Regulation of indoleamine 2,3 dioxygenase and its role in a porcine model of acute kidney allograft rejection

Youli Wang,[1] Todd D Merchen,[2] Xuexiu Fang,[1] Randi Lassiter,[2] Chak-Sum Ho,[3] Ryan Jajosky,[4] Daniel Kleven,[4] Thomas Thompson,[4] Eslam Mohamed,[1] Miao Yu,[1] Jennifer L Waller,[5] N Stanley Nahman Jr[1,6]

[1]Department of Medicine, Medical College of Georgia at Augusta University, Augusta, Georgia, USA
[2]Department of Surgery, Medical College of Georgia at Augusta University, Augusta, Georgia, USA
[3]Gift of Life Michigan, Ann Arbor, Michigan, USA
[4]Department of Pathology, Medical College of Georgia at Augusta University, Augusta, Georgia, USA
[5]Department of Population Health Sciences, Medical College of Georgia at Augusta University, Augusta, Georgia, USA
[6]Medicine, Charlie Norwood VAMC, Augusta, Georgia, USA

Correspondence to
Dr N Stanley Nahman Jr, Department of Medicine, Medical College of Georgia at Augusta University, Augusta, GA 30912, USA; nnahman@augusta.edu

Accepted 8 June 2018

To cite: Wang Y, Merchen TD, Fang X, *et al*. *J Investig Med* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/jim-2018-000742

ABSTRACT

In kidney transplantation acute allograft rejection is the most common cause of late allograft loss. Changes in indoleamine 2,3 dioxygenase (IDO) activity, which catabolizes the degradation of tryptophan to kynurenine, may predict rejection. However, exogenous IDO is immunosuppressive in rodent kidney transplantation. Thus, the increase in IDO activity observed in acute allograft rejection is insufficient to prevent rejection. To address this question, we assessed the regulation of IDO and its role in acute rejection in a porcine model of kidney transplant. In tissue samples from rejecting kidney allografts, we showed a 13-fold increase in IDO gene transcription and 20-fold increase in IDO enzyme activity when compared with autotransplanted kidneys. Allografts also demonstrated an over fourfold increase in tissue interferon (IFN)-γ, with marked increases in tumor necrosis factor (TNF)-α, TNF-β and interleukin 1β. Gene transcription and protein levels of kynurenine 3-monooxygenase (KMO) were decreased. KMO generates the immunosuppressive kynurenine, 3-hydroxykynurenine. The results of these studies demonstrate a clear association between rejection and increased allograft IDO expression, likely driven in part by IFN-γ and facilitated by other cytokines of the allogeneic response. Moreover, the loss of downstream enzymatic activity in the IDO metabolic pathway may suggest novel mechanisms for the perpetuation of rejection.

Significance of this study

What is already known about this subject?
- The indoleamine 2,3 dioxygenase (IDO) enzyme cascade generates immunosuppressive kynurenines through the action of kynurenine 3-monooxygenase (KMO).
- Induction of IDO before engraftment prevents kidney transplant rejection in rodents.
- IDO induction after engraftment does not prevent rejection and IDO is used as a biomarker of rejection.

What are the new findings?
- IDO's immunosuppressive properties are overwhelmed by cytokines from the allogeneic response.
- KMO is suppressed by rejection.
- Pigs are a good model for the study of IDO in rejection.

How might these results change the focus of research or clinical practice?
- This study addresses the clinical paradox of the IDO enzymatic cascade: it is a known immunosuppressant, but appears ineffective if not activated prior to transplant.
- We show that the allogeneic response leads to increased cytokines, including elevated interferon-γ, which drives IDO.
- We also demonstrate that KMO activity is suppressed by rejection, suggesting that the loss of immunosuppressive kynurenines may contribute to the loss of tolerance in this model.
- Clinically, activating IDO prior to engraftment may induce tolerance.
- The pig model is the most relevant platform for extrapolating this hypothesis into clinical transplantation.

INTRODUCTION

In kidney transplantation acute allograft rejection is the most common cause of late allograft loss,[1] is typically heralded by an increase in serum creatinine, and confirmed with renal biopsy. The rise in serum creatinine reflects a decrement in glomerular filtration from histologic damage; thus, the serum creatinine may be a late marker for parenchymal loss in this pathologic process. To address this issue, Brandacher *et al*[2 3] showed that in acute kidney allograft rejection, changes in serum and urine indoleamine 2,3 dioxygenase (IDO) activity predated an increase in serum creatinine, demonstrating that IDO activity is a predictor of rejection; findings that are also confirmed in pediatric transplant recipients.[4 5]

IDO is a heme-containing enzyme, is the rate-limiting step in the catabolism of tryptophan to kynurenine, and is associated with immunosuppression.[6] In this regard, placental IDO inhibits allogeneic rejection in pregnancy,[7] and IDO activity by metastatic cancer cells protects from immune recognition and clearance.[8] Experimentally, elevated levels of the human IDO transprotein prevent rejection of rodent cardiac, lung, islet cell, and renal transplants.[9–13] Tryptophan depletion, combined with the production of cytotoxic and/or immunosuppressive kynurenines, results in T cell apoptosis, the generation of regulatory T cells (Tregs), and modulation of allogeneic responses.[14–16]

IDO as a biomarker of rejection appears paradoxical: as an endogenous immunosuppressant, IDO should prevent rejection, not serve as a marker for a biologic process it purportedly prevents. However, it is clear that the increase in IDO activity observed in acute allograft rejection is not completely immunosuppressive, suggesting that IDO-dependent pathways of immunosuppression are insufficient to completely squelch the allogeneic response.[2] The blunting of IDO-dependent immunosuppression could result from incomplete tryptophan depletion and/or inadequate generation of immunomodulatory kynurenines. In this regard, kynurenine generated by IDO is a substrate for at least three distinct catabolic pathways: (1) kynureninase (KY) to anthranilic acid; (2) kynurenine aminotransferase (KAT) to kynurenic acid; and (3) kynurenine 3-monooxygenase (KMO) to 3-hydroxykynurenine (3-HK). 3-HK and kynurenic acid exhibit immunosuppressive properties, with 3-HK the more potent compound.[15 17]

IDO is not constitutively expressed, but is inducible.[18] In this regard, the IDO gene promoter contains an interferon (IFN) sequence response-like Cis element[19 20] and thus can be transcriptionally regulated by IFN-γ in solid organ transplantation.[21] IFN-γ is released by T cells after engagement of the T cell receptor complex in conjunction with major histocompatibility complex (MHC), and from natural killer (NK) cells following tumor necrosis factor (TNF)-α and IL-12 stimulation.[21] In rejection, IFN-γ also triggers the expansion of cytotoxic T cells,[22] opening an obvious window for IDO-independent pathways that favor rejection.

