

**Advanced Tertiary Lymphoid Tissues in Protocol Biopsies
are Associated with Progressive Graft Dysfunction in Kidney
Transplant Recipients**

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Abstract: **Background:** Tertiary lymphoid tissues (TLTs) are ectopic lymphoid tissues found in chronically inflamed organs. Although studies have documented TLT formation in transplanted kidneys, the clinical relevance of these TLTs remains controversial. We examined the impacts of TLTs on future graft function using our histological TLT maturity stages and the association between TLTs and Banff pathologic scores. We also analyzed the risk factors for the development of TLTs

Methods: Serial protocol biopsy samples (0-hour, 1-, 6-, and 12-months) without rejection were retrospectively analyzed from 214 patients who underwent living donor kidney transplantation. TLTs were defined as lymphocyte aggregates with signs of proliferation and their stages were determined by the absence (stage I) or presence (stage II) of follicular dendritic cells.

Results: Only 4% of patients exhibited TLTs at the 0-hour biopsy. Prevalence increased to almost 50% at the 1-month biopsy and then slightly further for 12 months. The proportion of advanced stage II TLTs increased gradually, reaching 19% at the 12-month biopsy. Presence of stage II TLTs was associated with higher risk of renal function decline after transplantation compared to patients with no TLT or stage I TLTs. Stage II TLTs were associated with more severe tubulitis and interstitial fibrosis/tubular atrophy at 12 months and predicted poorer graft function independently from the degree of interstitial inflammation. Pre-transplantation rituximab treatment dramatically attenuated the development of stage II TLTs.

Conclusions: TLTs are commonly found in clinically stable transplanted kidneys. Advanced stage II TLTs are associated with progressive graft dysfunction, independent of interstitial inflammation

Significance Statement

Tertiary lymphoid tissues (TLTs) are frequently found in transplanted kidneys, but their prevalence and clinical significance remain uncertain. Serial protocol kidney transplant biopsies without signs of rejection were collected and TLTs staged according to the presence of proliferating lymphocytes and follicular dendritic cells. TLTs rapidly developed within 1 month after kidney transplantation in approximately half the 214 patients. Advanced TLTs, defined as the presence of follicular dendritic cells, was associated with progressive decline in graft function independent of interstitial inflammation score. These findings suggest that advanced TLTs are strongly associated with late graft dysfunction even in the absence of rejection.

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4 **Progressive Graft Dysfunction in Kidney Transplant Recipients**
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Abstract

Background: Tertiary lymphoid tissues (TLTs) are ectopic lymphoid tissues found in chronically inflamed organs. Although studies have documented TLT formation in transplanted kidneys, the clinical relevance of these TLTs remains controversial. We examined the impacts of TLTs on future graft function using our histological TLT maturity stages and the association between TLTs and Banff pathologic scores. We also analyzed the risk factors for the development of TLTs.

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Conclusions: TLTs are commonly found in clinically stable transplanted kidneys. Advanced stage II TLTs are associated with progressive graft dysfunction, independent of interstitial inflammation.

Introduction

Kidney transplantation is an ideal treatment for patients with end-stage renal disease. Whereas short-term graft survival had been greatly improved in the last three decades, long term graft survival has changed only marginally^{1, 2}. Among various factors affecting the outcomes of transplanted kidneys, chronic intra-graft inflammation has been considered one of the most important components that contribute to persistent allograft injury³⁻⁶. Studies have consistently suggested that subclinical rejection frequently leads to deterioration of transplanted kidneys if left untreated⁷⁻¹⁰. Even mild tubulointerstitial inflammation was associated with poor graft outcomes after kidney transplantation, highlighting the relationship between unresolved inflammation and progressive functional decline¹¹. For these reasons, better understanding and proper management of graft inflammation is a prerequisite to long-term graft survival.

Persistent inflammatory stimuli often give rise to the development of tertiary lymphoid tissues (TLTs), i.e., inducible ectopic lymphoid tissues that arise in chronic inflammatory conditions such as aging, cancer, autoimmune diseases and in transplanted organs¹²⁻¹⁵. T and B lymphocytes are the main hematopoietic components of TLTs, and specialized fibroblasts provide structural support and produce homeostatic chemokines such as CXCL13¹⁶⁻²². Although the functional roles of TLTs are context-dependent, we have previously described a strong association between renal TLTs and maladaptive repair in rodent models²⁰.

To provide objective and standardized analytic methodology, we recently proposed a new TLT staging strategy based on the presence of follicular dendritic cells (FDCs) and germinal centers, both of which represent cellular components of advanced TLTs²³. TLT stages

positively correlated with the severity of kidney injury and inflammation, suggesting the potential to serve as additional histological markers of tissue inflammation.

The presence of TLTs in transplanted kidneys is well documented²⁴⁻³⁵. Nevertheless, their clinical relevance remains controversial. The main reasons for these conflicting results include that TLTs were not separated from concurrent rejection, and the definition of TLTs has been inconsistent across studies^{14, 36-38}. The facts that TLTs were frequently observed both in rejected and tolerated murine allografts further complicate their functional identity in transplanted kidneys³⁹⁻⁴¹. To overcome these issues and to clarify the impacts of TLTs on graft functions, we utilized two major strategies. First, we collected protocol biopsy samples from kidney transplant recipients without overt evidence of rejection to directly investigate the relationship between TLTs and graft function. Second, using our recently established TLT staging method²³, we classified TLTs based on their phenotypes, and analyzed the association between TLT stages and graft outcomes. Here, we demonstrate that TLTs developed in almost half of clinically stable patients and that the presence of advanced stage II TLTs was associated with progressive functional decline of renal allograft in comparison with stable graft function in patients without TLTs or with stage I TLTs.

Methods

Study population and protocol biopsy sample acquisition

An overview of the study design and patient recruitment strategy is given in **Figure 1**. We retrospectively screened 241 patients who underwent their first living donor kidney transplantation between July 2004 and December 2016 at Akita University in Japan. Four serial protocol biopsies were obtained from each patient during this period. A 0-hour protocol biopsy was performed during cold-saline perfusion after kidney explantation from the donor. Subsequently, recipients underwent protocol biopsies at 1 month, 6 months, and 12 months after transplantation. At least two cores of graft tissue were obtained at each biopsy, paraffin-embedded, and subjected to conventional histologic stains and immunofluorescence. Patients were excluded if they met one or more of the following criteria: 1) occurrence of biopsy-proven acute rejection within the first year of transplantation; 2) occurrence of BK virus-associated nephropathy within the first year of transplantation; 3) non-recovery of renal allograft function ($< 30 \text{ ml/min/1.73 m}^2$) over the first year of transplantation; or 4) loss to follow-up within the first year of transplantation. To be specific, recipients with subclinical rejection (biopsy-proven acute T cell-mediated or antibody-mediated rejection without an elevation in serum creatinine level) were excluded, while those with borderline T cell-mediated rejection ($t > 0$ with $i0$ or $i1$, or $t1$ with $i2$ or $i3$ without an elevation in serum creatinine levels) were included in this study⁴²⁻⁴⁴.

