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IDO2 is a critical mediator of autoantibody production and inflammatory pathogenesis in a mouse model of autoimmune arthritis[1]

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Abstract

Rheumatoid arthritis (RA) and other autoimmune disorders are associated with altered activity of the immunomodulatory enzyme indoleamine-2,3-dioxygenase (IDO). However, the precise contributions of IDO function to autoimmunity remain unclear. Here, we examine the effect of two different IDO enzymes, IDO1 and IDO2, on the development of autoimmune arthritis in the KRN preclinical model of RA. We find that IDO2, not IDO1, is critical for arthritis development, providing the first direct evidence of separate *in vivo* functions for IDO1 and IDO2. Mice null for *Ido2* display decreased joint inflammation relative to wild-type mice due to a reduction in pathogenic autoantibodies and antibody secreting cells. Notably, IDO2 appears to specifically mediate autoreactive, but not normal B cell responses, as total serum Ig levels are not altered and IDO2 ko mice are able to mount productive antibody responses to model antigens *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 the first insights into IDO2 function by defining its pathogenic contributions to autoantibody-mediated autoimmunity.

INTRODUCTION

Pathogenic drivers of autoimmunity remain a major focus of research aiming to reduce morbidity and mortality in patients that 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

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inflammation of the synovial joints and eventual degradation of cartilage and bone, represents one such autoimmune disease. While 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 have implicated the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (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), though its function is best established as a critical mediator of tumor immune evasion (reviewed in 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 (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 T cell receptor transgene, KRN, that when present in a genetic background expressing the I-A^{g7} 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 joint pathology, cellular infiltrates, pro-inflammatory cytokines, and autoantibody production (27, 28). However, as with all animal models, there are some differences. In particular, the specificity of the autoantibodies produced in K/BxN mice is to GPI rather than to rheumatoid factor or citrullinated protiens, 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 attenuated course of arthritis in the KRN-transgenic mouse model of RA (23, 32), have utilized the compound D/L-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). There are two closely related IDO genes, IDO1 and IDO2, which appear to be inhibited by the different stereoisomers of 1MT (34) and which 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 mouse mutants of IDO1 and IDO2.

In this study, we provide the first direct evidence of a specific pathogenic function for IDO2 in the establishment and development of autoimmune arthritis in the KRN transgenic 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 antibody, in IDO2-deficient mice. In contrast, IDO2 does not appear to affect normal B cell responses, as knockout mice are able to make high affinity, isotype-switched antibodies in response to immunization with a model antigen, as well as maintain *in vitro* B cell proliferation and antibody 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 antibodies 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^{g7} 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 similar kinetics as the original K/BxN mice (23). C57BL/6 IDO2 wt and ko mice lacking the TCR alpha chain (Cα) and carrying a single copy of the I-A^{g7} allele (Cα ko B6.g7/b and Cα ko IDO2 ko B6.g7/b) 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 2 copies of the I-A^b 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 LIMR Institutional Animal Care and Use Committee.

Administration of 1MT

Mice were given 400 mg/kg/dose (100μl total volume) of D/L-1MT (Sigma) diluted in Methocel/Tween (0.5% Tween 80, 0.5% methylcellulose (v/v in water)) twice daily by oral gavage (p.o.) 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 0.05mm. Data is represented as the change (Δ) in ankle thickness

compared to that measured at 3 wk of age. At the termination of the experiment, ankles were fixed in 10% buffered formalin for 48 hrs, decalcified in 14% EDTA for 2 wks, embedded in paraffin, sectioned, and stained with H&E. Histology sections were imaged using a Zeiss Axioplan microscope with a Zeiss Plan-Apochromat 10x/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 tissue from 6-8 week old KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice were harvested and passed through a 70μm 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 week 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 spectroscopy (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 LN (axillary, brachial, and popliteal LNs) from 6 week-old KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice were plated at 4×10^5 cells per well and diluted serially 1:4 in Multiscreen HA mixed cellulose ester membrane plates (Millipore) coated with GPI-his (10μg/ml). The cells were incubated on the Ag-coated plates for 4hr 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 (nitroblue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate; Sigma).

ELISA Assay

To measure serum autoantibody titers, serum samples from 6 week-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 (10μg/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>3x 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 to a standard curve of IgG_1 (BD Pharmingen).