In previous studies, we reported our experience with a porcine model of allogeneic kidney transplantation.[23] The current work extends those observations and investigates the regulation of IDO and its role in acute rejection. In kidney allografts with extensive rejection, we show marked increases in IDO activity that parallel tissue IFN-γ and other inflammatory cytokine levels (TNF-α, TNF-β and IL-1β). Rejecting allografts also demonstrated downregulation of KY, KAT, and KMO. The results of these studies demonstrate a clear association between rejection and increased allograft IDO expression, likely driven in part by IFN-γ and facilitated by other cytokines of the allogeneic response. Furthermore, the loss of downstream enzymatic activity in the IDO metabolic pathway may suggest novel mechanisms for the perpetuation of rejection in the early transplant period.

MATERIALS AND METHODS

Experimental design

Studies were performed on 30–40 kg outbred female Yorkshire piglets (Palmetto Research Swine, Reevesville, South Carolina) as we have described.[23] In brief, one pair of pigs was operated simultaneously, the left kidneys exchanged (allotransplants) or retransplanted (autotransplants). All transplants were ex vivo perfused at 4°C and orthotopically transplanted, followed by right nephrectomy (control tissue) and closure.[23] No immunosuppression was used. All autotransplants and allotransplants were recovered 72 hours after transplantation in a live organ harvest. Animals were sacrificed under general anesthesia and at the conclusion of the harvest. When indicated, swine leukocyte antigen (SLA) genotyping was performed, and histologic scoring according to Banff criteria was conducted as we described.[23] Tissues from control right kidneys, autotransplants, and allotransplants were probed for IDO and cytokine gene and protein expression, and IDO enzymatic activity.

Quantitative RT-PCR

Quantitative PCR primers of 90–120 bp amplicon for IFN-α_1, IFN-α_2, IFN-β, and IFN-γ; interleukin (IL) 1β; TNF-α and TNF-β; and the enzymes KY, KAT, and KMO were designed with Primer3 software (https://www.broadinstitute.org). Total RNA was isolated with TRIzol reagent (Invitrogen), first-strand cDNA was synthesized using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen), and PCR reactions performed with SsoAdvanced Universal SYBR Green Kit (Bio-Rad Laboratories, Hercules, California) on a CFX Connect real-time PCR detect system (Bio-Rad Laboratories). β-actin mRNA was used for normalization.

IDO enzyme activity assay

One hundred microliters of tissue homogenate were mixed with equal volume of substrate solution (100 mM phosphate buffered saline (PBS), pH 6.5, 50 µM methylene, 50 mM ascorbate, 0.4 mM L-Tryptophan and 20 µg catalase-fresh added), and 100 µL of the mixture was taken and mixed with 10 µL of 60% perchloric acid and saved on ice as the time 0 control; the remaining solution was incubated at 37°C on a plate shaker at 550 rpm for 2 hours, mixed well with 10 µL of 60% perchloric acid and the supernatant collected after spinning at 2000 g, and filtered with a Millipore MSRLN0410 filter plate before using high-pressure liquid chromatography (HPLC) for kynurenine assays.

Reverse-phase HPLC for tryptophan and kynurenine levels in serum, urine and tissue homogenates

Fifty microliters of serum, urine and tissue homogenate were mixed with an equal volume of PBS (pH 7.4), kept at room temperature for 2 min, followed by an addition of 10 µL of 60% perchloric acid, and vortexed. After incubation on ice, samples were centrifuged at 4°C for 10 min and the supernatant collected and filtered with a Millipore MSRLN0410 filter plate before using HPLC for kynurenine assays with the following conditions: C18 4.6×5 column with a 1.2 mL/min flow rate in buffer A (2.5% acetonitrile in 15 mM NaAc, pH 4.0)/buffer B (100% acetonitrile), with detectors at 360 nm for kynurenine, and 285 nm excitation/365 nm emission for tryptophan. Creatinine concentration in urine was determined using The Creatinine Companion Kit (Exocell, Philadelphia, Pennsylvania).

Immunohistochemistry

Immunohistochemistry for the detection of IDO using mouse monoclonal antihuman IDO (CalBioreagents, San Mateo, California) in 1:600 dilution in 1:600 dilution was conducted as we have described previously in detail.[23] In brief, 5 μm paraformaldehyde (4%)-fixed sections were deparaffinized and epitope retrieval achieved using Dako Target Retrieval Solution (Dako), blocked for non-specific staining and endogenous biotin, and incubated with affinity-purified primary antibody. Sections were incubated with biotinylated secondary antibody followed by an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, California), developed, counterstained with hematoxylin, and mounted for viewing.

Western blotting

Western blotting was performed as described in a previous report.[24] Briefly, tissues were lysed inradioimmunoprecipitation lysis buffer (RIPA) buffer with protease and phosphatase inhibitors (Roche Diagnostic Systems, Indianapolis, Indiana). Equal quantities of proteins determined by bicinchoninic assay (Pierce Biotechnology, Rockford, Illinois) were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions and transferred to plyvinylidene difluoride (PVD) membranes. Membranes were blocked with 5% non-fat milk and incubated with antiporcine IFN-γ antibody (R&D System, Minneapolis, Minnesota) or anti-KMO antibody (vendor), followed by horseradish peroxidase (HRP)-conjugated secondary antibody. Signals were detected with Pierce ECL (ThermoFisher Scientific), and quantitated with Image J from the National Institutes of Health and normalized with β-actin (Sigma-Aldrich).