Information regarding the baseline characteristics of the recipients and donors was obtained at the time of kidney transplantation and during visits to the outpatient clinic. Donor specific antibody was retrospectively measured in the sera at 1-year after transplantation stored until use using Luminex-based SAB kits (LABScreen® PRA and LABScreen® Single Antigen, Thermo Scientific, Waltham, MA). Positive evaluations were made as previously described⁴⁵. Indication biopsy was performed when patients had an unexplained rise in serum creatinine

($>25\%$ from baseline value) during the follow-up period, but the presence of TLTs in these samples was not assessed in this study. Kidney function was measured as the estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration formula for the Japanese population⁴⁶. All diagnoses and Banff pathologic scores were determined and rescored by a single experienced transplant nephrologist, in accordance with Banff 2017 criteria⁴².

All human specimens were analyzed after informed consent, and approval of the ethics committees at Akita and Kyoto University hospitals were obtained. This study adhered to the Declaration of Istanbul.

Outcome measures

The primary endpoint was the occurrence of death-censored renal function decline, defined as a decline of at least 30% in the eGFR from 1-year post-transplant graft function. The secondary endpoint was renal allograft function after kidney transplantation.

Immunosuppressive regimen

Protocols of immunosuppressive drugs are described elsewhere⁴⁷. Briefly, all patients received basiliximab (20 mg at day 0 and day 4) as induction immunosuppression, followed by maintenance immunosuppression with prednisolone, mycophenolate mofetil, and tacrolimus. Patients undergoing ABO-incompatible kidney transplantation received a single dose of rituximab (200 mg) 3 weeks before transplantation followed by plasma exchange or double-filtration plasmapheresis and the administration of intravenous immunoglobulin. Patients diagnosed with borderline T cell-mediated rejection in protocol biopsies were routinely treated with 10–20 mg/kg of intravenous methylprednisolone for 2–3 consecutive days depending on the degree of graft inflammation and patient status, unless contraindicated.

Identification and evaluation of TLTs in transplanted kidneys

In the present study, we defined TLTs as organized lymphocyte aggregates with the signs of proliferation as described previously^{23, 48}. Because TLT sizes in transplanted kidneys were variable (**Supplemental Figure 1A**), we defined organized lymphocyte aggregates as more than 60 lymphocytes (T cells or B cells) in this study (**Supplemental Figure 1A-C**). After diagnosis of TLTs, we determined stages of each TLT.

Identification and quantification of TLT stages was determined through two steps as follows:

1) identification of mononuclear cell infiltrates in permissive areas for TLT formation, which include subcapsular, periglomerular and perivascular area, with periodic acid–Schiff (PAS)-stained graft sections; and 2) assessment of the lymphocyte infiltrates using immunofluorescence of (a) CD3ε and CD20, and (b) Ki67 and CD21 in two serial sections for each individual, as described previously²³. After the diagnosis of TLTs, we determined TLT stages of each TLT.

TLT stages were defined as follows:

- i) TLT lacking either FDC or germinal center: stage I TLT
- ii) TLT containing FDC but lacking germinal center: stage II TLT
- iii) TLT containing both FDC and germinal center: stage III TLT

FDCs were defined as the cells strongly positive for non-hematopoietic CD21 signals within TLTs. Germinal centers were defined with Ki67-positive cell clusters, which contained more than 15 Ki67-positive cells per cluster, in B cell areas.

Renal immunofluorescence

Immunofluorescence studies were performed on tissues from the same block as were used for the preparation of PAS-stained slides. Immunofluorescence staining of biopsy tissues was

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performed as previously described⁴⁹. The following primary antibodies were used in these experiments: anti-CD3ε (catalog ab5690; Abcam, Cambridge, UK), anti-CD20 (catalog 14-0202; eBioscience, San Diego, CA), Ki67 (catalog ab16667; Abcam), anti-CD21 (catalog ab75985; Abcam, and catalog MA5-11417; Thermo Scientific, Waltham, MA), anti-CD45 (catalog 14-9457; eBioscience), anti-p75NTR (catalog AF1157; R&D, Minneapolis, MN), and anti-CXCL13 (catalog AF801; R&D). Staining was visualized using appropriate secondary antibodies. Cell nuclei were counterstained with DAPI. All immunofluorescence samples were analyzed using a confocal microscope (FV1000D; Olympus, Japan).

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 20.0 (IBM Corp., Armonk, NY) and STATA 14.1 (StataCorp, College Station, TX). Baseline patient characteristics and clinical parameters were expressed as mean $\pm$ standard deviation (SD) or as numbers of patients and percentages. Time on dialysis was expressed as median [1st and 3rd interquartile range] because it was non-normally distributed. Temporal changes in the TLT stages were assessed by trend analysis. Renal function decline, assessed in a time-to-event analysis, was analyzed by Cox proportional-hazards models with an adjustment for age, sex, the presence of diabetes after transplantation, ABO incompatibility, positive crossmatch, the presence of donor specific antibody at 1-year post-transplantation, and pre-transplantation donor eGFR. To further compare the differences in repeatedly measured eGFR during the follow-up period between groups, we used a linear mixed-effect model with robust variance estimation⁵⁰, adjusting for baseline covariates including donor and recipient age and gender, recipient body mass index, preemptive kidney transplantation, the presence of diabetes after transplantation, the number of HLA mismatching, positive crossmatch, ABO incompatibility, the use of immunosuppressants, baseline graft function, the presence of donor specific

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3 antibody at 1-year post-transplantation, and pre-transplantation donor eGFR. Baseline graft
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5 function was set as eGFR levels at each biopsy-time point. Logistic regression analysis was
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7 performed to identify risk factors for the development of TLTs. The relationship between the
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9 use of pre-transplantation rituximab and TLTs was analyzed using Pearson’s chi-square test
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11 or Fisher’s exact test as appropriate. Finally, the overall comparisons of Banff pathologic
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13 scores with TLT scores were performed using the Kruskal–Wallis test, and the Mann–
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15 Whitney test was used for the comparisons of each group. *P*-values less than 0.05 were
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Results

Baseline characteristics of enrolled patients

A total of 214 kidney transplant recipients were finally included in this study, and their baseline demographics and laboratory parameters are shown in **Table 1**. The most common cause of end-stage renal disease was chronic glomerulonephritis. Mean eGFRs were 66.3, 65.1, and 62.1 ml/min/1.73 m² at 1 month, 1 year, and 5 years after kidney transplantation, respectively. Approximately one-fourth (53/214, 24.8%) of the enrolled patients underwent ABO-incompatible kidney transplantation; they were older and more frequently received pre-transplantation rituximab than those who underwent ABO-compatible kidney transplantation (**Supplemental Table 1**). Acute rejection accounted for 63.0% (17/27) of the reasons for exclusions in our study; 13 and 4 cases were attributed to acute antibody-mediated rejection and acute T cell-mediated rejection, respectively (**Supplemental Table 2**).

