Received: 4 August 2021 | Revised: 14 October 2021 | Accepted: 15 October 2021

DOI: 10.1111/ajt.16880

ORIGINAL ARTICLE

Novel intragraft regulatory lymphoid structures in kidney allograft tolerance

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Funding information
National Institutes of Health, Grant/Award Number: P01-AI123086

Intragraft events thought to be relevant to the development of tolerance are here subjected to a comprehensive mechanistic study during long-term spontaneous tolerance that occurs in C57BL/6 mice that receive life sustaining DBA/2 kidneys. These allografts rapidly develop periarterial Treg-rich organized lymphoid structures (TOLS) that form in response to class II but not to class I MHC disparity and form independently of lymphotoxin α and lymphotoxin β receptor pathways. TOLS form in situ in the absence of lymph nodes, spleen, and thymus. Distinctive transcript patterns are maintained over time in TOLS including transcripts associated with Treg differentiation, T cell checkpoint signaling, and Th2 differentiation. Pathway transcripts related to inflammation are expressed in early stages of accepted grafts but diminish with time, while B cell transcripts increase. Intragraft transcript patterns at one week post-transplant distinguish those from kidneys destined to be rejected, that is, C57BL/6 allografts into DBA/2 recipients, from those that will be accepted. In contrast to inflammatory tertiary lymphoid organs (iTLOs) that form in response to chronic viral infection and transgenic *Lta* expression, TOLS lack high endothelial venules and germinal centers. TOLS represent a novel, pathogenetically important type of TLO that are in situ markers of regulatory tolerance.

Abbreviations: HEVs, high endothelial venules; PVN, polyoma virus nephropathy/nephritis; RIP-LTα, rat insulin promoter lymphotoxin α; TLOs, tertiary lymphoid organs; TOLS, Treg-rich organized lymphoid structures.

Ivy A. Rosales and Chao Yang are co-first authors; Alessandro Alessandrini and Robert B. Colvin are co-senior and co-corresponding authors.

KEYWORDS
animal models: murine, basic (laboratory) research/science, kidney transplantation / nephrology, molecular biology, molecular biology: mRNA/mRNA expression, pathology/ histopathology, tolerance: mechanisms

1 | INTRODUCTION

Intragraft events have been postulated to be important for the development of tolerance. Seminal studies demonstrated tolerogenic cells in mouse skin grafts could transfer tolerance to naïve recipients.[1] Lymph nodes have long been regarded as the source of these tolerogenic cells, rather than the graft itself.[2] Recent pathological studies of kidney allografts in mice with long-term spontaneous tolerance revealed $CD3^+Foxp3^+$ periarterial nodular aggregates in the renal cortex.[3-5] We have termed these aggregates Treg-rich organized lymphoid structures (TOLS), representing a novel member of the regulatory tertiary lymphoid organ (rTLOs) family. Depletion of Foxp3+ cells promoted dissolution of these lymphoid structures[5] and abolition of tolerance, indicating that the Foxp3+ cells are necessary for their integrity and that the tolerance is regulatory. Furthermore, Tregs isolated from spontaneously accepted kidney allografts can transfer tolerance of a skin graft to naïve recipients.[6]

Here, we describe the pathologic and molecular characteristics of TOLS and the mechanisms of their formation. TOLS differ in anatomy, cell type, and gene expression from inflammatory TLOs, which are ectopic collections of T and B cells arising in non-lymphoid tissue due to chronic inflammation or tissue injury from rejection, infection, neoplasia, and autoimmunity.[7-11]

2 | MATERIALS AND METHODS

2.1 | Mice

The C57BL/6J (B6, $H2^b$), DBA/2J (DBA, $H2^d$), C3H/HeJ (C3H, $H2^k$), B6.Foxp3-GFP, and B6.C-$H2^{d/bByJ}$ strains were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained under pathogen-free conditions in filter-top cages throughout the experiments with an automatic water system and were cared for according to methods approved by the American Association for the Accreditation of Laboratory Animal Care. Formalin-fixed paraffin-embedded (FFPE) tissue blocks from kidneys of Black Swiss mice infected with murine polyomavirus and sacrificed 4–20 weeks after infection were obtained from Dr. Volker Nickeleit. FFPE tissue blocks from kidneys of rat insulin promoter-lymphotoxin α (RIP-LTα) mice (B6, Thy1.2, H-2b)[12] were obtained through Dr. Fadi Lakkis.

2.2 | Kidney transplantation

Kidney transplantation was performed in the same way described in a previous report.[5] In brief, kidney allografts were prepared with the cuff of the aorta and the inferior vena cava. Anastomoses were performed in an end-to-side manner. The ureter was anastomosed to the urinary bladder. Bilateral nephrectomy was also simultaneously performed. Kidneys from DBA/2 donors were spontaneously accepted by C57BL/6 recipients as evidenced by normal BUN levels at 1-week posttransplant and up to 60 weeks posttransplant. C57BL/6 kidneys transplanted into DBA/2 recipients showed elevated BUN levels at 1-week posttransplant and were typically rejected in 10–14 days. One week samples from allograft kidneys that were destined to be accepted (DBA/2 to C57BL/6) were compared with 1 week samples from those destined to be rejected (C57BL/6 to DBA/2).

2.3 | Histological and immunopathological analysis

Sagittal sections of allografts were fixed in formalin, and sections were stained for H and E and PAS. TOLS were defined as an aggregate of mononuclear cells around an artery or arteriole and which upon immunohistochemical studies showed abundant Foxp3+ cells admixed with T cells, B cells, macrophages and dendritic cells. TOLS do not have B and T cell compartmentalization and germinal centers. TOLS do not have high endothelial venules (HEVs) (Table 1).

Routine immunohistochemistry using enzyme-conjugated antibodies was performed on paraffin embedded tissue sections to identify the cellular phenotype of TOLS using Foxp3 (FJK-16s, Invitrogen-ThermoFisher Scientific), individually and as double stain with CD3 (Polyclonal DAKO Agilent). Other stains included CD4 (4SM95 Invitrogen-Thermo Scientific), CD8 (4SM15 Invitrogen-Thermo Scientific), F4/80 (CI:A3-1 Abcam), Lyve-1 (Polyclonal, R&D Systems), PNAd (MECA-79, BD Biosciences), Ki-67 (16A8 Biolegend), Pax5 (Polyclonal Novus Biologicals), PDCA-1 (BST2 120G8.04, Novus Biologicals), Siglec-H (23M15C8, Novus Biologicals), B220 (RA3-652 BD Biosciences), CD138 (281–2 BD Biosciences), Prox1 (EPR 19273 Abcam), podoplanin (RTD4E10 Abcam), LAP (Polyclonal, R&D Systems), CD21 (EP3093 Abcam). Pathologic evaluation was done using an Olympus BX53 microscope (Olympus) equipped with a digital camera (DP76, Olympus). Immunofluorescence microscopy was performed using an immunofluorescent microscope (Eclipse 50i; Nikon) equipped with a digital camera (Spot RT KE; Diagnostics Instruments). Whole slide scans were performed at 20× magnification (Aperio CS; Aperio) and morphometric analysis was performed using Aperio Digital Pathology software (Leica Biosystems) image analysis algorithms.

2.3.1 | NanoString RNA analysis

mRNA was extracted from formalin fixed paraffin embedded kidney tissue samples of normal and allograft kidneys as described.[13,14] Gene expression was assessed with a comprehensive Mouse Cancer

TABLE 1 General differences between TOLS and tertiary lymphoid organs (TLOs)

Features	TOLS	TLOs
Location	Spontaneously accepted mouse kidney allografts	Sites of chronic inflammation
Morphology	Periarterial or periarteriolar, appear like sheaths on longitudinal sections	No preferential location; may be associated with mucosal epithelium
Blood vessels	Artery/arteriole	High endothelial venule
Lymphatics	Yes	Yes
B and T cell compartments	No	Yes
Germinal centers	No	Yes
B cell affinity maturation	Unknown	Yes
Cell traffic	Unclear; lymphatics	High endothelial venule
Dependence on LTα signaling	No	Yes
Formation	Independent of secondary lymphoid organs (spleen, lymph nodes, and thymus)	In response to chronic inflammation, infection, or malignancy

Immunology probe set, which includes 770 genes related to the immune system and major cell pathways, plus housekeeping genes. mRNA numbers were detected using the nCounter Max platform (NanoString Technologies). Normalized counts were analyzed with the NanoString nSolver software (NanoString Technologies). Cell type and pathway analysis were performed using built-in and custom pathways in the Advanced Analysis module.