ELISA for cytokines

Cytokines in serum, and kidney tissue lysate in PBS, were assayed according to vendor protocols—IFN-γ: Quantikine ELISA Porcine IFN-γ (R&D Systems); IL-1β: IL-1β Pig ELISA Kit (Abcam); IFN-α: RayBio Porcine IFN-α ELISA Kit (RayBiotech, Norcross, Georgia); and IFN-β: Porcine Fibroblast IFN-β1 ELISA Kit (BlueGene Biotech, Shanghai, China).

Statistical analysis

All statistical analyses were performed using SAS V.9.4 and data are expressed as mean±SEM. Due to the correlated nature of some of the cytokine measures, statistical significance was assessed using an alpha level of 0.01. Descriptive statistics within each group were determined. Associations between cytokine expressions within group were determined using analysis of covariance. To examine differences in cytokines between transplant groups (autograft, allograft), two-sample t-tests were used. IDO and cytokine expression data from control kidneys are also presented for reference purposes.

RESULTS

Histologic parameters and clinical characteristics of rejection in a porcine model of kidney transplant

Using orthotopic autotransplantation and allotransplantation, we defined grades of rejection and clinical responses from 16 pigs (6 autotransplants (Auto)

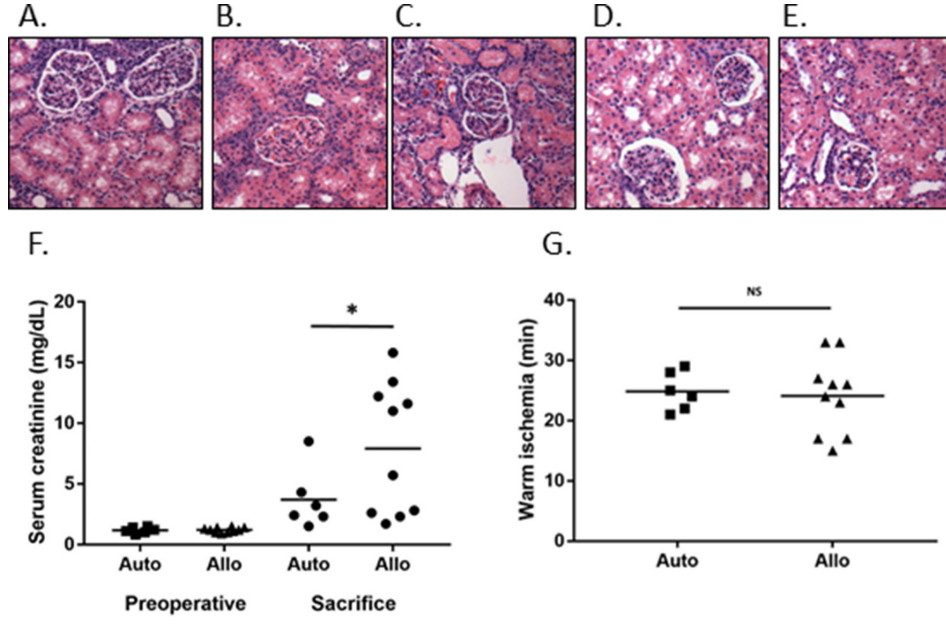

Figure 1 Renal histology, serum creatinine, and warm ischemia times from transplanted porcine kidneys. (A–C) Representative rejecting kidneys showing Banff IB (A), IIA (B), and IIB (C) rejection grades. (D) Autotransplant (Auto) with resolving acute tubular necrosis. (E) Control tissue from normal right kidney (white bar=200 µm). (F) Serum creatinine from autotransplants and allotransplants preoperatively and at sacrifice after 72 hours. There was a significant increase in serum creatinine at sacrifice in allotransplant (Allo) (*p<0.05 (to convert mg/dL to SI multiply by 88.42)). (G) Comparison of warm ischemia times between Auto and Allo. There was no difference (horizontal bars=mean for each group).

and 10 allotransplants (Allo)). Normal tissue from 11 right nephrectomy specimens from this cohort was also obtained. To confirm haplotype mismatching in rejecting Allos, tissue typing was performed and demonstrated SLA mismatches ranging from 2 to 4. All mismatched Allos demonstrated acute cellular rejection, with Banff scores of IA, IB, IIB, or IIIA. In two pairs of allotransplants, 3 of 4 kidneys showed 4 of 4 complete SLA matches, and resulted in graft acceptance without rejection (data not shown). Figure 1 indicates renal histology from five representative animals, and includes three rejecting kidneys with Banff level IB (figure 1A), IIA (figure 1B) and IIB (figure 1C) grades of rejection, a representative autotransplant with recovering tubular necrosis (figure 1D), and a right kidney control showing normal histology. Clinically, Allos had a significant elevation in serum creatinine at sacrifice when compared with Autos (figure 1F). Warm ischemia time did not differ between autotransplant and allotransplant groups, and averaged 24 min (figure 1G).

IDO expression and enzyme activity in allotransplantation

Rejection results from a self-amplifying cellular and cytokine cascade culminating in the destruction of foreign tissue. In this regard, pretransplant transformation of rodent allografts to express the IDO transgene and protein led to enhanced IDO activity of the allograft.[25] By transplanting IDO-enriched organs that antedate the above immune responses, rejection can be prevented.[12 25] In contrast, Brandacher *et al*[2] showed that increased IDO activity following transplantation of conventional (ie, untransformed) allografts was associated with rejection. These data suggest that the rejection cascades cited above both trigger IDO and overwhelm the immunomodulatory properties of the enzyme, and thus culminate in rejection, despite increased IDO. To further define IDO expression and activity in this setting, we investigated tissue and systemic IDO expression from conventional rejecting allografts. Figure 2 compares allograft IDO expression between Autos and Allos. Allos

