO2 ko KRN T cells induced robust arthritis in wt hosts starting 7–10 d after T cell transfer. In contrast, arthritis in IDO2 ko hosts was delayed in both time of onset and extent of severity, regardless of whether IDO2 ko or wt KRN T cells were transferred (Fig. 7B). A corresponding decrease in serum anti-GPI titers was also seen in IDO2 ko compared with wt hosts, again regardless of the IDO2 status of the transferred T cells (Fig. 7C). This finding confirms that arthritis is diminished in the absence of IDO2, whether it is induced spontaneously (IDO2 ko KRN.g7 mice) or through the adoptive transfer of KRN T cells. Together, these data demonstrate that IDO2 mediates autoantibody production and arthritis through a T cell–extrinsic role.

**Discussion**

Conflicting evidence exists for the role of IDO in modulating the immune system. In cancer, IDO has generally been considered immunosuppressive, allowing for the expansion of regulatory T cells and suppression of T cell activation (51, 52), although recent work hints at a more complex immunomodulatory role (reviewed in Ref. 19). The observation that IDO is upregulated in autoimmunity has been paradoxical, as increased immunosuppression would be predicted to be beneficial in this context, suggesting that our present understanding of the relationship between IDO activation and disease is incomplete. Unlike in cancer, in which IDO1 seems to be the major player in immune modulation (53), our results directly implicate a second, related enzyme, IDO2, in immune system regulation in the context of autoimmunity.

IDO2 is structurally related to IDO1, but its function is poorly established. Although IDO2 does catabolize tryptophan to kynurenine, it does so with substantially reduced efficiency compared with IDO1 (34, 40, 54). IDO2 is expressed in a smaller range of tissues than is IDO1, generally confined to liver, kidney, and epididymis, as well as APCs (e.g., dendritic cells) in immune tissues (34, 55). Even less clear is the mechanism by which IDO2 may influence the immune system. It is likely that the IDO pathway modulates the immune system indirectly, possibly through tryptophan depletion and sufficiency signals influencing GCN2 and mammalian target of rapamycin pathways (33), although the relative contribution of IDO1 versus IDO2 to these signals is unknown. Given the result in our study of a positive role for IDO2 in the development of an autoantibody-mediated disorder, its potential as a target molecule for development of therapies for autoimmune diseases warrants further investigation into its function.

Most previous studies evaluating the role of the IDO pathway in autoimmune responses used the small-molecule inhibitor D/L-1MT and have yielded conflicting results. Blocking IDO with 1MT exacerbates arthritis in collagen-induced arthritis (22, 56) and experimental autoimmune encephalomyelitis (21), but alleviates disease in K/BxN and KRN.g7 arthritis as well as inflammatory airway disease models (23, 24). It is unclear why 1MT alleviates autoimmunity in some models yet exacerbates it in others. Because 1MT can inhibit both IDO1 and IDO2, one possible explanation is that it results from varying contributions of IDO1 and IDO2 in the different disease models. The specificity of the respective 1MT isomers for IDO1 versus IDO2 has also been controversial, with one report demonstrating that L-1MT inhibits IDO1 and D-1MT inhibits IDO2 (34), and another showing the direct opposite (57). The L-isomer of 1MT appears to be a more direct enzymatic inhibitor of IDO in vitro; however, it is D-1MT, which affects IDO indirectly (33), that has a physiological effect on tumor progression in vivo (41). To avoid the pitfalls of interpreting the effect of an indirect inhibitor, in this study we used genetic ko models to examine the role of IDO in autoimmunity. Combining the use of 1MT and genetic ko models supports the idea that IDO may be inhibiting a pathway involved with IDO2, not the enzyme itself.

**FIGURE 7.** Effect of IDO2 is not intrinsic to T cells. Arthritis was induced by the adoptive transfer of purified CD4+ KRN T cells from wt or IDO2 ko C57BL/6 (I-A^b) mice into T cell–deficient wt or IDO2 ko C57BL/6 (I-A^b) hosts. (A) Schematic for reciprocal adoptive transfer strategy. (B) Rear ankles were measured as an indication of arthritis and represented as the mean change in ankle thickness ± SEM. (C) Serum anti-GPI titers were measured in recipient mice 14 d after T cell transfer. Data show the mean ± SEM of the reciprocal of serum anti-GPI titer. Data are from n = 14 wt into wt, n = 12 IDO2 ko into wt, n = 6 wt into IDO2 ko, and n = 7 IDO2 ko into IDO2 ko mice, pooled from five independent experiments. *p < 0.05, **p < 0.01.
Given these caveats to 1MT as an indirect inhibitor of an indirect modulator of immunity, caution must be exercised when interpreting the relationship between the effects of 1MT and the activity of IDO1 and/or IDO2. It will be important to breed the IDO1 and IDO2 ko alleles into other preclinical models of autoimmune disease to definitively determine the relative contributions of IDO1 and/or IDO2 to the underlying mechanisms mediating autoimmune responses.