2.3.2 | Digital spatial protein profiling

5-micron thick sections were cut from paraffin blocks of grafts from autopsy and were incubated with fluorescent-labeled antibodies to Foxp3, pan-cytokeratin, and alpha-smooth muscle actin at 4°C overnight. On the second day, the slides were scanned on a GeoMx™ Digital Spatial Profiler instrument and regions of interest (ROIs) were selected. Because of the fact that the anti-Foxp3 antibody used as morphology marker competed with the clone in the DSP panel, a secondary antigen retrieval was done to remove the Foxp3 fluorescent staining. The slides were incubated with a pre-commercial Mouse Immuno-Oncology panel of protein antibodies, which contained photocleavable indexing oligonucleotides and immunofluorescent markers pan-cytokeratin, and smooth muscle actin at 4°C overnight. Syto83 was added to stain the nucleus at room temperature for 5 min before loading to the GeoMx™ DSP instrument. The pre-selected ROIs were aligned and oligonucleotide tags from ROIs were collected and quantitated using the NanoString nCounter Max instrument. Normalized counts were analyzed using GraphPad Prism 9 (GraphPad Software Inc.).

2.4 | Statistical analysis

Results are given as the mean ± 95% CI. Variables among groups were compared using student's *t* test, with $p < .05$ considered significant. These analyses were performed with Prism versions 5 and 7 (GraphPad Software Inc.).

3 | RESULTS

3.1 | Characteristics of TOLS in accepted kidney allografts

In the first week after transplantation, accepted DBA/2 to C57BL/6 kidney allografts show widespread cortical interstitial infiltrates of T cells (Figure 1A) with focal collections around interlobular and arcuate arteries. At 2–3 weeks posttransplant, accepted grafts show progressive formation of peri-arterial TOLS. These are well defined at 6–8 weeks, with a mean area of 1.7 ± 0.3 mm^2 in cross section. TOLS are round to oval structures in cross section and form periarterial sheaths when viewed in longitudinal sections. The arteries and arterioles are normal, although mononuclear cells can sometimes be present underneath the endothelium and within the wall of interlobular arteries. Cells at the periphery of TOLS can be dispersed, occasionally invading tubules. The remainder of the kidney is generally unremarkable. TOLS persist in tolerant recipients up to 60 weeks posttransplant (Figure 1D).

TOLS have a predominant population of CD4+ cells with numerous Foxp3+ cells and a minor component of CD8+ cells (Figure S1A-C). B cells (Pax5+, B220+) (Figure S1D and S1E) are intermixed with T cells and do not show distinct compartmentalization. Follicular B cells (Pax5+, B220+, CD21+) are detected at 6–8 weeks, but follicles and defined germinal centers are not detectable. Plasmacytoid dendritic cells (PDCA1+, SiglecH+ pDCs) are found along the periphery of TOLS (Figure S1F), as well as CD11b+ cells, F4/80+ macrophages and CD138+ plasma cells. Podoplanin (Figures S1G and S2B) and Lyve-1 (Figure S1H) positive lymphatic channels form within TOLS and contain a heterogenous population of cells including Tregs, T cells, B cells, and plasmacytoid dendritic cells (Figure S2C-H).

At 6 weeks posttransplant, TOLS are significantly enriched in Foxp3+ cells (53.8 ± 6.5% of CD3+ cells) compared with periarteriolar sheaths (PALS) of spleens from the same recipients

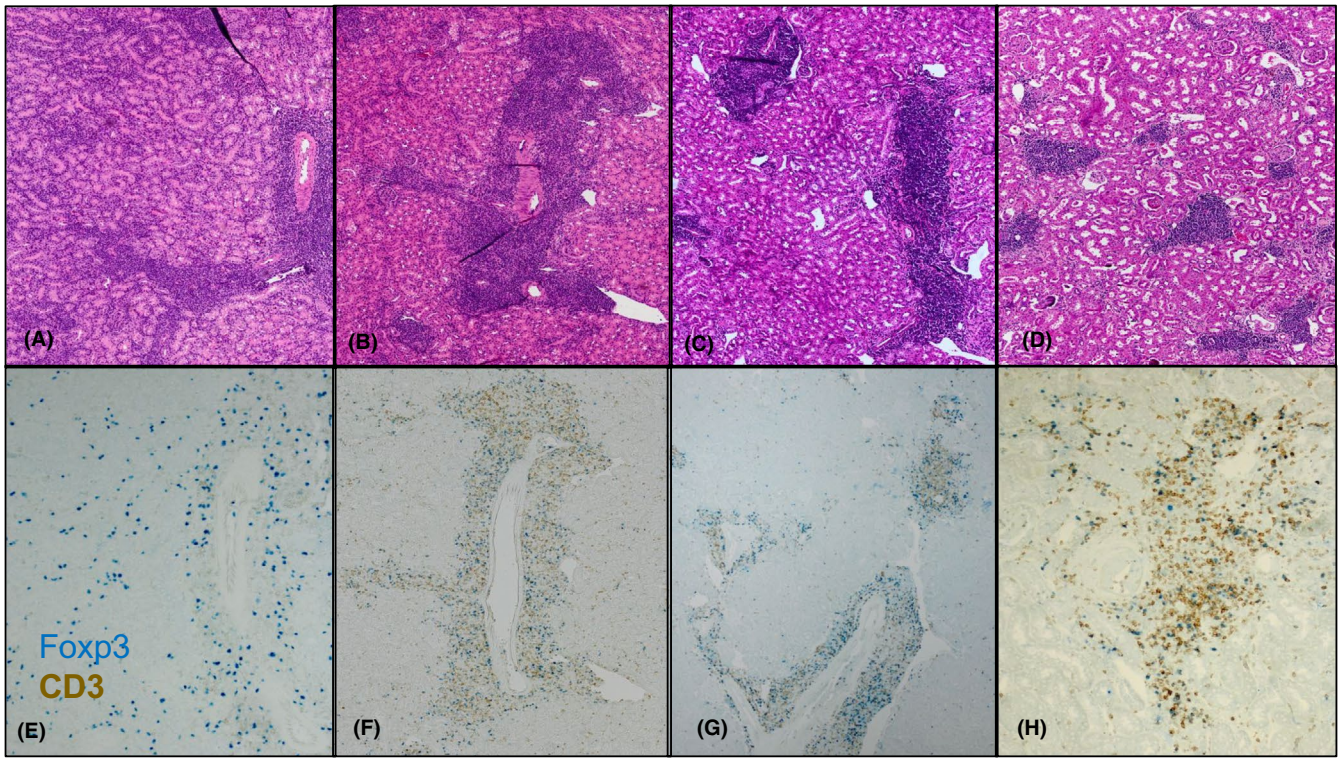


FIGURE 1 Treg-rich organized lymphoid structures (TOLS) form early at 1-week posttransplant and persist for up to 60 weeks (A-D, hematoxylin and eosin [H&E]; E-H, corresponding Foxp3-CD3 double stains). (A and E) At 1 week, TOLS form focally around an arcuate artery while the rest of the interstitial infiltrate is dispersed throughout the cortex (DBA/2 to B6, H&E 100×; Foxp3/CD3, 200×). (B and F) TOLS are relatively well formed at 2 weeks (DBA/2 to B6, H&E 100×; Foxp3/CD3, 100×). (C and G) TOLS in a kidney allograft at 30-40 weeks posttransplant (DBA/2 to B6, H&E 100×; Foxp3/CD3, 100×). (D and H) TOLS at 66 weeks posttransplant (DBA/2 to B6, H&E 100×; Foxp3/CD3, 200×).