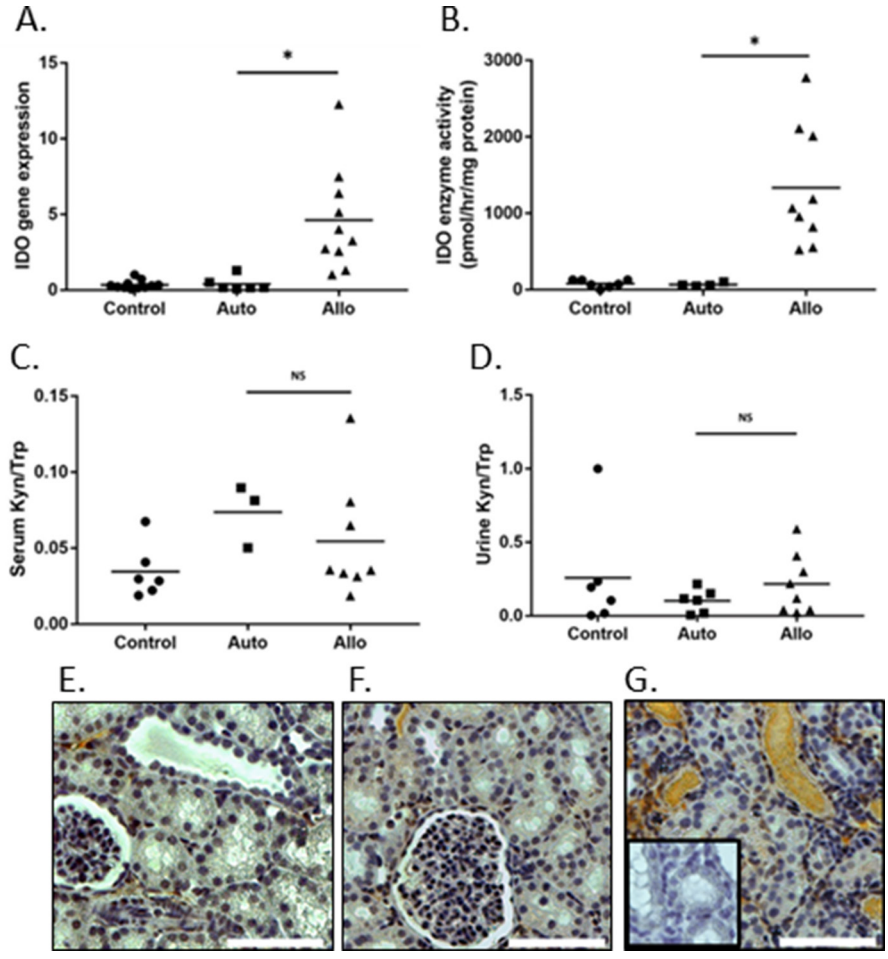

Figure 2 IDO gene, protein, and enzyme expression from transplanted pigs. IDO gene transcription (A) and enzyme activity (B) from transplanted kidneys were significantly increased in allotransplants (Allo) versus autotransplants (Auto) (*p<0.01). (C,D) Serum and urine IDO activities as measured by kynurenine (Kyn) to tryptophan (Trp) ratio. There were no differences between the Auto and Allo groups. (E,F) Representative immunohistochemical sections from normal kidney and autotransplanted kidney, respectively. There is minimal IDO protein staining (orange color). (G) Acutely rejecting allotransplant (Banff IIB) showing increased tubular cell and luminal IDO staining and a control section stained with secondary antibody only (inset) that was negative for IDO (white bar=200 µm). IDO, indoleamine 2,3 dioxygenase.

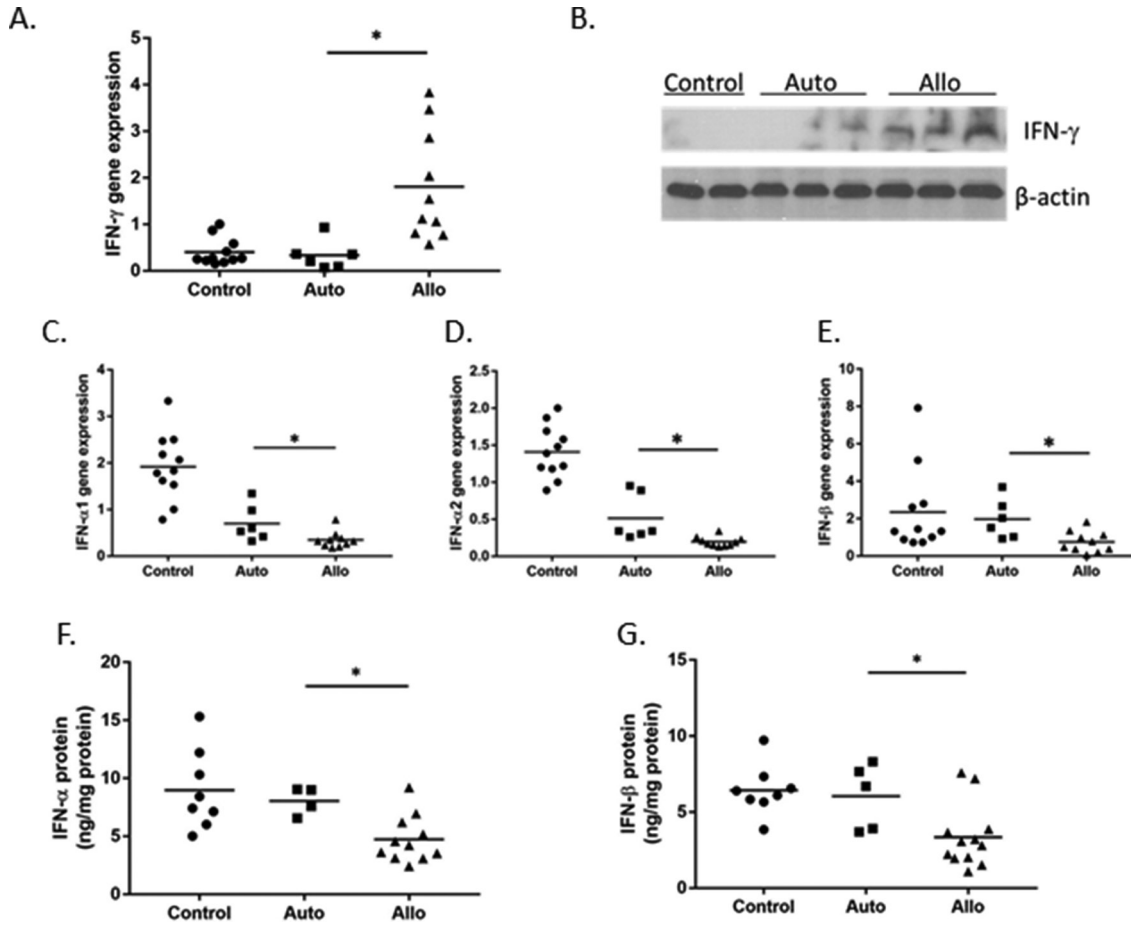

Figure 3 Interferon (IFN) gene and protein expression from transplanted pig kidneys. (A) IFN-γ gene expression was significantly elevated in allotransplants (Allo) versus autotransplants (Auto) (*p<0.0035). (B) Western immunoblot showing an increase in IFN-γ protein. (C–E) IFN-α_1, IFN-α_2, and IFN-β gene expression. Each cytokine was significantly decreased in Allo versus Auto (*p<0.05). (F,G) Kidney IFN-α and IFN-β protein levels. Both IFN-α and IFN-β were significantly decreased in Allo versus Auto (*p<0.05).