IDO2 appears to work specifically to promote the development of autoantibodies but does not play an important role in directing Ab responses in general. B cells from IDO2-deficient mice generate normal Ab responses to model Ags in vitro and in vivo. Thus, the mechanism for IDO2 may not be in the direct production of autoantibodies, but rather in providing T cell help to B cells to promote this autoantibody production. In support of this idea, IDO2 ko KRN.g7 mice have a general reduction in T cell help, with decreased Th1, Th2, and Th17 cell compartments and lower levels of the Th cytokines IL-4, IL-6, and IL-21. These cytokines have been shown to be important in driving B cell Ab responses, in particular by directing the differentiation and function of Th cells (e.g., Ref. 58). A trend toward lower levels of Th cells in IDO2 ko KRN.g7 mice was observed, although this did not reach statistical significance. IL-21, in addition to being associated with Th cells, is also produced by Th17 cells and is essential for the development of arthritis in the K/BxN model (59). The Th17 compartment is of particular interest in this situation, as IL-6, a regulator of Th17 cell differentiation, is reduced in IDO2 ko KRN.g7 mice, although IL-17 itself is not significantly altered. The role of Th17 cells in this model, however, has been difficult to elucidate. Wu et al. (60) have shown that the presence of segmented filamentous bacteria in the gut of these mice drives Th17 production and is required for arthritis development. However, adoptive transfer studies have yielded conflicting results on the contribution of Th17 cells to arthritis incidence. Hickman-Brecks et al. (49) demonstrated that adoptive transfer of Th17-polarized KRN T cells induces robust arthritis in recipient mice and that neutralization of IL-17A delays the onset of arthritis in this model. In contrast, Block and Huang (44) find that arthritis development proceeds normally with T cells transferred from a mouse with inactivated expression of RORγt, the transcription factor that directs Th17 development. Our data showing a general reduction in differentiated T cell populations and cytokines in IDO2 ko KRN.g7 mice suggest that IDO2 mediates arthritis and autoantibody production by regulating the overall quality of T cell help, rather than by affecting a specific T cell subpopulation.

Given the reduction in T cell help, we performed reciprocal adoptive transfer of T cells into T cell-deficient hosts to directly test the effect of wt and IDO2 ko T cells. In this study, we confirm that it is, in fact, the lack of IDO2 in the host mice that affects arthritis and autoantibody production, and not IDO2 in the T cells themselves. IDO2 may thus be acting in an APC in the host mice to influence both B and T cell activation. Although IDO1 is clearly important to dendritic cell function, especially in the ability of IDO-expressing dendritic cells to control the balance of effector and regulatory T cell populations required to maintain tolerogenic environments (reviewed in (61)), the role of IDO2 in dendritic cells and other APCs such as B cells is largely unknown. Recent reports in the literature demonstrate that cross-talk between B cells and Th cells is necessary to generate effective T cell help for B cell Ab production. This cross-talk involves both cell surface molecules and soluble factors, including PD-1, ICOS, and IL-21 and their respective ligands (62). It is possible that IDO2 is involved in directing one or more of these signals in autoreactive B cells. In support of this mechanism, CD4 T cells from IDO2 ko KRN.g7 mice express lower levels of IL-21. Another possibility is that IDO2 deletion in the recipient mice influences not just the activation, but also the survival, of the differentiated Th cell populations. For either scenario, in the absence of IDO2, T cell help and subsequent autoantibody production would be reduced, resulting in a diminished autoimmune response.

In summary, this study provides direct evidence of a pathogenic role for IDO2 in driving B cell–mediated autoimmune disease. Using the KRN preclinical model of RA, we show that IDO2 is required for the activation of CD4+ Th cells, production of pathogenic autoantibodies, and subsequent development of arthritis. IDO2 appears to specifically regulate autoreactive responses, but not normal B cell responses, as IDO2 ko mice are able to mount productive Ab responses to model Ags in vitro and in vivo. Reciprocal adoptive transfer studies confirmed that autoantibody production and arthritis are mediated by IDO2 expression in a cell type extrinsic to the T cell, most likely an APC. Together, our data demonstrate that IDO2 contributes to autoimmunity via its role in autoantibody production, implicating IDO2 as an exciting new therapeutic target for RA. In the future, it will be important to extend these findings to different models of autoimmunity to determine whether IDO2 plays a similar role in other autoantibody-mediated autoimmune diseases.

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Disclosures
J.B.D., R.M., and G.C.P. are inventors on issued U.S. patents claiming structure of matter and therapeutic uses of IDO inhibitors. These investigators are shareholders in NewLink Genetics Corp., which has licensed IDO patents granted to the investigators’ institution for clinical development of the technology. R.M. is currently employed by NewLink Genetics, and G.C.P receives compensation as an expert consultant for the company’s scientific advisory board. The other authors have no financial conflicts of interest.

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