Phenotypic characterization of TLTs in transplanted kidneys

PAS-stained graft tissues contained multiple TLT-like mononuclear cell infiltrates (**Figure 2A**), located in either subcapsular, perivascular, or periglomerular areas (**Figure 2B-D**), consistent with our previous study²³. These infiltrates were composed of T and B cells, (**Figure 2E-G**), some of which were proliferating (**Figure 2H**), meeting our definition of TLTs. Among lymphocyte infiltrates detected in PAS-stained samples, 86.8% were confirmed as TLTs based on immunofluorescence (**Figure 2I**). In many TLTs, T and B cells were intermingled with one another (**Figure 2E**), but some TLTs harbored densely packed B cell clusters (**Figure 2F and G**). CD21-positive FDCs, i.e. stromal cells in charge of organizing B cell homeostasis in TLTs²⁰, were also detected in some B cell clusters (**Figure 2J and 2K**). CXCL13 expression was also observed within TLTs and was colocalized with CD21 but not with CD45 (**Figure 2L and 2M**). Interestingly, T cell-dominant TLTs were

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detected in the graft tissues of patients treated with pre-transplantation rituximab (Supplemental Figure 2B-D) in permissive areas for TLT formation described above. TLTs in these patients harbored far fewer B cells (Supplemental Figure 2B-D) than TLTs in aged kidneys and the kidney with chronic kidney disease (Supplemental Figure 2A)^{20, 23}, but yet had proliferating lymphocytes inside and therefore met the definition of TLTs.

The prevalence and staging of TLTs in transplanted kidneys

We next categorized TLT phenotypes utilizing the TLT staging strategy we recently established (see **Methods** for details). We observed stage I and stage II TLTs, but not stage III TLTs in protocol biopsies of transplanted kidneys (Figure 3). Notably, the prevalence of TLTs as well as their stages significantly changed after transplantation (Figure 4). In 0-hour biopsies, TLTs were found in only 3.8% of the samples. This prevalence increased to 46.9% at 1 month after kidney transplantation, and then slightly further within the first year (53.4% and 58.4% in 6- and 12-month biopsies, respectively). By contrast, the development of stage II TLTs was more gradual; their prevalence in 1-month biopsies was comparable to that of 0-hour biopsies (1.4% and 3.6% in 0-hour and 1-month biopsies, respectively) and then began to increase steadily thereafter, reaching 8.6% in the 6-month biopsy samples (6.1-fold increase versus 0-hour) and 18.9% in the 12-month biopsy samples (13.5-fold increase versus 0-hour).

Renal allograft outcomes in relation to the presence and stage of TLTs

Next, we assessed renal allograft functions according to the presence and stages of TLTs at various time points of biopsies. The presence of TLTs, if stages were not taken into consideration, had no significant influence on late graft function (Supplemental Figure 3A-F). However, when patients were divided according to TLT stage, those with stage II TLTs in

the 6- or 12-month biopsies had significantly higher risk of death-censored renal function decline compared to those with no TLT (adjusted hazard ratios of 3.92 and 3.17 at the 6- and 12-month biopsies, $p = 0.02$ and 0.015 , respectively; **Figure 5B, 5C and Table 2**). eGFR over the 5 years after transplantation were also significantly lower in patients with stage II TLTs than in those with no TLT (adjusted mean differences of -14.35 and -11.71 ml/min/1.73 m² at the 6- and 12-month biopsies, $p = 0.008$ and 0.012 , respectively; **Figure 5E, 5F and Table 3**). In patients exhibiting stage II TLTs in the 1-month biopsy, the risk of late graft dysfunction was higher and mean eGFR at one-year post-transplantation was lower than in those without TLTs or in those with stage I TLTs, although without significant difference (**Figure 5A, 5D and Table 2, 3**). Sensitivity analyses of recipients who underwent ABO-compatible kidney transplantation consistently showed that the development of stage II TLT in the 6- or 12-month biopsy was associated with significantly higher risk of decline in graft function compared to those without TLTs (adjusted hazard ratios of 3.97 and 2.81 at the 6- and 12-month biopsies, $p = 0.030$ and 0.039 , respectively; **Supplemental Figure 4 and Supplemental Table 3, 4**).

Previous studies suggested a possible association between the presence of TLTs, especially B cell clusters, and the occurrence of allo-antibodies and subsequent antibody-mediated injury^{32, 33, 51}. In our cohort, donor specific antibodies at 1 year after transplantation were more frequently detected in patients with stage II TLTs than in those with no TLTs or stage I TLTs at the 12-month biopsies (**Supplemental Figure 5**). Nevertheless, no patient was diagnosed with biopsy-proven acute antibody-mediated rejection during 4 years of follow-up after the final protocol biopsy at 12 months. Seven patients had biopsy-proven acute T-cell mediated rejection during this period; however, these episodes were not associated with the prevalence of stage II TLTs at 12-month post-transplantation.

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About one-third of our patients (66/214) experienced at least one episode of borderline T cell-mediated rejection during the first year after kidney transplantation. Most of the patients were treated with steroid pulse therapy (63/66, 95.5%), and the trends of eGFR between patients with and without borderline rejection were not different (**Supplemental Figure 6**).

Risk factors for the development of stage II TLTs in the 12-month biopsies

Logistic regression analysis revealed that the use of pre-transplantation rituximab was the strong negative risk factor for the development of stage II TLTs in the 12-month biopsies (odds ratio of 0.17, 95% confidence interval of 0.04–0.72, $p = 0.016$; **Table 4**). Pre-transplant rituximab administration suppressed stage II TLTs but did not affect the prevalence of stage I TLTs, regardless of biopsy time point (**Figure 6 and Supplemental Table 5**). The prevalence of stage II TLTs was lower in ABO-incompatible subgroup than in ABO-compatible subgroup, although without statistical significance (**Supplemental Table 6**). eGFR levels were maintained at similar levels in patients treated with pre-transplantation rituximab compared with those who were not, despite ABO incompatibility (**Supplemental Figure 7**).

The presence of donor specific antibody at 1 year after transplantation was positively associated with 12-month stage II TLTs (odds ratio of 7.63, 95% confidence interval of 1.36–42.91, $p = 0.021$; **Table 4**). Borderline acute T cell-mediated rejection was not associated with the development of either stage I or II TLTs (**Table 4 and Supplemental Table 5**).