showed a ~13 fold increase in IDO gene transcription and an approximate 20-fold increase in IDO enzyme activity when compared with Autos (figure 2A,B). Moreover, increased expression of the IDO protein from Allos when compared with Autos was evident by immunohistochemistry (representative tissues in figure 2E–G). Serum IDO activity, as assessed by the ratio of kynurenine to tryptophan, was non-significantly increased in Autos over Allos (figure 2C), and urinary IDO activity, as measured by urinary kynurenine levels, was non-significantly increased in Allos when compared with Autos (figure 2D).

IFN-γ, IFN-α, and IFN-β expression, and IDO expression in kidney transplants

The IDO gene promoter contains an IFN responsive element, and IFN-γ drives IDO expression.[19–21] On this basis, IFN-γ, IFN-α, and IFN-β expression from Autos and Allos was investigated. Figure 3A,B demonstrates an over fourfold increase in kidney IFN-γ at the transcriptional level, with a corresponding increase in protein expression in Allos, when compared with Autos. In contrast, tissue IFN-α and IFN-β mRNA were downregulated, and protein expression by ELISA significantly decreased in Allos (figure 3C–G).

Cytokine activation in rejection and IDO expression

Rejection is associated with activation of antigen presenting cells, which stimulate $CD4^+$ lymphocytes, promoting the release of inflammatory cytokines that include IL-1β, TNF-α and IFN-γ, all of which further activate antigen presenting cells and amplify T cell responses.[26] TNF-α stimulates IDO independent of IFN-γ.[26] In this regard, kidney from Allos showed significant increases in the transcription and translation of the IL-β gene when compared with Autos (figure 4A,B). Transcription of the TNF-α and TNF-β genes was also significantly upregulated in Allos (figure 4C,D). Analysis of covariance (ANOCOVA) analysis, controlling for transplant type, was used to assess for associations between cytokine and IDO gene transcription (figure 5). IDO gene expression correlated positively with TNF-α, and TNF-β mRNA, and negatively with IFN-β (figure 5A–C), but showed no relationship in association with IFN-α_1 (figure 5D).

Kynurenine degradative enzyme gene expression

IDO is the rate-limiting step in the degradation of tryptophan into kynurenine.[6] Kynurenine is further degraded by at least three major enzymes into potentially immunosuppressive metabolites, including the product of KMO, 3-HK.[15 17] Loss of immunosuppressive kynurenines

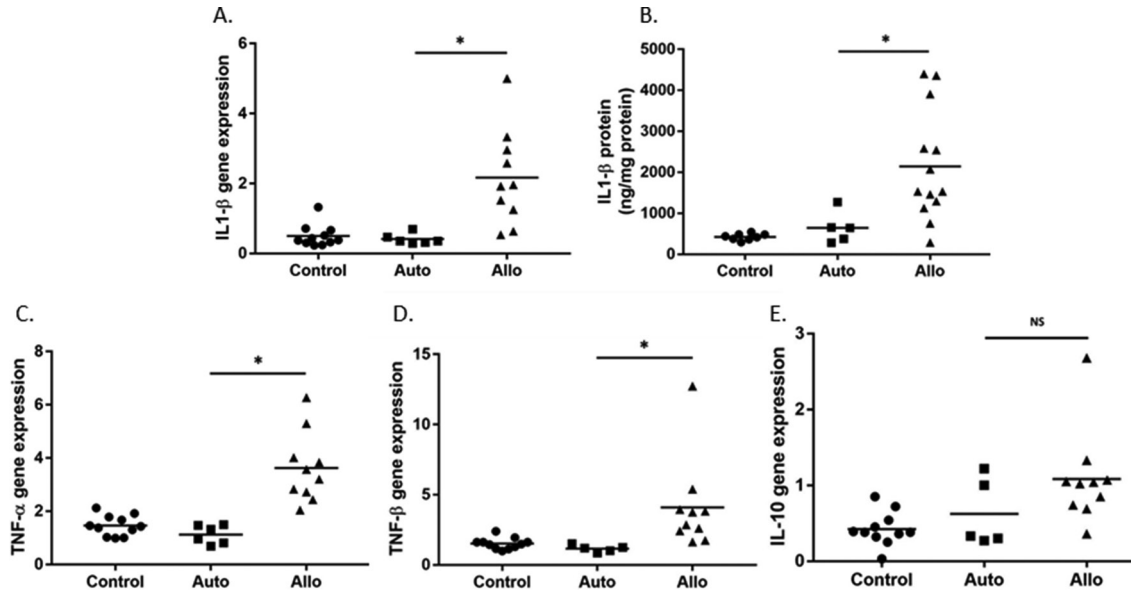

Figure 4 Cytokine expression from control, auto and allotransplanted pig kidneys. (A,B) IL-1β gene and protein expression from the kidney tissue. There was a significant increase in allotransplant (Allo) versus autotransplant (Auto) (*p<0.05). (C–E) TNF-α and TNF-β gene expression. TNF-α and TNF-β were significantly increased in Allo versus Auto (*p<0.02). IL, interleukin; TNF, tumor necrosis factor.

could promote rejection despite elevated IDO levels. To investigate this question we compared KY, KAT, and KMO mRNA levels from Autos and Allos. There was no difference between Autos and Allos for KY and KAT (figure 6A,B), but KMO gene transcription was significantly decreased in Allos (figure 6C). Similarly, KMO protein expression was decreased in Allos when compared with Autos (figure 6D).