Serum Transfer

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

NP-KLH Immunization

C57BL/6 or IDO2 ko C57BL/6 mice were immunized i.p. with 100 μg (4-Hydroxy-3-Nitrophenyl)Acetyl Keyhole limpet hemocyanin (NP-KLH) (Biosearch Technologies) precipitated in alum as described previously (37). Mice were boosted 10 weeks after initial immunization with 100μg NP-KLH precipitated in alum. Mice were bled 8 days following the primary and secondary immunizations and NP titers 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 NP_3-BSA (Biosearch Technologies). The serum titer was defined as the reciprocal of the last dilution that gave an OD>3x 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

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

Analysis of T helper subsets

Joint draining LN cells from 6 week old KRN.g7 and IDO2 ko KRN.g7 mice were harvested and stained for $CD4^+$ T cells (BioLegend clone GK1.5) and the following markers to distinguish Th subsets: bcl6 (Tfh, BD Pharmingen Clone K112g1), foxP3 (Treg, Biolegend clone 150D), gata3 (Th2, eBioscience clone TWAJ), rorγt (Th17, eBioscience clone AFKJS-9), T-bet (Th1, eBioscience clone 4B10). The samples were acquired on a BDFACSCanto II flow cytometer using FACSDiva Software (BD Bioscience) and analyzed using FlowJo Software (TreeStar).

Cytokine Secretion

Cells from the joint draining LNs of 6 week 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, TNFα, IFNγ, and MCP-1 by cytometric bead array (BD Biosciences). The samples were stained according to manufacturer instructions and analyzed on a BDFACSCanto II flow cytometer using FACSDiva software. Cytokine concentrations were calculated by comparing to standard curves using FACS array analysis software (BD Biosciences).

Intracellular IL-21

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

Adoptive 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 (Miltyeni Biotec). Following purification, 3×10^5 $CD4^+$ T cells were adoptively transferred i.v. into Cα ko B6.g7 or Cα ko IDO2 ko B6.g7 hosts. Arthritis was measured as described above. Mice were sacrificed after 2 weeks and anti-GPI titer measured by ELISA.

Statistical Analysis

Statistical significance was determined using an unpaired Student's t test or the Mann-Whitney nonparametric test and Instat Software (GraphPad Software, Inc).

RESULTS

Deletion of IDO2, but not IDO1, ameliorates arthritis

To determine if 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 their KRN.g7 counterparts. IDO2 ko KRN.g7 mice, however, had a significantly delayed arthritis onset and overall reduction in ankle swelling compared to 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 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 catabolize 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 (e.g. 6, 7, 11) and mice (23), suggesting an association between the IDO pathway and autoimmunity. To determine if 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. While 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 if the effect of 1MT was due to inhibition of IDO1, IDO2, or both, 1MT was administered to wt

KRN.g7 and IDO1 and IDO2 ko KRN.g7 mice beginning at 21 days 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 anti-arthritic 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, while 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 antibody 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 determine if 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 (ASC) in the joint draining lymph nodes 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 were reduced in the IDO2 ko compared to IDO1 ko and wt KRN.g7 mice (Fig. 3B). To determine whether the reduced antibody response is specific to GPI-reactive Ig, or reflected an overall reduction in antibody titers, total serum Ig levels were measured by ELISA. No significant differences in total Ig were seen between the 3 groups (Fig. 3C). Thus, deletion of IDO2 specifically reduces the number of autoantibody producing cells and serum autoantibody titers, but does not affect the total antibody response in arthritic mice.

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 determine if 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 non-arthritic mice (28). Here, IDO2 ko or wt C57BL/6 mice were administered K/BxN serum and followed for the development of arthritis. Within two days of serum transfer, wt mice developed joint inflammation that peaked approximately 1 week 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 suggests 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 antibody 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 toll-like receptor ligand LPS as a polyclonal stimulus (Fig. 5A). IDO2 ko B cells proliferated and secreted Ig to both stimuli at levels indistinguishable from wt B cells. Next,

IDO2 ko and wt C57BL/6 mice were immunized with the model antigen NP-KLH and followed for their ability to mount a high affinity isotype-switched immune response *in vivo* (Fig. 5B). High titers of anti-NP IgG were generated in both wt and IDO2 ko mice following primary immunization. Furthermore, both IDO2 ko and wt mice generated robust secondary immune responses (Fig. 5C). Collectively, these data demonstrate that IDO2 is not required for B cell antibody responses to model antigens *in vitro* or *in vivo* and suggest that IDO2 plays a more specific role in the B cell responses to autoantigens.