The association between TLTs and Banff pathologic scores

Finally, we investigated the relationship between TLT stages and Banff pathologic scores obtained at 12 months after kidney transplantation (**Table 5**). The presence of TLTs

correlated with more pronounced interstitial inflammation at 12 months, presumably because TLTs themselves are regarded as interstitial inflammation according to the definition of Banff scores⁴². Nevertheless, small stage II TLTs were occasionally found against the background of trivial Banff i scores (**Supplemental Figure 8**). Importantly, patients in the stage II TLT group exhibited significantly worse tubulitis, tubular atrophy, and interstitial fibrosis than did patients in the no TLT group (t score of 0.12 ± 0.36 vs. 0.60 ± 0.91 , ct score of 0.77 ± 0.72 vs. 1.29 ± 0.75 , and ci score of 0.70 ± 0.63 vs. 1.17 vs. 0.92 ; $p < 0.001$, < 0.001 , and 0.008 , respectively; **Table 5**). Patients with the stage I TLTs also showed worse tubulointerstitial inflammation, tubular atrophy, and interstitial fibrosis scores compared to those without TLTs (**Table 5**), although late graft function was similar between these groups (**Figure 5F**). Moreover, the presence of stage II TLTs at 12-month biopsies might be associated with future graft dysfunction, even in patients with quantitatively mild interstitial inflammation (adjusted hazard ratio of 2.60 and mean eGFR differences of -11.2, $p = 0.048$ and 0.054 , respectively; **Figure 7 and Table 6**). Banff pathologic scores at 12-month biopsy were not significantly different between ABO-compatible and ABO-incompatible subgroups, except for C4d, whose scores were clearly higher in recipients who underwent ABO-incompatible transplantation (**Supplemental Table 7**).

2
3 **Discussion**
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5 In the present study, we investigated the prevalence and clinical relevance of TLTs in
6 transplanted kidneys without the signs of rejection. We found that TLTs were frequent in
7 rejection-free protocol biopsies and that their cellular and molecular phenotypes were similar
8 to those found in aged patients²⁰. By contrast to stage I TLTs that appeared as early as 1
9 month after kidney transplantation, stage II TLTs developed gradually over time and were
10 independently associated with progressive graft dysfunction. These data suggest that
11 advanced TLTs may help to stratify stable renal allografts without rejection into those with
12 and without risk of functional deterioration.
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26 The presence of lymphocyte clusters in the absence of rejection was first described in heart
27 and lung allografts, in which protocol biopsies are performed more frequently; in these hearts
28 and lungs, the prevalence of lymphocyte clusters ranged from 39 to 58%⁵²⁻⁵⁷, similar to that
29 of TLTs in our study (**Figure 4**). The clinical impacts of lymphocyte clusters on these
30 allografts have been debated and controversial⁵²⁻⁵⁷. In the previous study, we showed that
31 TLTs in the kidneys develop through at least three developmental stages irrespective of
32 etiologies, and the developmental progression are associated with the severity of kidney
33 injury in human utilizing surgically resected kidney samples²³. In the present study, utilizing
34 renal biopsy samples, we showed that the presence of stage II TLTs was associated with
35 functional decline in graft function, while the presence of stage I TLTs was not. These results
36 suggest that the presence of FDC, not of B cell infiltrations, is the determinant for future graft
37 dysfunction, and may partly explain the inconsistent results of the clinical significance of
38 graft-infiltrating B cells in transplanted kidneys^{24-29, 32, 34, 58}. We therefore propose evaluating
39 TLTs using our staging strategy, especially focusing on the presence or absence of FDCs.
40 Given that FDCs are found in heart allografts^{59, 60}, application of our TLT staging strategy
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may clarify hitherto unrevealed functional roles of lymphocyte clusters in other transplanted organs.

Time-dependent changes in the distribution of TLT stages provide valuable information regarding their evolution in renal allografts (**Figure 4**). Stage I TLTs rapidly developed within 1 month after kidney transplantation. By contrast, the prevalence of stage II TLTs did not change at this time point; rather, it increased in the 6- and 12-month biopsy samples. These findings are consistent with our rodent experimental data, where the proportion of advanced TLTs increased in a time-dependent manner after the injury²³. Notably, we could not find histologic evidence of stage III TLTs, which had been documented in chronically rejected allograft explanted at more than 5 years after KT^{26, 32, 35}. We speculate that 12 months were too short for ectopic germinal centers to be established and the intensity of graft inflammation in our cases was substantially lower than that in transplanted kidneys with chronic rejection.

Our TLT staging strategy distinguished between progressors and non-progressors, even among patients categorized as having Banff i scores of 0 or 1. The paradox of kidney allograft with advanced TLTs being categorized as having minimal or mild interstitial inflammation is explained by differences in the grading systems used to score TLTs and interstitial inflammation in the Banff classification. Because Banff i scores depend on the percentage of the area with inflammatory cell infiltration, biopsy samples with small, but FDC-containing TLTs would be classified as minimal or mild interstitial inflammation (**Supplemental Figure 8**). Another possible explanation is that interstitial infiltrates in subcapsular cortex and in areas of interstitial fibrosis were included in the assessment of TLTs, but not in the determination of Banff i score. Our data suggest that TLTs are

inflammatory lesions that are qualitatively different from simple interstitial inflammation and therefore should be assessed in a different manner.

Although the underlying mechanisms were not investigated here, analysis of Banff pathologic scores identified sustained tubular injury as a possible cause of progressive graft dysfunction in patients with stage II TLTs (**Table 5**). Consistent with this hypothesis, a rodent kidney transplantation model study demonstrated that B cells in TLTs promoted tubulointerstitial fibrosis, possibly by secreting fibrosis-related cytokines⁶¹. Other studies demonstrated that TLTs were associated with the formation of alloantibodies^{26, 62}, and the intensity of antibody-mediated alloimmune responses correlated with the maturation status of TLTs^{32, 33}, suggesting a link between advanced TLTs and antibody-mediated graft injury. In the present study, however, no patient showed biopsy-proven antibody-mediated rejection, although donor-specific antibodies were more frequently observed in patients with stage II TLTs (**Table 4** and **Supplemental Figure 5**). Moreover, stage II TLTs were not associated with any pathologic features suggestive of antibody-mediated injury such as glomerulitis (g score), peritubular capillaritis (ptc score), or C4d staining at 12 months post-transplantation (**Table 5**), consistent with findings of a previous report⁶³. Taken together, these data suggest that stage II TLTs contributed to graft dysfunction presumably via tubular inflammation and fibrosis, at least in the first year after kidney transplantation. The association between advanced TLTs and antibody-mediated rejection should be clarified in further investigations.

The effects of rituximab on the development of TLTs have rarely been investigated. In the present study, the administration of pre-transplantation rituximab dramatically reduced stage II TLTs up to a year after kidney transplantation (**Figure 6**). These findings are mechanistically reasonable, given the capacity of rituximab to deplete circulating B cells for

12 months or longer⁶⁴. Interestingly, a retrospective study showed that post-transplantation rituximab did not result in the clearance of intra-graft TLTs⁵⁸. Similar findings were consistently reported in other conditions such as autoimmune diseases⁶⁵⁻⁷¹, suggesting the importance of the timing of rituximab infusion for controlling TLT formation. Furthermore, patients treated with pre-transplantation rituximab maintained similar graft function over 5 years after kidney transplantation, comparable to those who did not, even though the immunologic risk was higher in the ABO-incompatible, rituximab-treated group (**Supplemental Figure 7**). Nevertheless, it remains uncertain whether this effect was due to the reduction of stage II TLTs or other unrevealed mechanisms of rituximab. It is also possible that plasma exchange and/or intravenous immunoglobulin, administered along with rituximab, may be associated with suppression of advanced TLTs.