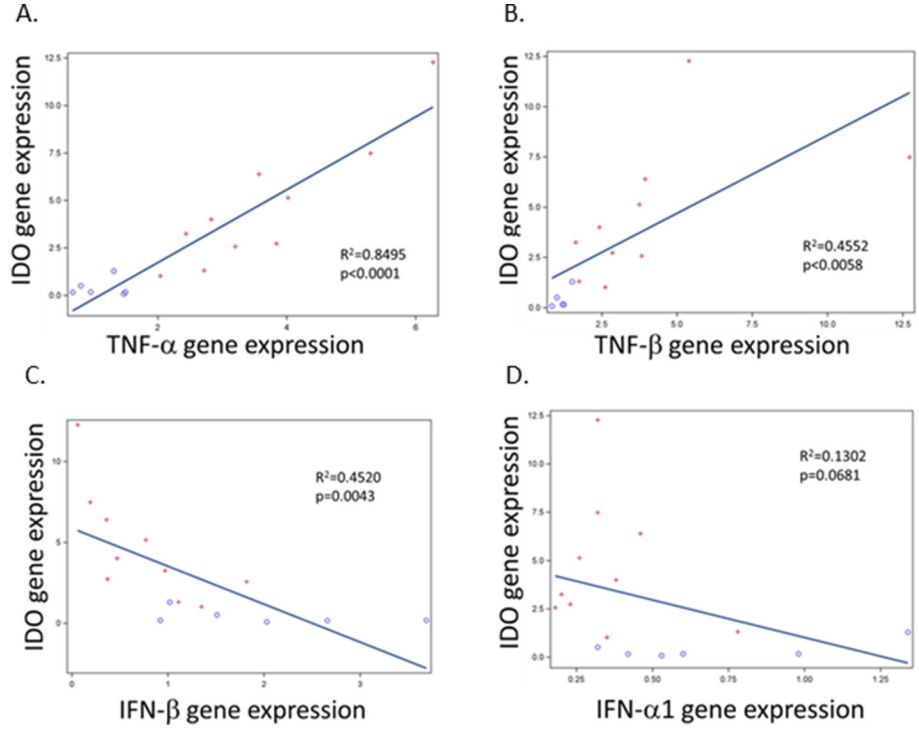

Figure 5 Association between IDO and cytokine gene transcriptions from Auto (open circles) and Allo (crosses) transplanted groups. (A–D) IDO gene expression correlated positively with TNF-α and TNF-β mRNA, and negatively with IFN-β (figure 5A–C), and showed no relationship in association with IFN-α1 (figure 5D). IDO, indoleamine 2,3 dioxygenase; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

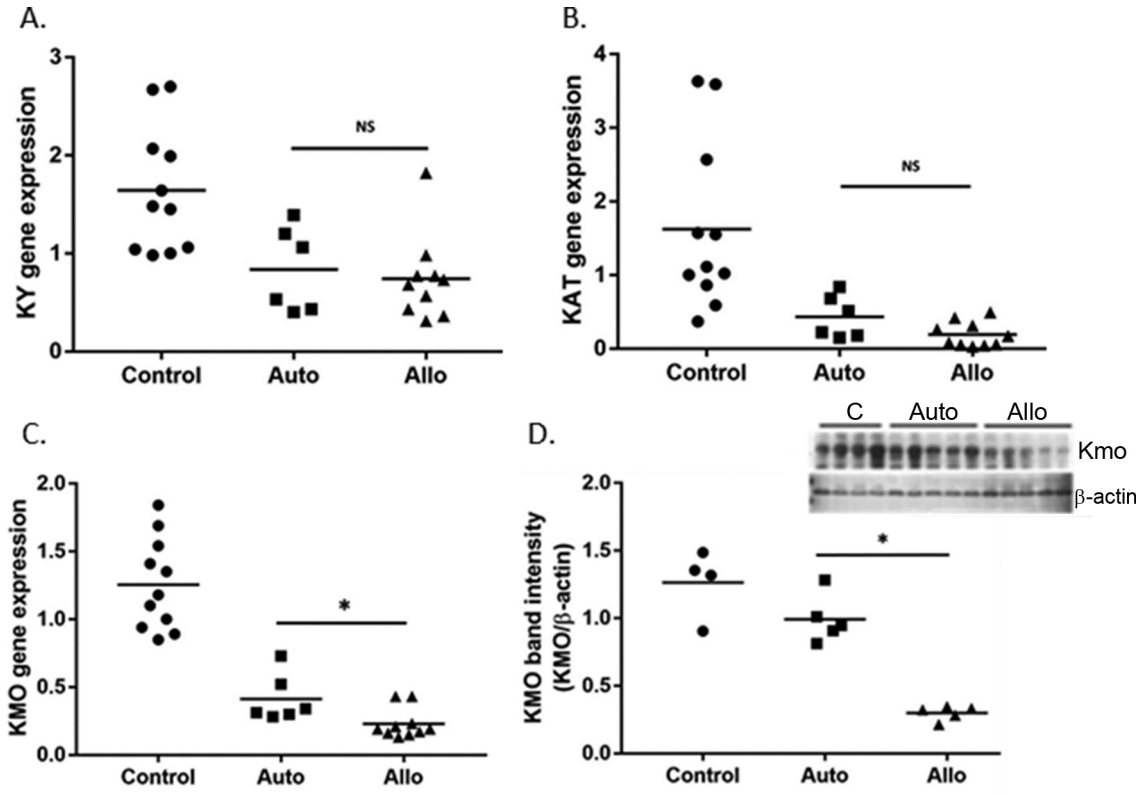

Figure 6 Kynurenine degradative enzyme expression from pig kidneys. (A) Kynureninase (KY). (B) Kynurenine aminotransferase (KAT). (C) Kynurenine 3-monooxygenase (KMO). There was no difference between autotransplant (Auto) and allotransplant (Allo) for KY and KAT; however for KMO, Allo was significantly depressed (*p=0.0216). (D) KMO protein expression from 14 representative animals (4 controls (C), 5 Autos, and 5 Allos). Each bar represents the group mean of the KMO to β-actin ratio from the indicated western immunoblot (inset). KMO expression was significantly decreased in Allo when compared with Auto (*p<0.05).