(23.1 ± 10.9% of the CD3+ cells; $p < .01$) (Figure 2A,B). The Foxp3+ cells in TOLS express TGFβ-related latency associated peptide (LAP), a marker of activated Foxp3+ cells.[15] LAP in TOLS is expressed at higher frequency than in recipient spleens ($p < .0001$) (Figure 2C,D). Foxp3+ cells in TOLS also show marked proliferative activity (>50% Ki67+), similar to Foxp3+ cells in the spleen from the same recipients. TOLS do not have HEVs as evidenced by morphology and lack of MECA79+ vessels (an antibody to peripheral node addressin/PNAd).[10,16]

3.2 | Digital spatial protein profiling

To complement immunohistochemical analysis, we performed multiplex spatial protein quantification using the NanoString GeoMx Digital Spatial Profiler, by comparing TOLS with non-TOLS cortex from the same kidney sections. TOLS-specific differential expression is revealed for T cell (CD3e, CD8a, CD4), Treg (GITR, Foxp3) and T cell checkpoint molecules (Lag3, PD-1, Vista) at 1 week and 6–8 weeks posttransplant (Figure 3). This characteristic feature within the periarterial structures suggest the roles of T cell checkpoint and regulation in tolerance. Increased B cell (CD19, CD27) and dendritic cell (CD11c) molecules are also present in TOLS at both timepoints.

3.3 | Dynamic transcript changes in accepted grafts over time

To determine molecular pathways involved in graft acceptance, we assessed bulk mRNA transcripts by NanoString nCounter analysis of kidney grafts at selected time points after transplantation. Accepted grafts at 1 week compared with long-term accepted grafts show an increase in cell-specific transcripts such as T cell ($p < .000005$), CD8 T cell ($p < .0000001$), cytotoxic cell ($p < .00001$), and NK cell ($p < .0002$) scores, which diminish over time. In contrast, Treg cell scores rise early and are sustained (Figure 4). B cell scores ($p < .0003$) show progressive increase over 60 weeks posttransplant. T follicular regulatory (Tfr) cell score (*Bcl6*, *Cxcr5*, *Cxcl13*, *Foxp3)* parallels the B cell data. Individual genes associated with inflammatory cytokines, chemokines, and cytotoxic CD8 T cells are differentially expressed but show a significant reduction by 40 to 60 weeks (Figure S3).

Pathway analysis shows varying degrees of reduction in mean scores of inflammatory pathways in accepted graft samples over time. Several pathways were prominent initially, but then markedly diminished, including cytokine signaling, innate and adaptive immune systems, toll-like receptor signaling and apoptosis pathways. Treg differentiation pathway scores are less reduced. MHC Class II antigen and B cell receptor signaling pathways are not reduced over time.

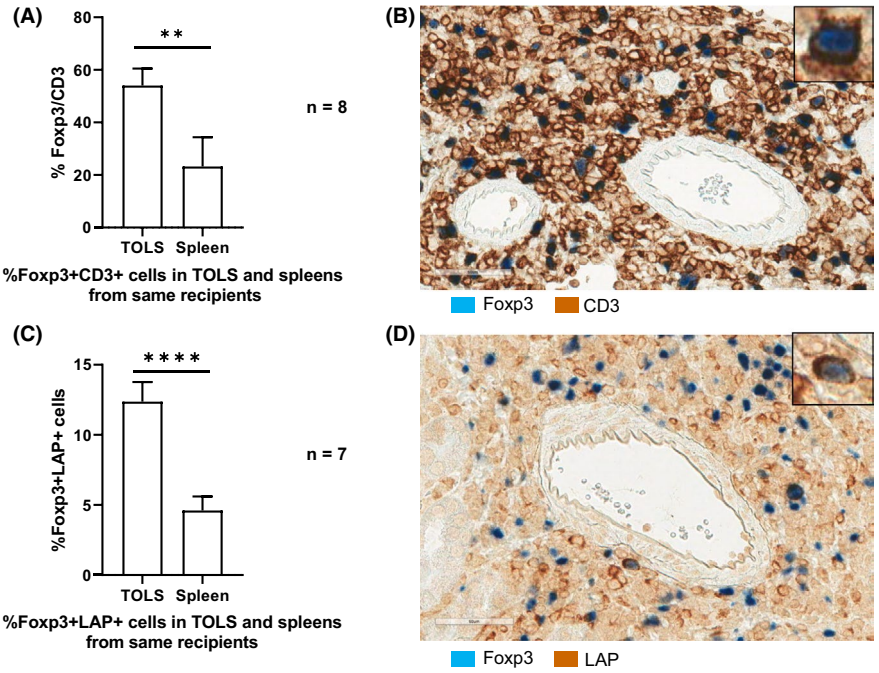


FIGURE 2 Treg-rich organized lymphoid structures (TOLS) show increased Foxp3+ cells that are positive for latency associated peptide (LAP). Foxp3+CD3+ and Foxp3+LAP+ cells are increased in TOLS in accepted kidney allografts when compared with spleens from the same animals. TOLS also show an increase in mitotic activity as evidenced by Fop3+Ki67+ expression on immunofluorescence. (A) Foxp3+CD3+ cells are displayed as a percentage of all CD3+ cells per TOLS analyzed by morphometric software (Aperio). (B) Simultaneous detection of Foxp3 and CD3 by immunoperoxidase in TOLS. Inset shows a double positive cell with the nucleus (blue) staining for Foxp3 and the cytoplasmic membrane (brown) for CD3 (400× and inset). (C) LAP+Foxp3+ cells are displayed as percentage of all Foxp3+ cells analyzed manually. (D) Simultaneous detection of Foxp3 and CD3 by immunoperoxidase in TOLS. Inset shows a double positive cell with the nucleus (blue) staining for Foxp3 and the cytoplasm (brown) for LAP (400× and inset).

3.4 | Transcriptional analysis of kidney allograft samples of early acceptance versus early rejection

Rejecting grafts are allograft kidneys of DBA/2 recipients from C57BL/6 mice. This strain combination shows elevated BUN at 1 week posttransplant. In contrast, grafts that are destined to be tolerant are allograft kidneys of C57BL/6 recipients from DBA/2 mice, a strain combination that shows spontaneous acceptance and normal BUN at 1 week and up to 60 weeks posttransplant. Accepted and rejecting grafts at 1 week have similar histology of diffuse infiltrating leukocytes. Rejecting grafts do not form TOLS. There are similar levels of infiltrating leukocytes, as judged by *Cd45* transcripts, as well as T cell, B cell, NK cell, and dendritic cell function scores, complement pathway and chemokine pathway scores.

Differential expression of individual genes shows accepted grafts have elevated transcripts related to Treg cells (*Foxp3*, *Tcf7*, *Ikzf1*, *Ikzf2*, *Cxcr3*), NK cells (*Klrd1*, *Klrc2*, *Cd244*, *Slamf6*, *Slamf7*), and B cells (*Cxcl13*). In contrast, grafts with early rejection have increased transcripts related to macrophages (*F13a1*, *Msr1*, *Clec4n*, *S100a8*, *Cxcl14*), myeloid cells (*Cd33*, *Trem2*, *Fpr2*), and matrix (*Fn1*, *Ppbp*) (Figure 5). Elevation of *Klrd1* is due to a mutation in DBA/2, not present in B6.[17]

Pathways that are elevated in early acceptance compared with early rejection include T cell checkpoint signaling ($p = .02$), Th2 differentiation ($p = .03$), and mTOR signaling ($p = .01$). Pathways lower in grafts that will be accepted include IFNγ ($p = .01$), Th1 differentiation ($p = .04$), and MHC class I antigen presentation ($p = .04$) as well as cytotoxic T cell ($p < .02$), neutrophil ($p < .002$) and pDC ($p < .002$). Treg subtypes, as defined transcriptionally by Miragaia and colleagues[18] were assessed using custom cell-type panels in the NanoString nCounter software. Accepted grafts at 1 week show elevated pan-Treg ($p < .001$), central Treg (cTreg, $p < .000015$), effector Treg (eTreg, $p < .000002$) and tumor-infiltrating Treg (Treg-tumor, $p < .00037$), T cell ($p < .003$), B cell ($p < .03$) transcript scores, compared with rejecting grafts at 1 week posttransplant (Figure 6).