It is noteworthy that most patients treated with pre-transplantation rituximab were ABO-incompatible subgroups (50/57, 87.7%), indicating that the significant differences in the prevalence of stage II TLTs between ABO-compatible and ABO-incompatible subgroups might be due to the different baseline demographics and immunologic risks rather than the use of pre-transplantation rituximab. It was difficult to investigate the effects of pre-transplantation rituximab on stage II TLTs among recipients who underwent ABO-compatible transplantation, because of the small number of rituximab-treated recipients in this population (7/161, 4.3%). Given that pre-transplantation rituximab is prescribed exclusively for patients with high immunologic risks, distinguishing the effects of rituximab and immunologic profiles on stage II TLTs would be extremely difficult in real world.

Older age, an important risk factor for developing TLTs²⁰, was not associated with their formation in the present study (**Table 4**). One of the reasons for this discrepancy could be the

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fact that both kidney donor and recipients were much younger compared with those recruited in our previous study²⁰ (i.e. a mean age of 58 (donor) and 48 (recipients) vs. 70 years (previous study)). We speculate that sustained inflammatory stimuli from immunologic differences, rather than the age of patients, appear to be a more powerful inducer of TLTs in the setting of kidney transplantation.

A limitation of this study should be mentioned. Approximately 12% (108/856) of biopsy samples were missing in this study, mostly because of refusals by recipients (4 [1.9%], 22 [10.3%], 51 [23.8%], and 31 [14.5%] missed samples at 0-hour, 1-, 6-, and 12-month post-transplantation, respectively); these missingness raises a possibility of unexpected selection bias. Nonetheless, we speculate that the proportion of missing cases were relatively small, considering the difficulties in obtaining serial protocol biopsies from recipients maintaining stable graft function. Furthermore, the baseline characteristics and clinical parameters were mostly comparable between recipients who underwent protocol biopsy and those who did not (data not shown). Therefore, the impacts of the missing cases on the overall results might be trivial.

In conclusion, we demonstrated that the intra-graft detection of stage II TLTs was independently associated with progressive decline in renal allograft function. Prospective studies are needed to confirm whether our novel TLT staging strategy has the potential to serve not only as a valuable tool for systematic classification, but also as a predictor of transplant functional decline. Further investigations are also needed to determine whether therapeutic strategies to prevent the development and maturation of TLTs could lead to better long-term graft outcomes in kidney transplant recipients.

Author contributions

Y.H.L., Y.S. and M.Y. designed the study; Y.H.L. and Y.S. carried out experiments; Y.H.L., Y.S., M.S., M.S., S.Y., A.K., N.F., S.S., T.H. and M.Y. collected and analyzed the data; Y.H.L. and S.F. performed statistical analyses; Y.H.L., Y.S., S.H.L., B.P., F.J. and M.Y. drafted and revised the paper; all authors approved the final version of the manuscript.

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Table 1. Baseline characteristics and clinical parameters of enrolled patients

Number of patients	214
Age (year)	48.8 ± 12.5
Gender (Male, %)	139 (65.0)
Body mass index (kg/m ²)	22.4 ± 3.7
Etiology of end-stage renal disease (n, %)	
Chronic glomerulonephritis	122 (57.0)
Diabetes mellitus	36 (16.8)
Hypertension	16 (7.5)
Polycystic kidney disease	14 (6.5)
Others ^a	26 (12.2)
Time on dialysis (month)	18.0 [5.0, 48.8]
Preemptive kidney transplantation (n, %)	40 (18.7)
Number of HLA mismatching (n)	3.3 ± 1.5
Positive crossmatch	14 (6.5)
ABO-incompatible kidney transplantation (n, %)	53 (24.8)
Pre-transplantation rituximab (n, %)	57 (26.6)
Cold ischemic time (minute)	144.0 ± 37.6
Warm ischemic time (minute)	5.0 ± 3.7
Induction immunosuppressant (n, %)	
Basiliximab	214 (100)
Maintenance immunosuppressant (n, %) ^b	
Prednisolone	171 (79.9)
Tacrolimus	214 (100)
Mycophenolate mofetil	214 (100)
Borderline T cell-mediated rejection (n, %) ^c	66 (30.8)
Donor specific antibody at 1-year post-transplantation (n, %) ^d	13/207 (6.3)
Class I	7 (53.8)

Class II	7 (53.8)
Post-transplant eGFR (ml/min/1.73 m ²)	
1-month	66.3 ± 20.6
1-year	65.1 ± 18.9
5-year	62.1 ± 21.6
Donor age (year)	58.3 ± 9.9
Donor gender (Male, %)	82 (38.3)
Pre-transplantation donor eGFR (ml/min/1.73 m ²)	103.0 ± 10.8

Data are expressed as mean ± standard deviation or the number of patients (percentage). Time on dialysis is non-normally distributed and is expressed as median [1st and 3rd interquartile range].

^a Others include chronic tubulointerstitial nephritis, gestational hypertension, vesicoureteral reflux disease, sepsis, cystinuria, and bone marrow transplant nephropathy.

^b Data obtained at the time of outpatient visit 1 year after transplantation.

^c At least one episode during the first year after transplantation.

^d Not assessed in 7 recipients.

Abbreviations: HLA, human leukocyte antigen; eGFR, estimated glomerular filtration rate.

Table 2. Hazard ratios of the stages of tertiary lymphoid tissues for death-censored renal function decline

	TLT stages	No. of events ^a (%)	Adjusted HR ^b (95% CI)	<i>p</i> value
1-month	No TLT (n=102)	19 (18.6)	Reference	-
	Stage I (n=83)	18 (21.7)	1.44 (0.72 to 2.87)	0.309
	Stage II (n=7)	3 (42.9)	3.60 (0.96 to 13.50)	0.058
6-month	No TLT (n=76)	10 (13.2)	Reference	-
	Stage I (n=73)	15 (20.5)	1.49 (0.63 to 3.53)	0.370
	Stage II (n=14)	6 (42.9)	3.92 (1.23 to 12.47)	0.020
12-month	No TLT (n=77)	11 (14.3)	Reference	-
	Stage I (n=73)	13 (17.8)	1.05 (0.44 to 2.51)	0.914
	Stage II (n=35)	14 (40.0)	3.17 (1.25 to 8.02)	0.015

^a Renal function decline was defined as a decline of at least 30% in the eGFR from 1-year post-transplant graft function.

^b The comparisons between groups are performed by Cox regression analysis with multiple adjustments for confounders including age, sex, the presence of diabetes after transplantation, ABO incompatibility, positive crossmatch, the presence of donor specific antibody at 1-year post-transplantation, and pre-transplantation donor eGFR.

Abbreviations: TLT, tertiary lymphoid tissue; HR, hazard ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate.