DISCUSSION

These studies demonstrate that the allogeneic response induces significant increases in allograft IDO gene, protein, and enzyme activity in mismatched pig kidney transplants. IFN-γ and TNF-α tracked with the observed increase in IDO expression as did other cytokines known to facilitate acute rejection.[27 28] Finally, suppression of KMO, with presumed loss of 3-HK,[15] may offer an additional, IDO-dependent pathway for promoting rejection. Taken together, this work indicates that the rejecting allograft is a source of significant IDO generation occurring in association with an inflammatory cytokine surge and suppression of KMO. These data offer possible explanations for the inability of elevated allograft IDO activity to inhibit rejection.

In the present work, our outbred pig model was used to induce kidney transplant rejection.[23] All allografts were SLA-mismatched and showed clinical and histologic evidence of rejection within 72 hours. There was no difference in warm ischemia time between autotransplanted and allotransplanted animals, effectively excluding ischemia as an explanation for any observed differences between the two groups.

The current studies demonstrated striking increases in kidney allograft IDO expression 3 days post-transplant, with increases in gene expression, protein levels, and IDO enzyme activity. These data are consistent with untreated mismatched murine cardiac transplants, where graft IDO mRNA was increased 100-fold by postoperative day 6.[21] In addition, histologic expression of tubular cell IDO in our pig allotransplants was substantially increased, similar to what others have reported.[2 29]

Our data did not demonstrate significant increases in serum or urine IDO activity from pigs with rejecting allografts, and contrasts with data showing elevated levels in patients with rejection.[2] The explanation for this observation likely stems from clinical differences in the two populations. In this regard, all serum and urine samples from the pigs were obtained under general anesthesia, and unlike patients the animals were not receiving immunosuppression. It is unknown how immunosuppressive medication or anesthetics may influence IDO blood or urine activity. The smaller sample size of the pig study group, as well as the short time course between the transplant and sampling (72 hours), may have also influenced these data. For example, the urinary kynurenine levels were numerically elevated in the allotransplant group, but the difference did not reach significance.

The present studies demonstrated elevated cytokine levels in rejecting allografts, including IFN-γ, IL-1β, and TNF-α and TNF-β. IFN-γ is the primary cytokine triggering IDO,[21] is synthesized by lymphocytes and macrophages,[30 31] and is stimulated by TNF-α.[21] IL-1β, TNF-α and TNF-β are integral to inflammatory allogeneic responses,[22 28 32] and along with IFN-γ were strongly associated with IDO gene transcription (figure 5). Not surprisingly, the expression of IFN-α and IFN-β was decreased in rejecting allografts. Both

cytokines are produced mainly as a reaction to infection, immunologically are antiproliferative, and may clinically be used to treat viral infections.[27]

The current studies present the clinical conundrum of acute rejection with elevated allograft IDO activity. We have theorized that the immunosuppressive properties of enhanced IDO activity in the rejecting allograft are mitigated by both IDO-independent and/or IDO-dependent processes. In this regard, the predominant IDO-independent factor is represented by the overwhelming cytokine surge of the allogeneic response that includes TNF-α.[21] A possible IDO-dependent process is the suppression of KMO and the potential loss of 3-HK. 3-HK has been shown to strongly suppress T cell responses in rodents,[15] suggesting any decline in KMO catabolism of kynurenine during rejection may blunt the immunosuppressive effects of IDO. It is unknown how rejection may suppress KMO, but the enzyme may be sensitive, at least in part, to ischemia, since autotransplants also exhibited depression of the enzyme. In addition, figure 6 suggests a disconnect between KMO gene expression and KMO protein levels. We would speculate this is related, at least in part, to mRNA stability (with degradation) combined with a longer protein half-life. Alternatively, differential expression between the gene and the protein may be affected by as yet undefined factor or factors associated with allogeneic responses. Finally, KMO suppression during rejection might be expected to increase serum kynurenine levels as a result of decreased degradation (figure 2). It is unclear why the loss of KMO activity did not raise kynurenine concentrations; however, we would speculate that the ongoing metabolic activity of KY and KAT may be contributing to ongoing degradation of kynurenine.

IDO remains an attractive, protolerant enzyme for mitigating the immune response in solid organ transplantation. IDO exerts its immunosuppressive effects through tryptophan depletion and kynurenine generation.[33] Exhaustion of local tryptophan stores increases stress-response protein kinase GCN2, and in the presence of kynurenine promotes induction of Foxp3$^+$ Tregs.[33 34] Further, kynurenine pathway products bind and activate the aryl hydrocarbon receptor on T cells,[33 35] and promote immunosuppression by increasing the expression of Tregs.[33] The protolerant effects of IDO are manifest in pregnancy, where high levels of placental IDO protect the allogeneic fetus from maternal attack[7]; cancer, in which elevated IDO levels promote local immunosuppression in the tumor environment[33]; and in rodent models of heart, lung, and kidney transplantation treated with the IDO transprotein.[9–12] Harnessing the immunosuppressive properties of IDO in kidney transplantation may offer a novel approach to either induction protocols or long-term tolerance.

In summary, using a porcine model of kidney allograft rejection, we have shown that the allogeneic response triggers an inflammatory cytokine surge in association with marked increases in allograft IDO expression. The work also revealed the possible role of KMO, a downstream enzyme in the IDO cascade, for facilitating the protolerant properties of IDO. This experimental platform may be harnessed to further define IDO biology in allogeneic kidney transplantation, as well as test novel therapeutic options.

Acknowledgements Our thanks to Anatolij Horuzsko, PhD, for his critical review of the manuscript, and Yan Cui, PhD, for assistance with high-pressure liquid chromatography.

Contributors All authors have contributed to the preparation of this manuscript.

Funding This work was supported in part by grants from the Carlos and Marguerite Mason Charitable Trust (TDM), and Reserve Research grant fund #S-2444 from Dialysis Clinic (NSN).

Competing interests None declared.

Patient consent Not required.