3.5 | TOLS are distinguished from inflammatory tertiary lymphoid organs (iTLOs) in the kidney

3.5.1 | TLOs in RIP-LTα transgenic mouse

The RIP-LTα transgenic mouse expresses LTα in kidney tubular cells and characteristically develops TLOs in the cortex.[10,19] Native kidneys from RIP-LTα mice show cortical interstitial (Figure 7A) and subcapsular nodular aggregates of T and B cells, with distinct B cell follicles and germinal centers (Figure 7B) and MECA-79+ HEVs (Figure 7C). Periarterial sheaths are not formed.

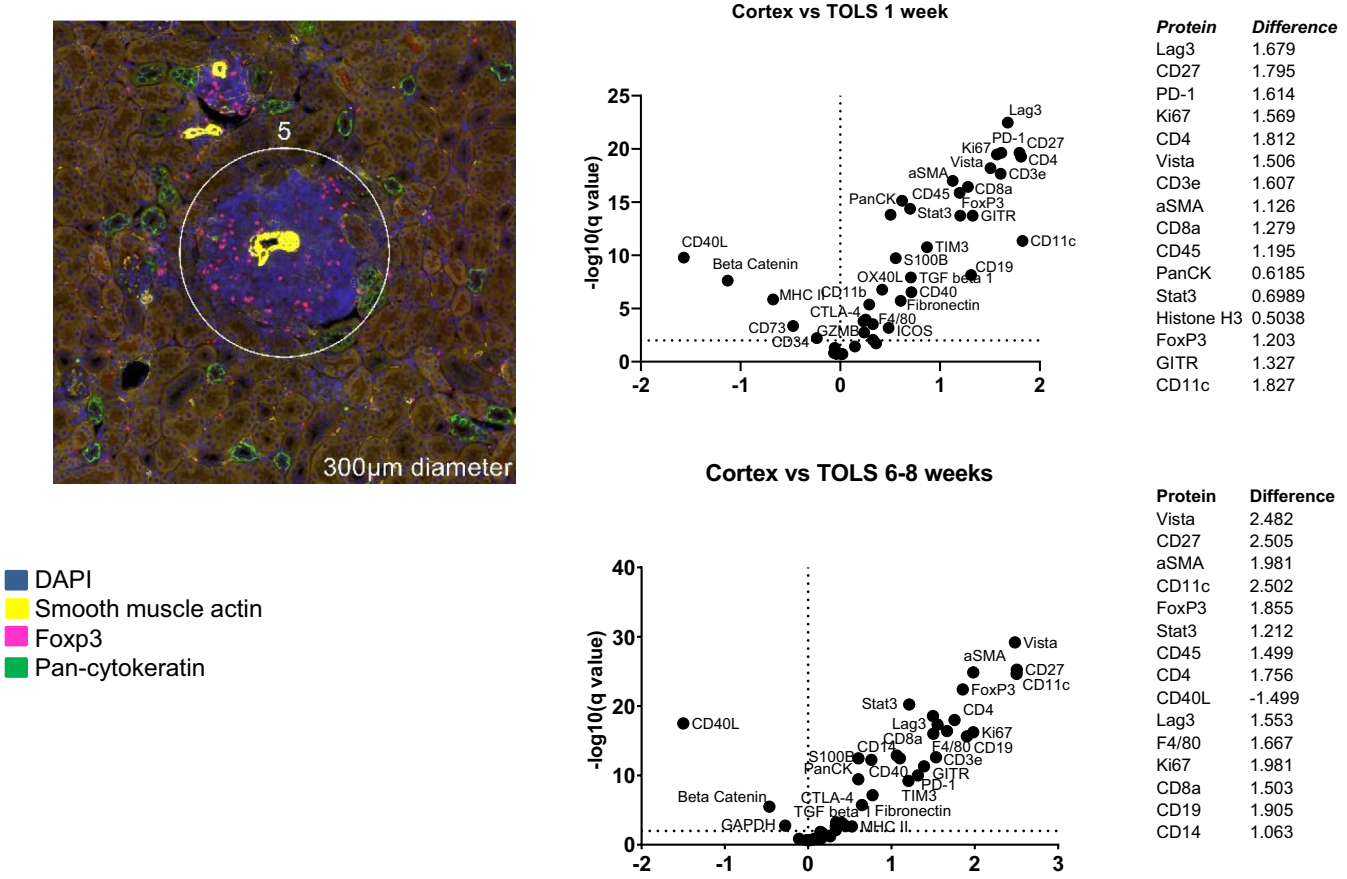


FIGURE 3 Digital spatial protein profiling (far left) of accepted kidney graft with Treg-rich organized lymphoid structures (TOLS) encircled as region of interest. The tissue is stained with fluorescent-labeled markers (DNA, smooth muscle actin, pan-cytokeratin, and Foxp3). Differential expression of proteins show Treg, T cell, T cell regulation, and checkpoint signaling proteins in TOLS.

3.5.2 | TLOs in polyomavirus nephropathy

Mice infected with murine polyomavirus develop interstitial nephritis over several weeks, and TLOs in the cortical parenchyma. The TLOs are randomly distributed in the cortex without specific localization, although some periarterial aggregates are present. The TLOs are associated with marked tubular injury and presence of epithelial nuclear viral inclusions. Numerous plasma cells in clusters are predominant, admixed with CD4+, CD8+, and B220+ cells. T cells and B cells are in distinct compartments, and germinal centers are evident as well as HEV.

3.5.3 | TLOs vs TOLS transcripts

The level of *Cd45* transcripts is not significantly different in allograft kidneys with TOLS at 40–60 weeks compared with native kidneys with TLOs due to LTA Tg or PVN. However, transcripts related to Tregs, B cells, cytotoxic cells, macrophages, and NK cells are increased in kidneys with TOLS.

Transcript analysis shows several differences between kidneys with TOLS and those with TLOs. TOLS show elevated pathways of Treg, T cell checkpoint signaling and Th2 differentiation, T and B cell receptor signaling, MHC Class I and II antigen presentation, and interferon-signaling pathways compared with kidneys with TLOs due to either LTA Tg or PVN (Figure 8). Higher levels of pathway transcripts related to Th1 and Th17 cell function and TNF and NF-κB signaling characterize kidney with TLOs due to LTA Tg compared with PVN.

Accepted kidney allografts with TOLS also have increased transcripts that correlate with cTreg, eTreg, and non-lymphoid tissue-infiltrating Treg (NLT-Treg) compared with native kidneys with TLOs. Normal thymus and spleens have higher cTreg than accepted grafts with TOLS. Accepted grafts also have higher levels of eTreg and tissue infiltrating Treg, including those associated with tumors (Figure S4).