Table 3. Association between the stages of tertiary lymphoid tissues and graft function

Biopsy time point	TLT stages	Adjusted difference in eGFR ^a (95% CI)	<i>p</i> value
1-month	No TLT (n=102)	Reference	-
	Stage I (n=83)	-0.24 (-6.04 to 5.56)	0.935
	Stage II (n=7)	-13.16 (-32.60 to 6.29)	0.185
6-month	No TLT (n=76)	Reference	-
	Stage I (n=73)	-4.07 (-10.07 to 1.93)	0.183
	Stage II (n=14)	-14.35 (-24.93 to -3.76)	0.008
12-month	No TLT (n=77)	Reference	-
	Stage I (n=73)	-1.16 (-5.78 to 3.47)	0.624
	Stage II (n=35)	-11.71 (-20.84 to -2.58)	0.012

^a The comparisons between groups are performed by linear mixed effect models with multiple adjustments for confounders including recipient age and gender, donor age and gender, recipients body mass index, preemptive kidney transplantation, the presence of diabetes after transplantation, the number of HLA mismatching, positive crossmatch, the use of immunosuppressant, baseline graft function, the presence of donor specific antibody at 1-year post-transplantation, and pre-transplantation donor eGFR. Baseline graft function was set as eGFR levels for each time point. The differences in eGFR are calculated by comparing eGFR at baseline and eGFR at 4-5 years after kidney transplantation.

Abbreviations: TLT, tertiary lymphoid tissue; eGFR, estimated glomerular filtration rate; CI, confidence interval; HLA, human leukocyte antigen.

Table 4. Multivariable analyses of risk factors for the development of stage II tertiary lymphoid tissues in 12-month protocol biopsies

Variables	OR (95% CI)	<i>p</i> value
Recipient age (per 10-year increase)	1.08 (0.74 – 1.59)	0.696
Donor age (per 10-year increase)	0.69 (0.38 – 1.24)	0.210
Recipient gender (male)	0.93 (0.30 – 2.85)	0.892
Donor gender (male)	1.84 (0.63 – 5.32)	0.263
Body mass index	0.97 (0.85 – 1.11)	0.679
Diabetes mellitus	1.70 (0.63 – 4.55)	0.292
Number of HLA mismatching (per one mismatch increase)	0.90 (0.65 – 1.24)	0.519
Positive crossmatch	0.86 (0.11 – 7.06)	0.891
Pre-transplantation rituximab	0.17 (0.04 – 0.72)	0.016
Steroid maintenance therapy at 1-year post-transplantation	0.55 (0.20 – 1.57)	0.265
Cold ischemic time (per ten-minute increase)	1.02 (0.95 – 1.10)	0.568
Warm ischemic time (per an minute increase)	0.91 (0.76 – 1.09)	0.299
Donor specific antibody at 1-year post-transplantation	7.63 (1.36 – 42.91)	0.021
12-month eGFR (per 10 ml/min/1.73 m ² increase)	0.96 (0.71 – 1.23)	0.779
Donor eGFR (per 10 ml/min/1.73 m ² increase)	0.61 (0.35 – 1.06)	0.080
Borderline T cell-mediated rejection	1.88 (0.73 – 4.85)	0.192

ABO incompatibility was not used as variables because of its significant correlation with the use of pre-transplantation rituximab.

Abbreviations: OR, odds ratio; CI, confidence interval; HLA, human leukocyte antigen; eGFR, estimated glomerular filtration rate.

Table 5. Banff pathologic score at 12-month post-transplantation by the stages of tertiary lymphoid tissues

12-month protocol biopsy				p value			
	No TLT	Stage I	Stage II	Overall comparison ^a	No TLT vs. stage I ^b	No TLT vs. stage II ^b	Stage I vs. stage II ^b
i	0.44 ± 0.64	0.97 ± 0.87	1.06 ± 0.94	0.029	<0.001	<0.001	0.639
t	0.12 ± 0.36	0.38 ± 0.68	0.60 ± 0.91	0.013	0.004	<0.001	0.272
v	0 ± 0	0 ± 0	0 ± 0	1.000	-	-	-
g	0.03 ± 0.16	0 ± 0	0.03 ± 0.29	0.525	-	-	-
ptc	0.08 ± 0.32	0.14 ± 0.39	0.17 ± 0.45	0.387	-	-	-
ct	0.77 ± 0.72	1.01 ± 0.62	1.29 ± 0.75	0.003	0.007	<0.001	0.079
ci	0.70 ± 0.63	0.99 ± 0.68	1.17 ± 0.92	0.050	0.008	0.008	0.390
cv	0 ± 0	0 ± 0	0 ± 0	1.000	-	-	-
cg	0 ± 0	0 ± 0	0 ± 0	1.000	-	-	-
C4d	0.63 ± 1.07	0.84 ± 1.12	0.39 ± 0.76	0.097	-	-	-

^a Kruskal-Wallis test and ^b Mann-Whiney test were used for overall and between-group comparisons, respectively.

Abbreviations: TLTs, tertiary lymphoid tissues; i, interstitial inflammation; t, tubulitis; v, intimal arteritis; g, glomerulitis; ptc, peritubular capillaritis; ct, tubular atrophy; ci, interstitial fibrosis; cv, chronic fibrous intimal thickening; cg, transplant

glomerulopathy.

Table 6. Association between the stages of tertiary lymphoid tissues and cumulative incidence rate of death-censored renal function decline and post-transplant eGFR among patients with mild interstitial inflammation in the 12-month biopsies

TLT stages at 12-month	No. of events ^a (%)	Adjusted HR ^b (95% CI)	p value	Adjusted difference in eGFR ^c (95% CI)	p value
No TLT or stage I (n=129)	22 (17.1)	Reference	-	Reference	-
Stage II (n=23)	9 (39.1)	2.60 (1.01 to 6.70)	0.048	-11.2 (-22.7 to 0.20)	0.054

^a Renal function decline was defined as a decline of at least 30% in the eGFR from 1-year post-transplant graft function.