T helper cells and cytokines are reduced in IDO2 ko mice

The B cell response in the KRN transgene model is absolutely dependent on T cell help (reviewed in 43). Since deletion of IDO2 does not appear to have a general effect on B cell responses, we then established whether differences in T cell help to B cells between KRN.g7 and IDO2 ko KRN.g7 mice might contribute to a functional difference in autoantibody production. We first examined T helper (Th) cell subsets by examining intracellular transcription factors in T cells isolated from the draining LNs (dLNs) of KRN.g7 and IDO2 ko KRN.g7 mice. Correlating with the observed decrease in autoimmunity, $CD4^+$ helper T cell subsets are reduced in IDO2 ko KRN.g7 mice, particularly Th1, Th2, and Th17 cells (Fig. 6A). Total $CD4^+$ T cell populations were not significantly different between the wt and IDO2 ko KRN.g7 mice ($CD4^+$ cells constitute 6.5 ± 0.6% and 6.6 ± 0.5% of wt (n=15) and IDO2 (n=12) ko KRN.g7 lymphocytes, respectively). This suggests that T cell differentiation is reduced or impaired in IDO2 ko mice. To determine whether this decrease in differentiated helper T 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 draining lymph node cells. No significant differences were found in the inflammatory cytokines IL-10, IL-17, IFNγ, TNFα, MCP-1, or RANTES. Significant decreases were found in IL-4 and IL-6, both of which can influence B cell proliferation, differentiation, and antibody production (Fig. 6B). IL-21, which can direct B cell antibody 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). While the role of IL-6 is less clear in this system (46), it is undoubtedly relevant to human disease given the success of IL-6 inhibitors in the treatment of RA (47, reviewed in 48). Overall, the data here suggest that the reduced autoantibody production and subsequent arthritis in IDO2 ko KRN.g7 mice is due to a diminished T helper cell response.

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

The reduced T helper response in IDO2 ko mice could be due to a T cell-intrinsic role for IDO2 or a T cell-extrinsic role that IDO2 plays in another cell type, which then influences T helper 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 (Cα ko B6.g7/b or Cα ko IDO2 ko B6.g7/b) (Fig. 7A). Both wt and IDO2 ko KRN T cells induced robust arthritis in wt hosts starting 7-10 days 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 to wt hosts, again regardless of the IDO2 status of the transferred T cells (Fig. 7C). This 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

There is conflicting evidence 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 cell populations and suppression of T cell activation (51, 52), though recent work hints at a more complex immunomodulatory role (reviewed in 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, where 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 to IDO1 (34, 40, 54). IDO2 is expressed in a smaller range of tissues than IDO1, generally confined to liver, kidney, and epididymis, as well as antigen presenting cells (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 mTOR pathways (33), though the relative contribution of IDO1 versus IDO2 to these signals is unknown. Given the result here 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 (CIA) (22, 56) and experimental autoimmune encephalomyelitis (EAE) (21), but ameliorates disease in the 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 is due to 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), whereas another report shows 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 interpretation of the effect of an indirect inhibitor, here we utilized genetic knockout models to examine the role of IDO on autoimmunity. Combining the use of 1MT and genetic knockout models supports the idea that 1MT may be inhibiting a pathway involved with IDO2, not the enzyme itself. 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 antibody responses in general. B cells from IDO2 deficient mice generate normal antibody responses to model antigens *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, 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 T helper cytokines IL-4, IL-6, and IL-21. These cytokines have been shown to be important in driving B cell antibody responses, in particular by directing the differentiation and function of T follicular helper (Tfh) cells (e.g. 58). There was a trend toward lower levels of Tfh cells in IDO2 ko KRN.g7 mice, although this did not reach statistical significance. IL-21, in addition to being associated with Tfh 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 here, as IL-6, a regulator of Th17 cell differentiation, is reduced in the IDO2 ko KRN.g7 mice, though 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. Here, 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 antigen-presenting cell (APC) in the host mice to influence both B and T cell activation. While IDO1 is clearly important to dendritic cell (DC) function, especially in the ability of IDO-expressing DCs to control the balance of effector and regulatory T cell populations required to maintain tolerogenic environments (reviewed in (61)), the role of IDO2 in DC and other antigen presenting cells such as B cells is largely unknown. Recent reports in the literature demonstrate that cross-talk between B cells and T helper cells is necessary to generate effective T cell help for B cell antibody 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 T helper 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 the first 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 $CD4^+$ T helper cells, production of pathogenic autoantibodies, and subsequent development of arthritis. IDO2 appears to specifically regulate autoreactive, but not normal B cell responses, as IDO2 ko mice are able to mount productive antibody responses to model antigens *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 antigen presenting cell. 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 if IDO2 plays a similar role in other autoantibody-mediated autoimmune diseases.