3.6 | Mechanisms of TOLS formation

3.6.1 | Antigenic stimulus

TOLS do not develop in syngeneic kidney grafts (B6 to B6), indicating allogeneic stimulation is necessary for their formation.[5] To test whether MHC Class I or Class II antigenic differences are sufficient to induce TOLS formation, B6.C-H-2^{bm1} (bm1) and B6.C-H-2^{bm12} (bm12) kidneys[20] were transplanted to wild type C57BL/6 recipients and

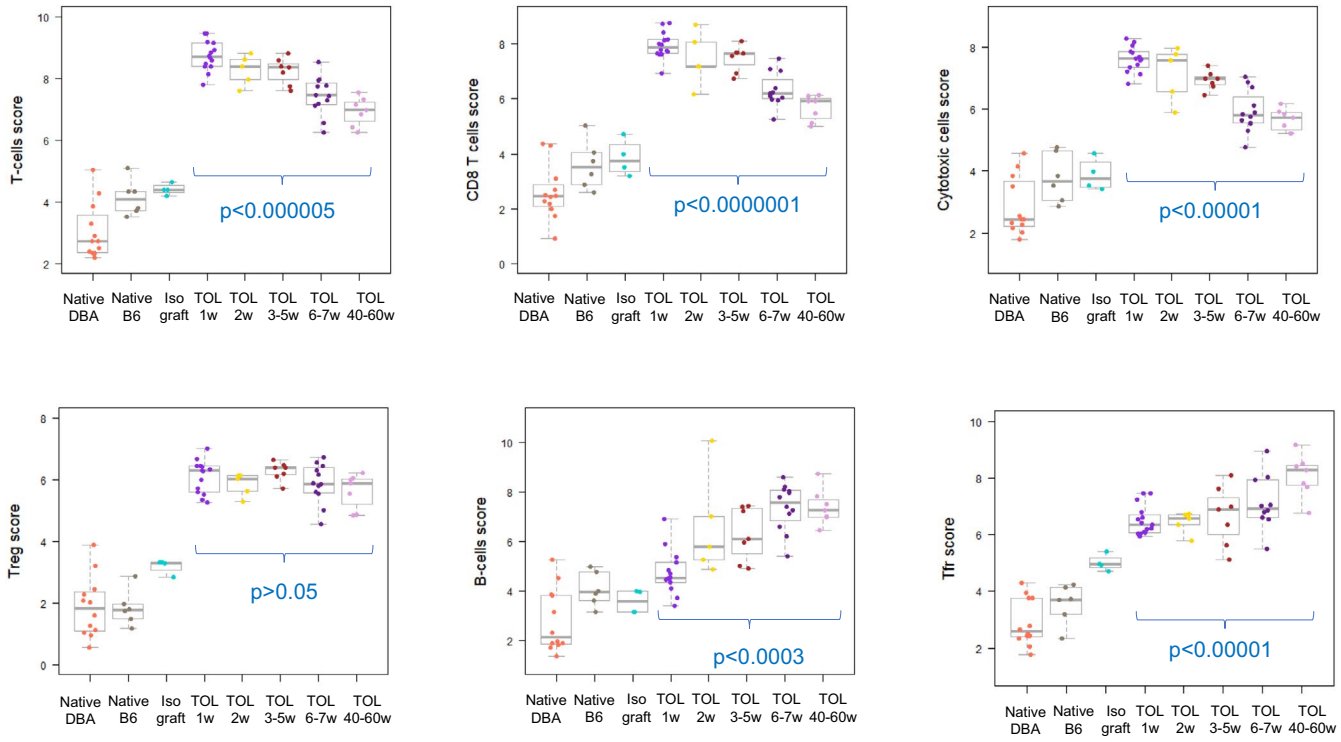


FIGURE 4 Many cell types including T cells, including cytotoxic cells and CD8 T cells, are maximal at 1 week posttransplant and diminish over time up to 60 weeks posttransplant. Relative to all leukocytes, Tregs remain abundant but CD8 T cells and cytotoxic cells show decrease with time. B cells rise significantly with time.

analyzed 56 days posttransplant at a time when the serum creatinine was normal. Well-formed TOLS developed in bm12 kidney allografts, but not bm1 kidney allografts, which had minimal perivascular infiltrates along a few arcuate arteries and arterioles (Figure 9B).

3.6.2 | Role of lymphotoxin β signaling

To test the dependence of TOLS on LTα or LTβ, we treated recipient animals with antagonistic LTβR-Ig for 28 days, using a dose schedule that is known to inhibit TLO induction.[21,22] This regimen did not inhibit TOLS formation in kidney allografts at 28 days. Bulk transcript pathway analysis showed no quantitative differences compared with wild-type C57BL/6 recipients (data not shown). These data indicate that the LTβR signaling pathway, which is necessary for the formation of TLOs,[21,22] is dispensable in the formation of TOLS (Figure 9C).

3.6.3 | Role of primary and secondary lymphoid organs

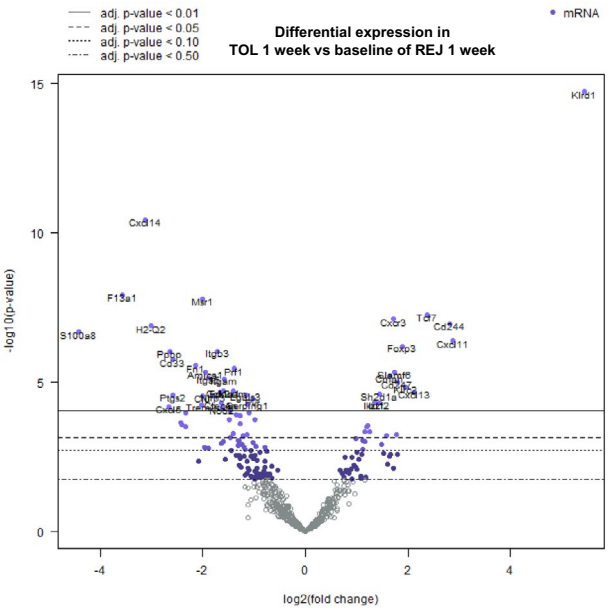
The site of the development of peripheral Foxp3+ T cells has long been considered to be secondary lymphoid organs.[2,23] To test whether lymph nodes are also necessary for the development of TOLS, kidneys from DBA/2 were transplanted into C57BL/6 LTα KO mice, which fail to develop lymph nodes and cannot form TLOs.[24] Kidney allografts were spontaneously accepted. The histology showed well-formed TOLS at 56 days posttransplant in the absence of identifiable lymph nodes in the recipient. Bulk transcript pathway analysis showed no quantitative differences compared with wild-type C57BL/6 recipients (data not shown). Similar results were observed in splenectomized C57BL/6 LTα KO mice recipients (Figure 9D). TOLS also form in DBA/2 kidneys in thymectomized C57BL/6 recipients, which are accepted long term (Figure 9E). These results indicate that spontaneous acceptance and formation of TOLS are independent of lymph node, spleen, and thymic presence and function.

4 | DISCUSSION

We have shown that the lymphoid infiltrates in accepted kidney allografts, which we refer to as TOLS, are novel members of rTLO and are distinct from lymphoid infiltrates associated with chronic inflammation and rejection (iTLOs). There are similarities in the cellular makeup of TOLS and TLOs such as the presence of B and T cells, Tregs, conventional and plasmacytoid DCs, CD11b+ cells, and plasma cells. However, among the differences seen in TOLS are lack of HEVs and germinal centers and a well-defined anatomical distribution along small arteries. Joshi and colleagues have recently described TLOs that are associated with the tumor microenvironment of lung cancers in mice that, like TOLS, are regulatory in nature. But these structures differ from TOLS in that they exhibit CD31+PNAd+ HEV-like structures with distinct B and T cell zones.[25] Li and

Genes increased in rejected grafts (DBA/2 kidneys from C57BL/6 mice) at 1 week

Gene	Log2 fold change	std error (log2)	BY p value
S100a8	-4.43	0.686	9.97E-05
F13a1	-3.58	0.48	1.71E-05
Cxcl14	-3.12	0.327	7.78E-08
H2-Q2	-3.02	0.457	7.14E-05
Cxcl5	-2.66	0.588	0.00743
Ppbp	-2.64	0.444	0.000321
Cd33	-2.58	0.45	0.000547
Ptgs2	-2.58	0.533	0.00382
Nos2	-2.44	0.591	0.0197
Il1b	-2.41	0.592	0.0224
Ccl3	-2.34	0.536	0.011
Lcn2	-2.34	0.584	0.025
Fn1	-2.14	0.382	0.000808
Trem2	-2.03	0.442	0.0065
Msr1	-2.01	0.274	1.72E-05
Clu	-2.01	0.418	0.00382
Amica1	-1.95	0.36	0.0011
Itga5	-1.9	0.361	0.00157
Nlrp3	-1.8	0.374	0.00382
Itgb3	-1.71	0.286	0.000321
Fpr2	-1.66	0.336	0.00339
Clec4n	-1.65	0.355	0.00596
Nod2	-1.62	0.36	0.00767
Col1a1	-1.61	0.325	0.00336
Itgam	-1.58	0.302	0.00161
Fcgr3	-1.49	0.356	0.0165
Thbs1	-1.47	0.332	0.00968
Col3a1	-1.47	0.395	0.0478
Tgfb2	-1.41	0.369	0.0395
Mcam	-1.4	0.283	0.00336
Prf1	-1.39	0.251	0.000959
Lrp1	-1.36	0.314	0.0119
Plaur	-1.28	0.298	0.0123
Anxa1	-1.28	0.313	0.0214
H2-Ea-ps	-1.22	0.324	0.0439
Tlr4	-1.22	0.326	0.047
Lgals3	-1.16	0.24	0.00382
Cdh5	-1.14	0.302	0.0435
Serping1	-1.11	0.24	0.00603
Il4ra	-1.1	0.25	0.0104
Pvr	-1.02	0.215	0.00486
Cd97	-0.987	0.235	0.0164