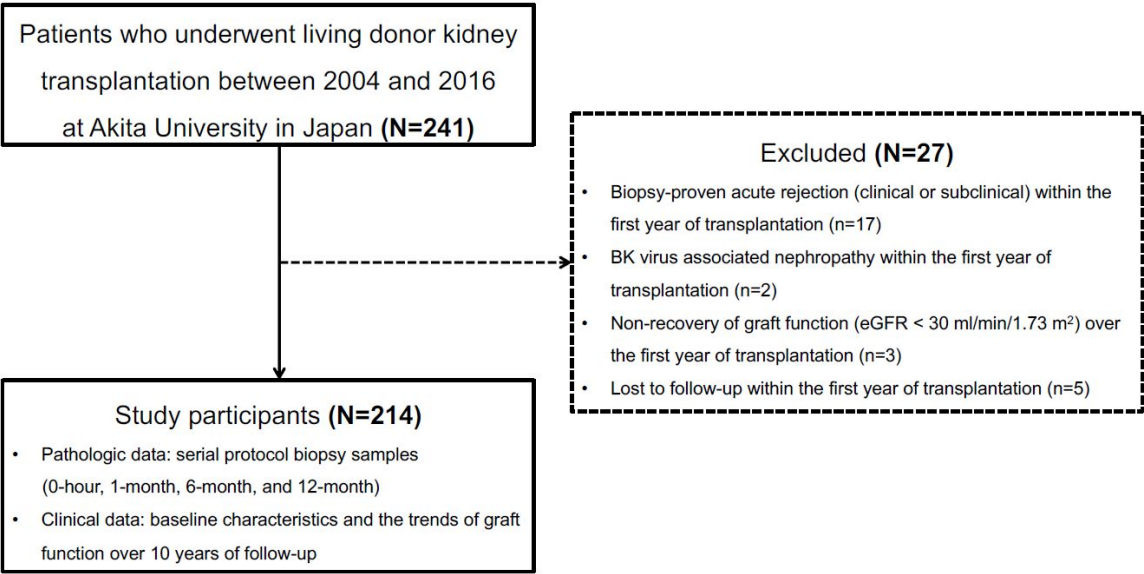
^b The comparisons between groups are performed by Cox regression analysis with multiple adjustments for confounders including age, sex, the presence of diabetes after transplantation, ABO incompatibility, positive crossmatch, the presence of donor specific antibody at 1-year post-transplantation, and pre-transplantation donor eGFR.

^c The comparisons between groups are performed by linear mixed effect models with multiple adjustments for confounders including recipient age and gender, donor age and gender, recipients body mass index, preemptive kidney transplantation, the presence of diabetes after transplantation, the number of HLA mismatching, positive crossmatch, the use of immunosuppressant, baseline graft function, the presence of donor specific antibody at 1-year post-transplantation, and pre-transplantation donor eGFR, Baseline graft function was set as 12-month eGFR levels. The differences in eGFR are calculated by comparing eGFR at baseline and eGFR at 4-5 years after kidney transplantation.

Abbreviations: eGFR, estimated glomerular filtration rate; TLT, tertiary lymphoid tissue; HR, hazard ratio; CI, confidence interval; HLA, human leukocyte antigen.

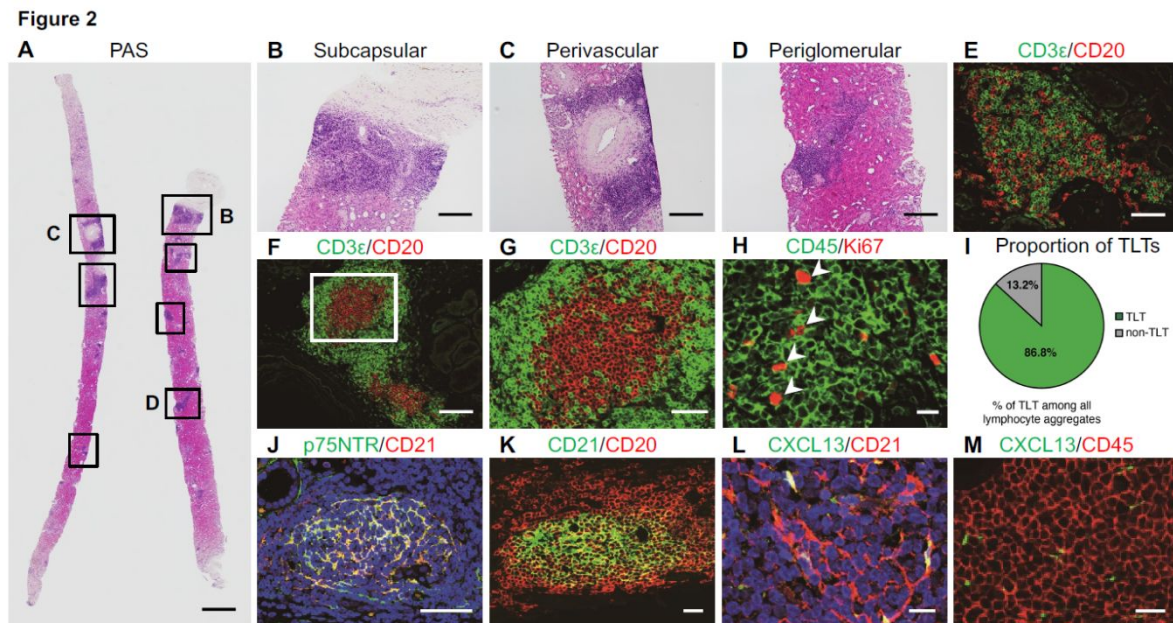
Figure 1. A flowchart of the study participant selection

Figure 1



We retrospectively screened 241 patients who underwent living donor kidney transplantation between 2004 and 2016 at Akita University in Japan. After 27 patients with known risk factors for poor graft outcome and those lost to follow-up within a year of kidney transplantation were excluded, the remaining 214 kidney transplant recipients were enrolled. Serial protocol biopsy samples were obtained and processed for immunofluorescence to determine the presence and staging of tertiary lymphoid tissues.

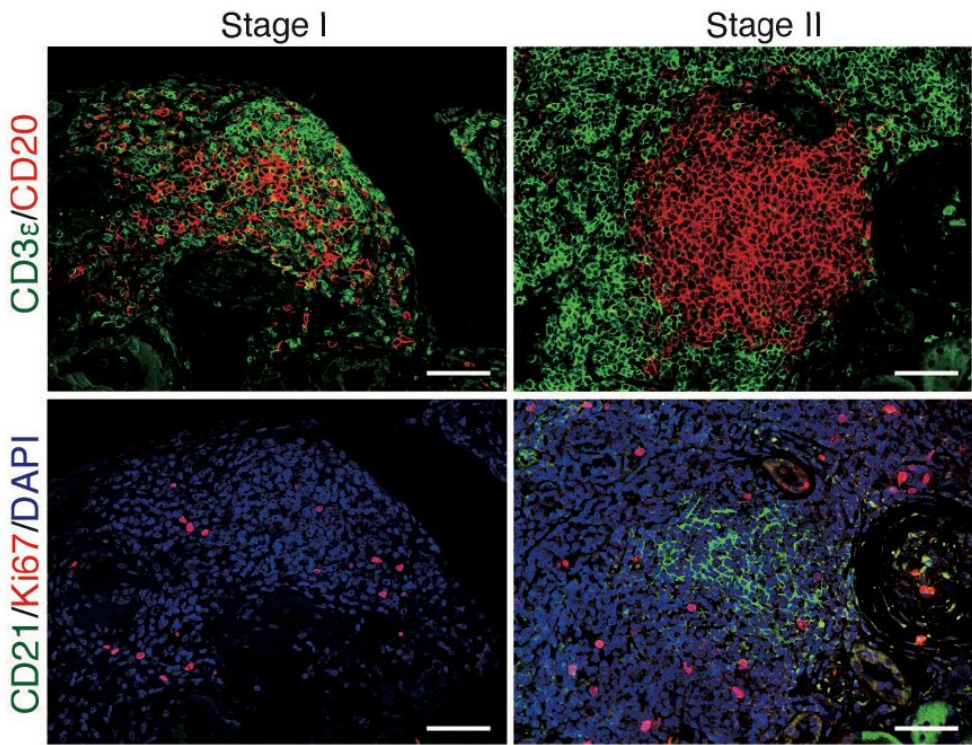
eGFR, estimated glomerular filtration rate.