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Nonstandard abbreviations

ASCs	antibody secreting cells
Cα−/−	TCR alpha chain deficient
dLN	draining LN
GPI	glucose-6-phosphate isomerase
K/BxN	KRN (C57BL/6 × NOD)F_1
1MT	1-methyl-tryptophan
RA	rheumatoid arthritis

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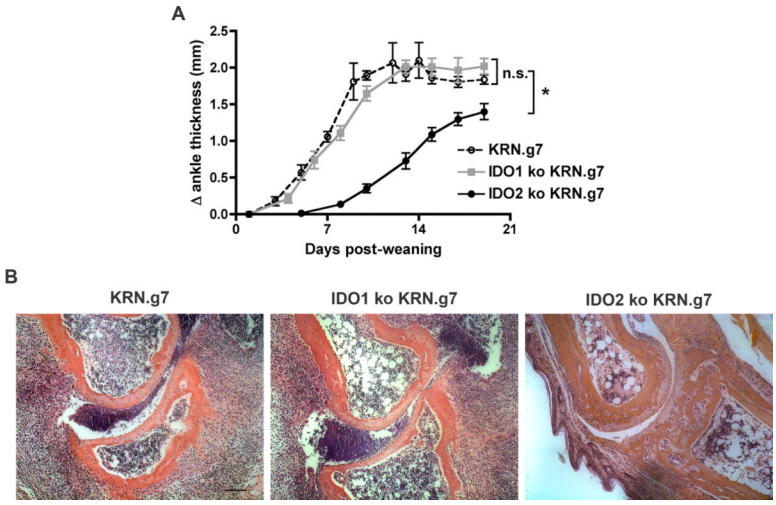

Figure 1. Deletion of IDO2, but not IDO1 ameliorates arthritis

(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 3 independent litters per genotype. (B) Metatarsal joint from KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice at 42d 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 = 100μm. *$p<0.05$. n.s., not significant.

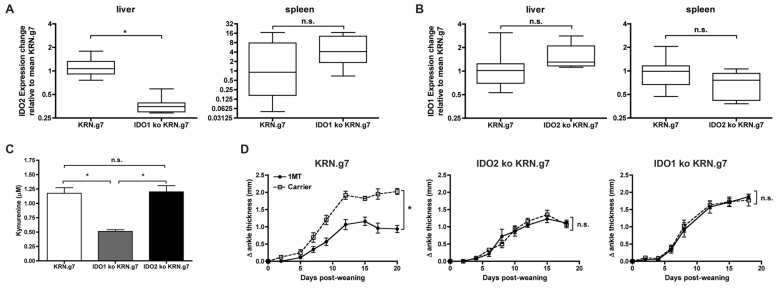

Figure 2. IDO expression, kynurenine production, and the effect of the IDO inhibitor 1MT in IDO1 ko and IDO2 ko KRN.g7 mice

(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 3 independent litters of each genotype. (D) KRN.g7, IDO2 ko KRN.g7, and IDO1 ko KRN.g7 mice were treated with 400mg/kg D/L 1-MT or carrier alone beginning at 21 days 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 2 independent experiments. *$p<0.05$. n.s., not significant.

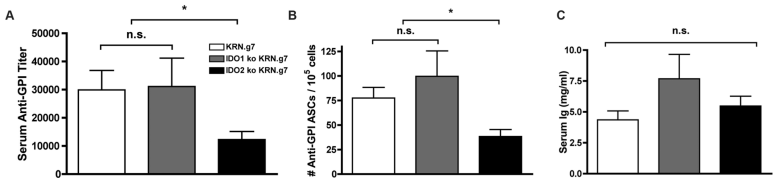

Figure 3. IDO2 affects anti-GPI B cell response but not total antibody levels

(A) Anti-GPI Ig titer in serum from KRN.g7, IDO1 ko KRN.g7, and IDO2 ko KRN.g7 mice was determined by ELISA. Data are represented as mean titer of Ig ± SEM, n=8 mice per group, pooled from 3 independent litters of each genotype. (B) The number of anti-GPI ASCs from joint dLN was determined using an ELISPOT assay. Data shows the mean number of ASC ± SEM for n=27 KRN.g7, n=11 IDO1 ko KRN.g7, and n=12 IDO2 ko KRN.g7 mice, pooled from 3 independent litters of each genotype. (C) Total serum Ig level was measured by ELISA. Mean Ig ± SEM is shown for n=12 mice/group, pooled from 3 independent litters of each genotype. *$p<0.05$. n.s., not significant.