Genes increased in accepted grafts (C57BL/6 kidneys from DBA/2 mice) at 1 week

Gene	Log2 fold change	std error (log2)	BY p value
Klrd1	5.44	0.396	8.13E-12
Cxcl11	2.87	0.46	0.000178
Cd244	2.83	0.423	6.79E-05
Tcf7	2.37	0.342	4.72E-05
Cxcl13	2.13	0.434	0.00339
Klrc2	1.95	0.386	0.00268
Foxp3	1.9	0.312	0.000246
Cd247	1.8	0.346	0.00188
Ctla4	1.77	0.466	0.0421
Slamf6	1.73	0.319	0.0011
Cxcr3	1.71	0.252	5.59E-05
Cmah	1.64	0.308	0.00145
Slamf7	1.58	0.419	0.0439
Sh2d1a	1.45	0.298	0.00372
Ikzf2	1.45	0.314	0.00596
Ikzf1	1.35	0.29	0.00593
Btla	1.26	0.325	0.0364
Tlr1	1.21	0.3	0.0241
Cd83	1.19	0.298	0.025
Cd7	1.16	0.299	0.0361

FIGURE 5 Volcano plot showing differentially expressed genes between accepted grafts (C57BL/6 kidneys from DBA/2 mice) (TOL) at 1 week and rejected grafts (DBA/2 kidneys from C57BL/6 mice) (REJ) at 1 week. Accepted grafts show increased Treg transcripts (*Foxp3*, *Tcf7*, *Ikfz1*) and natural killer (NK) cell-associated transcripts (*Klrd1*, *Cd244*, *Klrd2*) while rejected grafts show macrophage-associated transcripts (*S100a8*, *F13a1*, *Msr1*, *Clec4n*, *Nos2*, *Arg1*) and myeloid and monocyte-associated markers (*Trem1*, *Cd33*, *Cxcl14*). (BY - Benjamini–Yekutieli correction method for multiple comparisons)

colleagues have described HEV+ bronchus-associated lymphoid tissue (BALT) that are regulatory in nature in mouse lung allografts, following the induction of tolerance by costimulatory blockade.[26] These have been termed BALT, but a periarterial distribution has been illustrated, similar to TOLS.

The Foxp3+ cells in TOLS have a high proliferative rate and express LAP more abundantly than those in the spleen. Because Foxp3 cells are essential for the maintenance of TOLS and tolerance, as previously reported,[5] and the in vitro induction of Foxp3 cells is dependent on class II and not class I MHC disparity,[27] we hypothesized that class II MHC disparity would be the likely stimulus. Indeed, isolated class II mismatch (bm12 to B6) was sufficient to stimulate the formation of TOLS, which did not occur in class I mismatched allografts or isografts.

Although TOLS lack HEV, they develop prominent Lyve+Prox-1+Podoplanin+ lymphatic structures. Lymphatics are normally present in the kidney along the arterial tree and transport fluid and cells to regional lymph nodes. The expansion of lymphatic vessels within the TOLS may be important in the egress of lymphocytes and maintenance of tolerance. This fits with the recent study of Pedersen and colleagues who reported that the induction of lymphangiogenesis in kidney allografts in mice resulted in increased survival of the graft.[28]

Several studies have implicated the lymphotoxin β receptor signaling pathway in the development of both lymph nodes and TLOs, as demonstrated by lack of lymph nodes and inducible TLOs in LTα KO, LTβ KO, and LTβR KO mice[21,22,24] and absence of TLOs in LTβR signaling blockade in chronically rejecting heart allografts.[29] Transplantation of DBA/2 kidneys into C57BL/6 LTα KO mice, with and without splenectomy, showed spontaneous acceptance of kidney grafts and TOLS formation. To further eliminate the possibility that donor kidneys are the source of lymphotoxin to activate LTβR signaling, recipient animals were treated with antagonistic LTβR-Ig for 28 days. There was spontaneous acceptance of kidney allografts and TOLS appeared normally.

The differences detected by transcripts between early rejection and early tolerance not only provides distinguishing characteristics but also reveals transcripts involved in early TOLS formation. The early presence of *Tcf7*, *Foxp3*, and *Ikzf1* suggests early T cell differentiation into a regulatory phenotype. Bulk transcript analysis also suggests that increased T cell checkpoint signaling, Th2 differentiation and mTOR signaling pathways may be important in the genesis of tolerance. Transcripts associated with macrophages and myeloid cells indicate the role of innate immune cells in rejection or their downregulation during early acceptance. Our analysis using the GeoMx digital spatial profiler confirmed protein expression of Foxp3, GITR, and PD-1 in cells within TOLS as early as 1 week, validating our findings from bulk transcript analysis, as well as T cell checkpoint and B cell protein markers.