Ethics approval All experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and approved and monitored by the Augusta University Institutional Animal Care and Use Committee (IACUC).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Stegall MD, Gaston RS, Cosio FG, *et al*. Through a glass darkly: seeking clarity in preventing late kidney transplant failure. *J Am Soc Nephrol* 2015;26:20–9.
2 Brandacher G, Cakar F, Winkler C, *et al*. Non-invasive monitoring of kidney allograft rejection through IDO metabolism evaluation. *Kidney Int* 2007;71:60–7.
3 Brandacher G, Margreiter R, Fuchs D. Clinical relevance of indoleamine 2,3-dioxygenase for alloimmunity and transplantation. *Curr Opin Organ Transplant* 2008;13:10–15.
4 Dharnidharka VR, Al Khasawneh E, Gupta S, *et al*. Verification of association of elevated serum IDO enzyme activity with acute rejection and low CD4-ATP levels with infection. *Transplantation* 2013;96:567–72.
5 Al Khasawneh E, Gupta S, Tuli SY, *et al*. Stable pediatric kidney transplant recipients run higher urine indoleamine 2, 3 dioxygenase (IDO) levels than healthy children. *Pediatr Transplant* 2014;18:254–7.
6 Pallotta MT, Orabona C, Volpi C, *et al*. Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nat Immunol* 2011;12:870–8.
7 Munn DH, Zhou M, Attwood JT, *et al*. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 1998;281:1191–3.
8 Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 2004;4:762–74.
9 Li J, Meinhardt A, Roehrich ME, *et al*. Indoleamine 2,3-dioxygenase gene transfer prolongs cardiac allograft survival. *Am J Physiol Heart Circ Physiol* 2007;293:H3415–H3423.
10 Swanson KA, Zheng Y, Heidler KM, *et al*. CDllc+ cells modulate pulmonary immune responses by production of indoleamine 2,3-dioxygenase. *Am J Respir Cell Mol Biol* 2004;30:311–8.
11 Ebrahimi A, Kardar GA, Teimoori-Toolabi L, *et al*. Inducible expression of indoleamine 2,3-dioxygenase attenuates acute rejection of tissue-engineered lung allografts in rats. *Gene* 2016;576:412–20.
12 Vavrincova-Yaghi D, Deelman LE, Goor H, *et al*. Gene therapy with adenovirus-delivered indoleamine 2,3-dioxygenase improves renal function and morphology following allogeneic kidney transplantation in rat. *J Gene Med* 2011;13:373–81.
13 Jalili RB, Forouzandeh F, Rezakhanlou AM, *et al*. Local expression of indoleamine 2,3 dioxygenase in syngeneic fibroblasts significantly prolongs survival of an engineered three-dimensional islet allograft. *Diabetes* 2010;59:2219–27.
14 Munn DH, Shafizadeh E, Attwood JT, *et al*. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J Exp Med* 1999;189:1363–72.
15 Bauer TM, Jiga LP, Chuang JJ, *et al*. Studying the immunosuppressive role of indoleamine 2,3-dioxygenase: tryptophan metabolites suppress rat allogeneic T-cell responses in vitro and in vivo. *Transpl Int* 2005;18:95–100.
16 Maddison DC, Giorgini F. The kynurenine pathway and neurodegenerative disease. *Semin Cell Dev Biol* 2015;40:134–41.
17 Dai X, Zhu BT. Suppression of T-cell response and prolongation of allograft survival in a rat model by tryptophan catabolites. *Eur J Pharmacol* 2009;606:225–32.
18 Mbongue JC, Nicholas DA, Torrez TW, *et al*. The role of indoleamine 2, 3-dioxygenase in immune suppression and autoimmunity. *Vaccines* 2015;3:703–29.
19 Konan KV, Taylor MW. Importance of the two interferon-stimulated response element (ISRE) sequences in the regulation of the human indoleamine 2,3-dioxygenase gene. *J Biol Chem* 1996;271:19140–5.

20 Chon SY, Hassanain HH, Pine R, *et al*. Involvement of two regulatory elements in interferon-gamma-regulated expression of human indoleamine 2,3-dioxygenase gene. *J Interferon Cytokine Res* 1995;15:517–26.
21 Brandacher G, Margreiter R, Fuchs D. Implications of IFN-gamma-mediated tryptophan catabolism on solid organ transplantation. *Curr Drug Metab* 2007;8:273–82.
22 Dinarello CA. Historical insights into cytokines. *Eur J Immunol* 2007;37(Suppl 1):S34–S45.
23 Lassiter R, Wang Y, Fang X, *et al*. A model of acute renal allograft rejection in outbred Yorkshire piglets. *Transpl Immunol* 2017;42:40–6.
24 Wang Y, Zhou J, Minto AW, *et al*. Altered vitamin D metabolism in type II diabetic mouse glomeruli may provide protection from diabetic nephropathy. *Kidney Int* 2006;70:882–91.
25 Liu H, Liu L, Fletcher BS, *et al*. Novel action of indoleamine 2,3-dioxygenase attenuating acute lung allograft injury. *Am J Respir Crit Care Med* 2006;173:566–72.
26 Hall BM. T Cells: Soldiers and Spies--The Surveillance and Control of Effector T Cells by Regulatory T Cells. *Clin J Am Soc Nephrol* 2015;10:2050–64.
27 Parkin J, Cohen B. An overview of the immune system. *Lancet* 2001;357:1777–89.
28 Pizarro TT, Malinowska K, Kovacs EJ, *et al*. Induction of TNF alpha and TNF beta gene expression in rat cardiac transplants during allograft rejection. *Transplantation* 1993;56:399–404.
29 Cook CH, Bickerstaff AA, Wang JJ, *et al*. Spontaneous renal allograft acceptance associated with "regulatory" dendritic cells and IDO. *J Immunol* 2008;180:3103–12.
30 Taylor MW, Feng GS. Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. *Faseb J* 1991;5:2516–22.
31 Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity* 1995;2:271–9.
32 Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003;3:745–56.
33 Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends Immunol* 2013;34:137–43.
34 Fallarino F, Grohmann U, Puccetti P. Indoleamine 2,3-dioxygenase: from catalyst to signaling function. *Eur J Immunol* 2012;42:1932–7.
35 Stockinger B, Hirota K, Duarte J, *et al*. External influences on the immune system via activation of the aryl hydrocarbon receptor. *Semin Immunol* 2011;23:99–105.