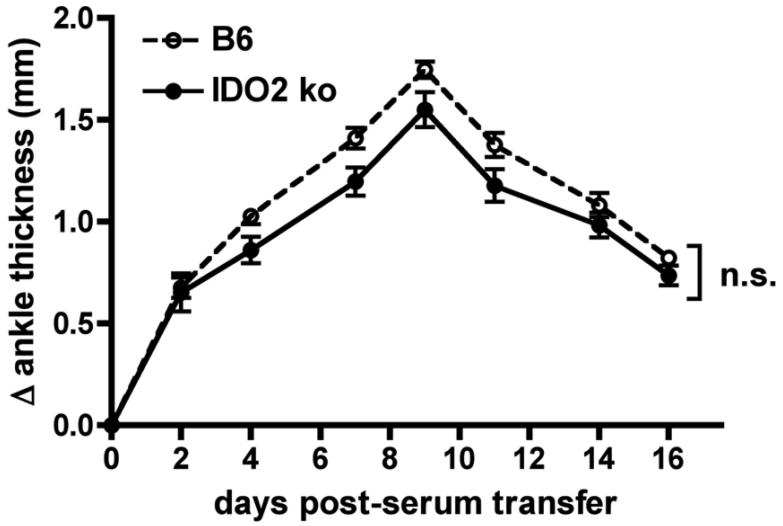

Figure 4. IDO2 deficient mice develop arthritis in response to transferred arthritogenic antibodies

Arthritis was induced in wt and IDO2 ko C57BL/6 mice by transfer of arthritic serum on day 0. Data show mean change in ankle thickness ± SEM for n=12 wt and n=9 IDO2 ko mice, pooled from 2 independent experiments. n.s., not significant.

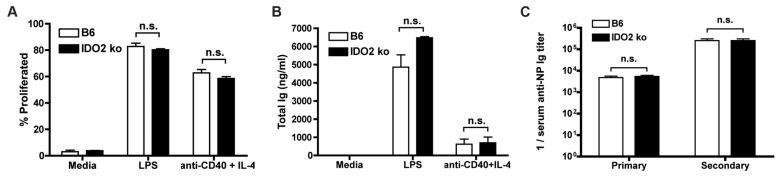

Figure 5. IDO2 does not affect B cell response to model antigens

Purified B cells, pooled from 2 each of C57BL/6 or IDO2 ko C57BL/6 mice, were labeled with CFSE and cultured for 3 days in either media alone, 25μg/ml LPS, or 2μg/ml anti-CD40 + 50ng/ml IL-4. (A) Proliferation was measured by flow cytometry as a decrease in CFSE intensity. Graphs show mean ± SEM of percent proliferated cells, gated on $B220^+$ cells, pooled from 3 independent experiments. (B) Total Ig levels were measured in the culture supernatants. Plots show the mean ± SEM, pooled from 3 independent experiments. (C) wt and IDO2 ko C57BL/6 mice were immunized with NP-KLH precipitated in alum and high affinity anti-NP Ig titer measured by ELISA 8 days after initial immunization to measure primary response and again 8 days following a second immunization to quantify secondary response. Graph shows mean ± SEM of the reciprocal of serum anti-NP titer from n=10 mice per group, pooled from 2 independent experiments. n.s., not significant.