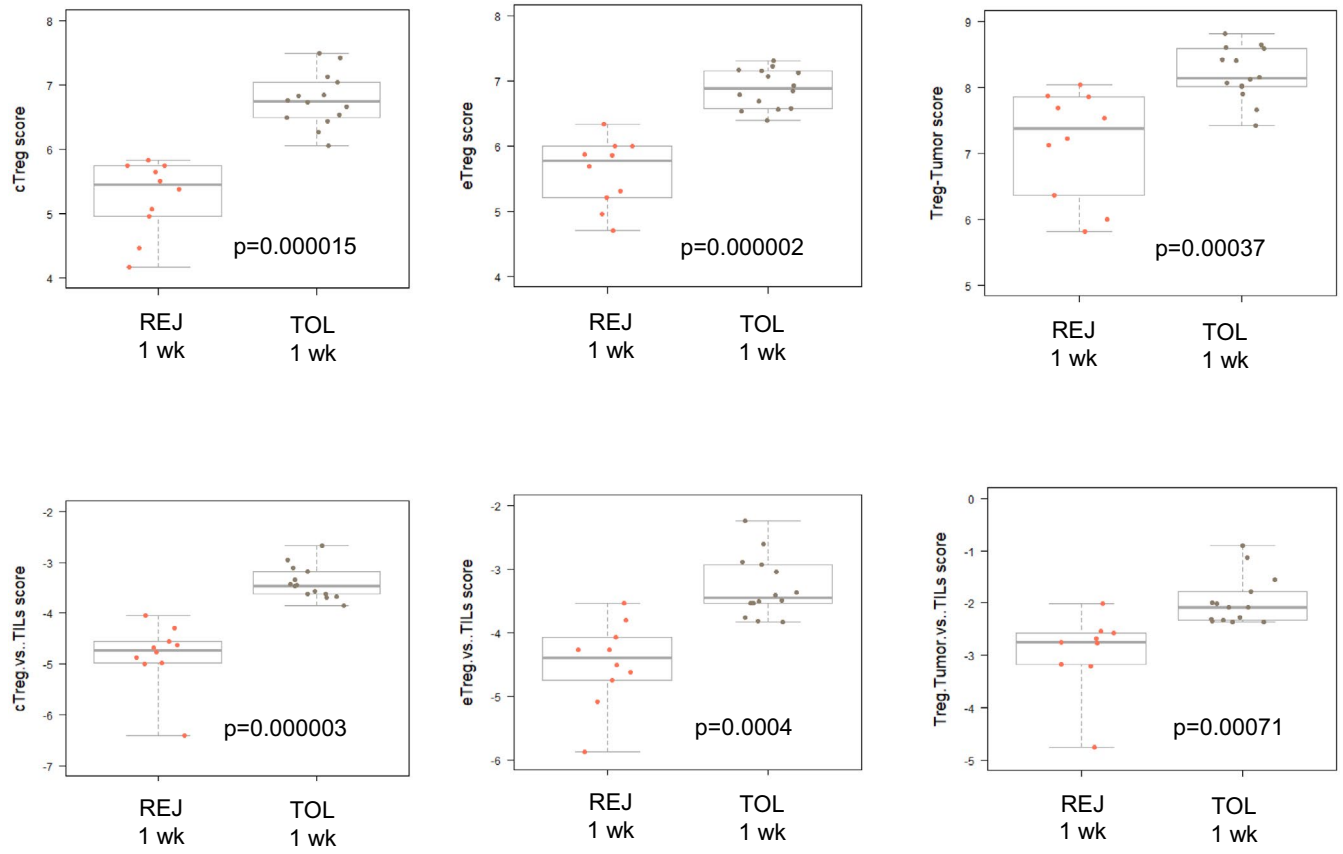


FIGURE 6 Box plots showing T reg subtype scores between rejected (DBA/2 kidneys from C57BL/6 mice) and spontaneously accepted grafts (C57BL/6 kidneys from DBA/2 mice) at 1 week. Central Treg (cTreg), effector Treg (eTreg), and tumor-associated Treg (Treg-tumor) cell subtype scores are higher in accepted grafts.

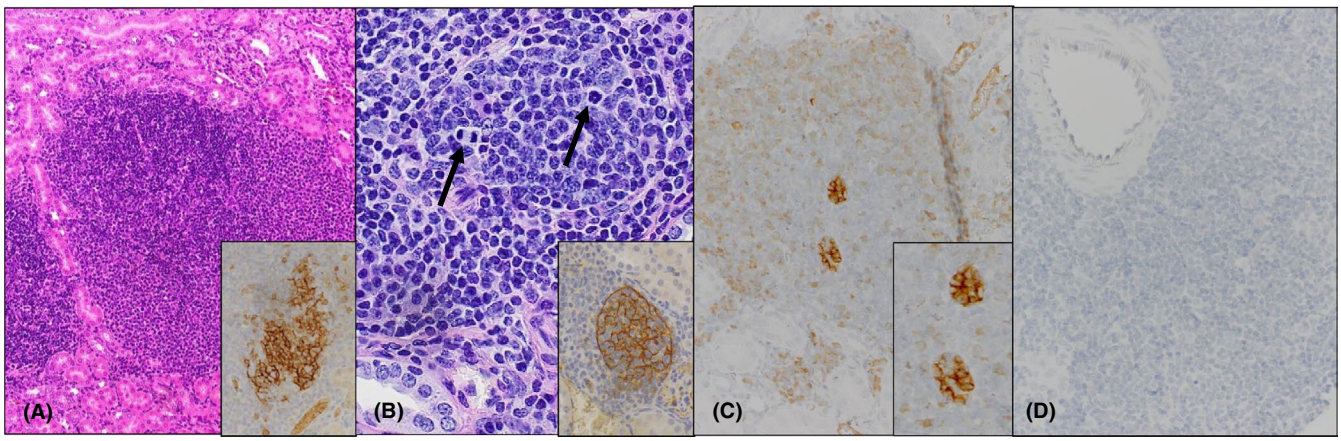


FIGURE 7 Tertiary lymphoid organs (TLOs) in other models of chronic inflammation in native kidneys. A, TLOs in transgenic mice bearing lymphotoxin alpha show follicles and dark zones. Inset shows germinal centers that stain for CD21 (200x, inset original image at 400x). B, Native kidneys from a polyoma virus interstitial nephritis model shows germinal centers in areas of inflammation, here showing mitotic figures (arrows). Inset shows germinal centers highlighted by CD21 (original image at 400x, inset original image at 400x). C, TLOs in transgenic mice bearing lymphotoxin alpha showing high endothelial venules identified by peripheral node addressin (PNAd; MECA79-A). D, TOLS in accepted kidney allografts at 6 weeks posttransplant showing no staining for PNAd.

One of the controversies in transplantation is the site where peripheral induction of Foxp3+ cells takes place, with considerable evidence that induction occurs in lymph nodes.[23] However, the lack of dependence on lymph nodes and spleen in the present model argues that the induction of Foxp3+ cells can occur in the allograft itself.[30] We show a high rate of proliferation of the Foxp3+ cells in TOLS, but we have no direct proof that Foxp3+ cells arise in the TOLS from non-Foxp3+ cells.

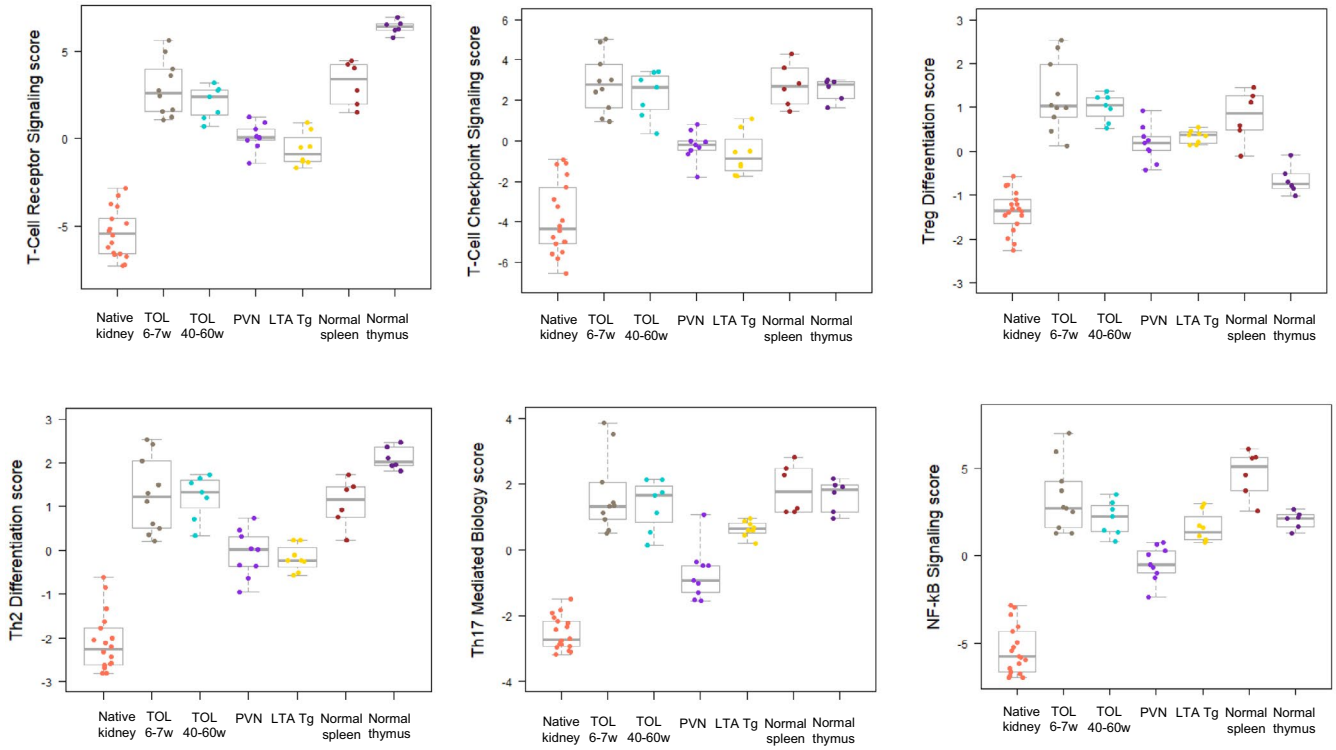


FIGURE 8 Pathway analysis scores show higher T cell receptor signaling, T cell checkpoint signaling, Treg differentiation, and Th2 differentiation scores in kidney grafts with Treg-rich organized lymphoid structures (TOLS) than in tertiary lymphoid organsTLOs in polyomavirus nephritis (PVN) and lymphotoxin alpha transgenic mice (LTaTg). Higher levels of pathway transcripts related to Th1 and Th17 (not shown) cell function and TNF (not shown) and NF-κB signaling characterize kidney with TLOs due to LTA Tg compared with PVN.