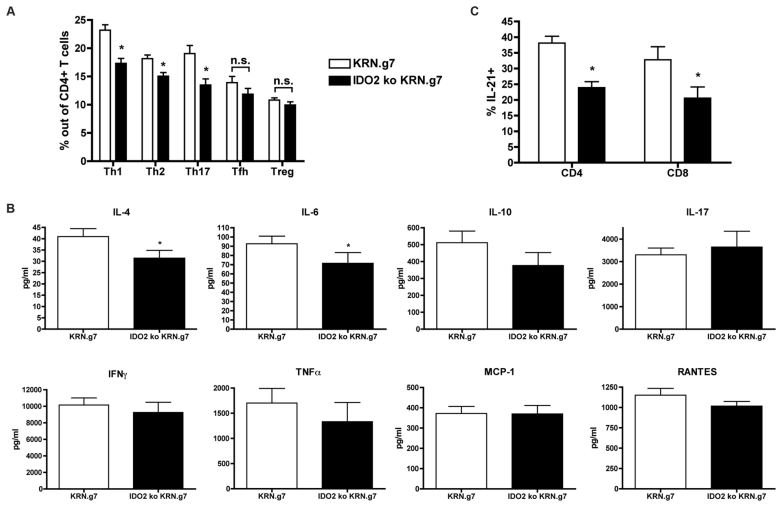

Figure 6. IDO2 alters helper T cell populations and cytokine levels

(A) Frequency of $CD4^+$ T helper cell subpopulations were measured by flow cytometry by intracellular staining for the transcription factors T-bet (Th1), GATA-3 (Th2), RORγt (Th17), Bcl-6 (Tfh), and FoxP3 (Treg). Graphs show mean % ± SEM of each subpopulation out of total $CD4^+$ T cells for n=14 IDO2 ko KRN.g7 and n=13 KRN.g7 mice, pooled from 3 independent experiments. (B) Cells from the joint dLNs (popliteal, axillary, and brachial LNs) were cultured overnight in PMA (50ng/ml) + ionomycin (500ng/ml). Cytokines were measured in culture supernatants by cytometric bead array. Graphs show the mean concentration ± SEM from n=12 IDO2 ko KRN.g7 and n=22 KRN.g7 mice (except n=21 for MCP-1 and RANTES), pooled from 3 independent experiments. (C) Cells from the joint dLNs were cultured for 4h in PMA (50ng/ml) + ionomycin (500ng/ml) + 3μg/ml brefeldin A. Intracellular IL-21 was measured by flow cytometry. Graph shows % $IL\text{-}21^+$ cells ± SEM out of total $CD4^+$ and $CD8^+$ T cell populations from n=12 IDO2 ko KRN.g7 and n=15 KRN.g7 mice, pooled from 4 independent experiments. *p<0.05. n.s., not significant.

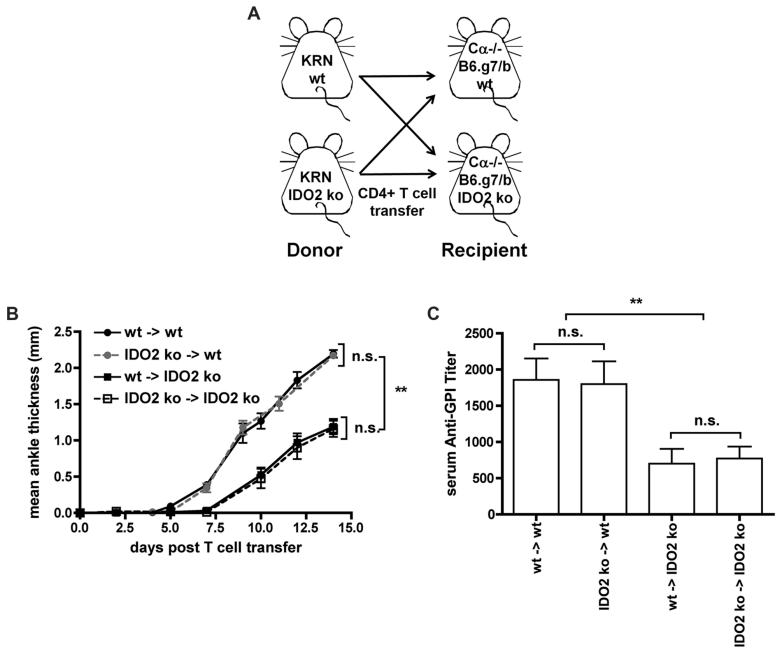

Figure 7. Effect of IDO2 is not intrinsic to T cells

Arthritis was induced by the adoptive transfer of purified $CD4^+$ KRN T cells from wt or IDO2 ko C57BL/6 ($I\text{-}A^b$) mice into T-cell deficient wt or IDO2 ko C57BL/6 ($I\text{-}A^{g7/b}$) 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 days after T cell transfer. Data shows the mean ± SEM of the reciprocal of serum anti-GPI titer. Data is 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 5 independent experiments. *$p<0.05$, **$p<0.01$. n.s., not significant.