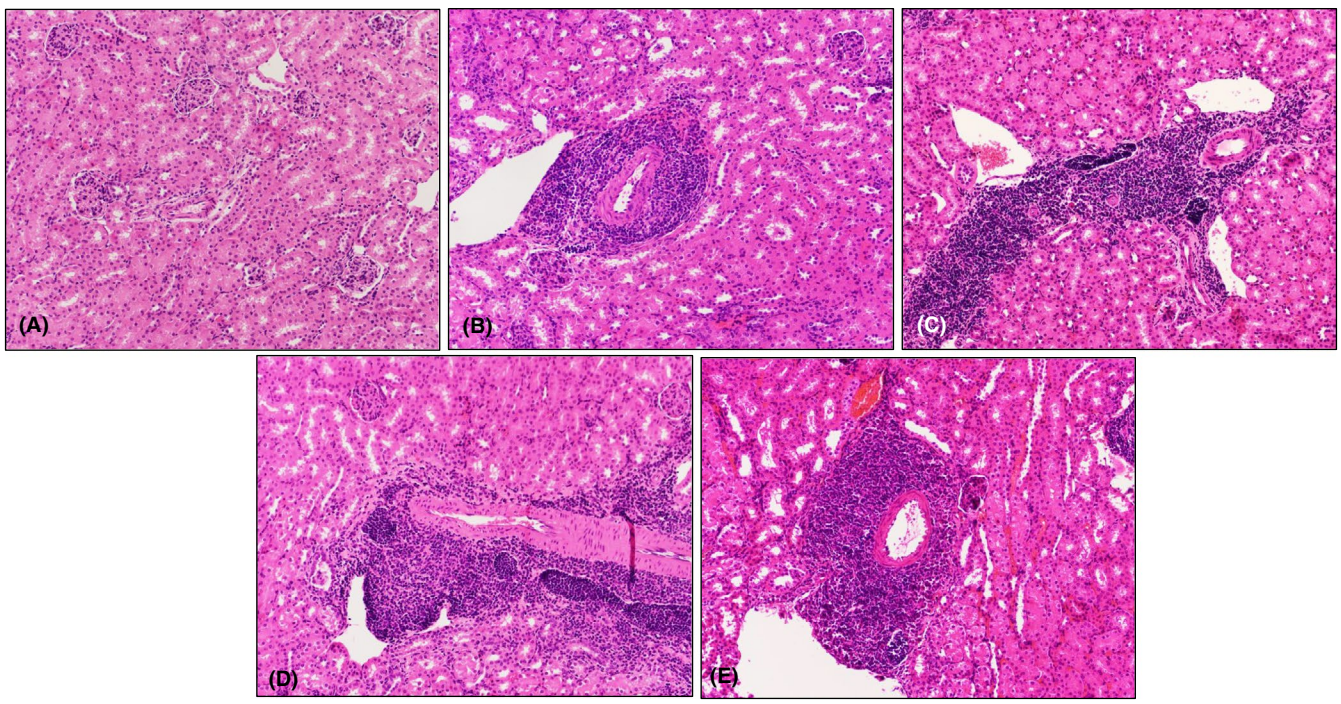


FIGURE 9 (A) Treg rich organized lymphoid structures (TOLS) do not arise in isografts (hematoxylin and eosin [H&E], 200×). (B) TOLS are present when kidney grafts from B6.C-H-2bm12 (bm12) donors are transplanted into wild-type C57BL/6 recipients (H&E, 200×). (C) TOLS are also present despite treatment against lymphotoxin beta receptor (LTβR; DBA/2 to wild-type C57BL/6 recipients) and (D) form in the absence of lymph nodes in a splenectomized lymphotoxin-α knockout ($LT\alpha^{-/-}$) mouse (DBA/2 to splenectomized C57BL/6 $LT\alpha^{-/-}$). (E) Thymectomized C57BL/6 recipients also form TOLS.

We do show that this form of tolerance does not require the continued presence of the thymus for the generation of natural Tregs.[31]

Tolerance is manifested in the graft by a progressive shift from cytotoxic/CD8 cells to Treg/CD4 cells and B cells. The time-dependent induction of B cell–specific genes and protein markers in the accepted mouse kidney allografts are consistent with previous reports in human studies that renal transplant acceptance is associated with a B cell signature.[32-34] Many transcripts (n = 130) in 1-week samples were significantly reduced at 40–60 weeks, including a variety of transcripts related to cytokines, chemokines, and cytotoxic CD8 T cells. Over time Foxp3 transcripts persist at high levels and MHC class II antigen presentation and B cell receptor signaling pathways were not reduced.

Transcripts characteristic of Treg subtypes, specifically cTreg, eTreg, and tumor-infiltrating Tregs, show a sustained level of expression following kidney allograft transplantation. Interestingly, the Tfr signature mirrors that of B cells, showing an increased expression of transcripts specific for Tfr cells with time. What the relationship between Tfr and B cells is, especially in the context of the Tfh signature, which also parallels that of Tfr and B cells, is a focus of future studies. One possibility is that there is an interplay between these cells, mediating the function of B cells to a more regulatory phenotype and less so towards one that favors the development of anti-donor antibody producing plasma cells.

This study has several limitations. Only two forms of TLOs were included and there is probably substantial heterogeneity of the phenotype. Nonetheless, consistent differences in TOLS were detected that probably apply to at least some TLOs. Furthermore, TOLS themselves may be heterogeneous. Foxp3+ rich aggregates in neoplasia have been reported to have HEV.[25] One report of TOLS in murine kidney grafts described HEV as judged by immunohistochemistry.[35] Similar results were observed by Tse and colleagues in a bm12 to B6 kidney transplant model using unilaterally nephrectomized recipients, where they observed HEV and chronic allograft damage.[36] In confirmation, we found that keeping a native kidney in B6 recipients of DBA/2 allografts led to histologic signs of rejection by 30 to 40 days posttransplant and development of HEV (unpublished data).

Our study did not establish the generality of TOLS in other conditions of tolerance. However, we have observed aggregates similar to murine TOLS in renal allografts in non-human primates with tolerance induced by mixed chimerism.[37] Although Foxp3+ cells are critical for maintaining TOLS and preventing rejection,[5] further studies will be needed to determine whether these intragraft structures per se mediate or predict outcomes and if the presence of HEV predicts susceptibility to future rejection episodes.

In conclusion, we have identified novel regulatory lymphoid organs, TOLS, in renal allografts in tolerant recipients that can be distinguished anatomically, phenotypically, and genetically from other ectopic lymphoid structures that are inflammatory in nature, iTLOs. The formation of TOLS is dependent on class II MHC differences and does not require generation of Foxp3+ cells in lymph nodes, spleen, or thymus. Foxp3+ cell proliferate in TOLS, and we hypothesize that TOLS are the site of Foxp3+ induction, functionally different from iTLOs, and a marker of regulatory tolerance.

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health Grants P01-AI123086 (A.A., R.B.C., J.C.M.), T32-AI007529 (E.A.F.) and R01-AI081734 (R.B.C.). T.A. was supported by King Saud University College of Medicine, Department of Pathology, Riyadh, Saudi Arabia.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

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How to cite this article: Rosales IA, Yang C, Farkash EA, et al. Novel intragraft regulatory lymphoid structures in kidney allograft tolerance. *Am J Transplant*. 2022;22:705–716. doi:10.1111/ajt.16880