Figure 2. Characterization of tertiary lymphoid tissues in transplanted kidney

(A-D) Analyses of periodic acid-Schiff (PAS)-stained graft tissues revealing multiple lymphocyte infiltrates in protocol biopsy samples, as indicated by black boxes (A). The clusters were located either (B) under the renal capsule, (C) around blood vessels, or (D) in the periglomerular area. (E-H) Immunofluorescence of (E, F, G) CD3ε (a T cell marker) and CD20 (a B cell marker); and (H) CD45 (a common leukocyte marker) and Ki67 (a proliferation marker). (I) Proportion of tertiary lymphoid tissues among lymphocyte infiltrates found in PAS-stained samples. (J-M) Immunofluorescence of (J) p75 neurotrophin receptor (p75NTR) and CD21; (K) CD21 and CD20; (L) CXCL13 and CD21; and (M) CXCL13 and CD45. Note that p75NTR and CD21 are expressed in follicular dendritic cells in tertiary lymphoid tissues, and CXCL13 is a main chemoattractant for B cells. **Figure 1G** shows a magnified view of the white box in **Figure 1F**. Arrowheads in **Figure 1H** indicate Ki67-positive proliferating lymphocytes. Scale bars; (A) 1 mm; (B-D) 200 μm; (F) 100 μm; (E, G, J, K) 50 μm; (H, L, M) 10 μm.

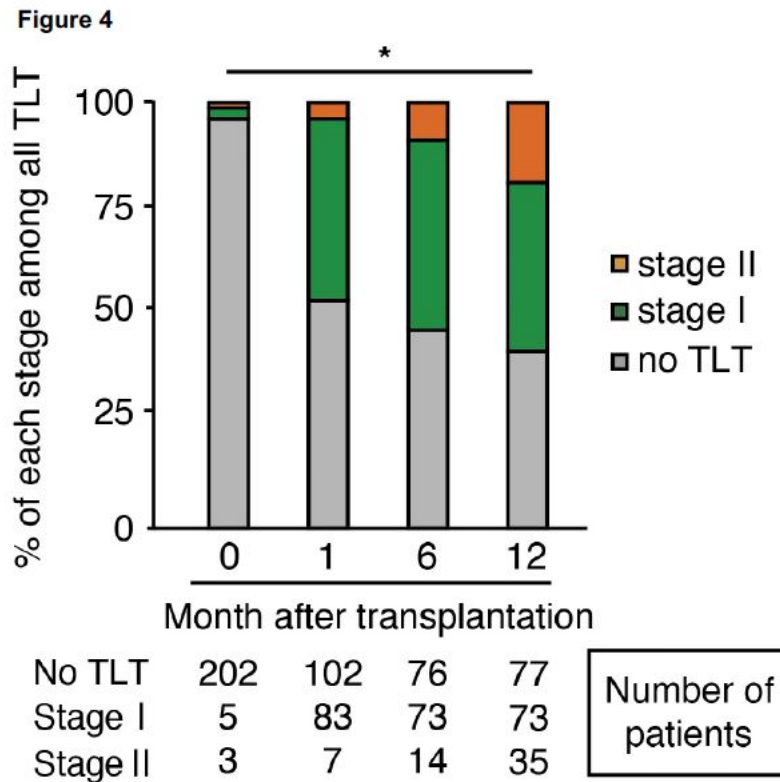
Figure 3. The staging of tertiary lymphoid tissues in transplanted kidney

Figure 3



Representative immunofluorescence of different TLT stages as determined by the expression patterns of CD3ε, CD20, CD21, and Ki67. Stage I TLTs were defined by the presence of lymphocyte clusters (CD3ε⁺ and CD20⁺) with signs of proliferation (Ki67⁺), and the absence of FDC (CD21⁻). Stage II TLTs were defined by the presence of lymphocyte clusters (CD3ε⁺ and CD20⁺) with signs of proliferation (Ki67⁺) along with the presence of FDCs (CD21⁺). TLTs, tertiary lymphoid tissues; FDCs, follicular dendritic cells.

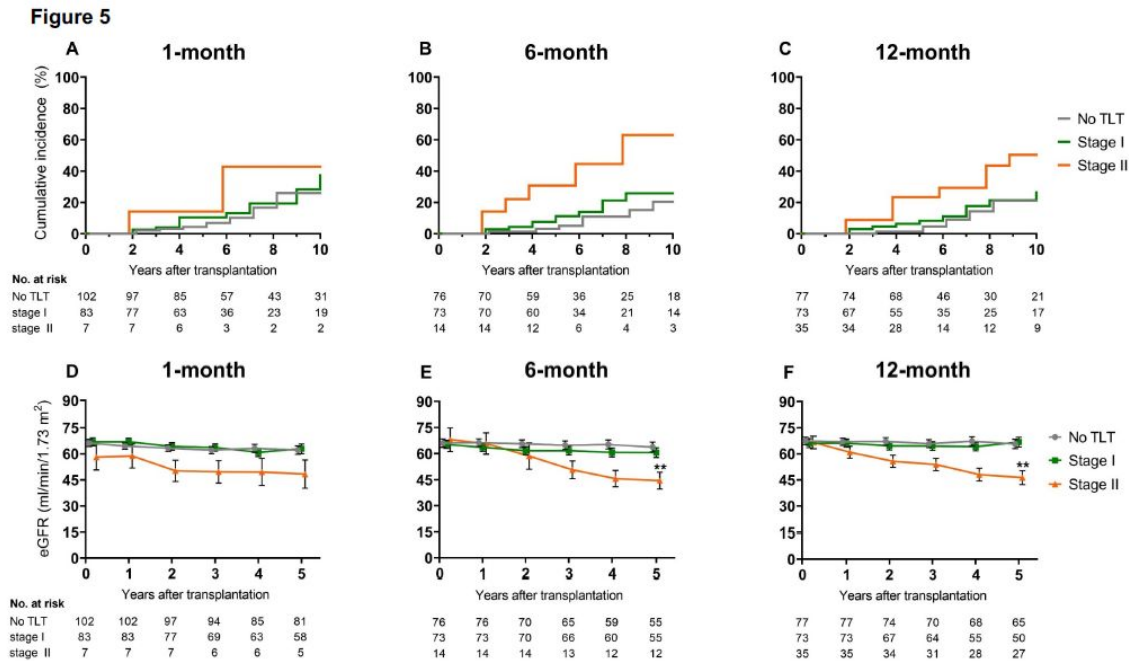
Scale bars; 100 μm.

Figure 4. The prevalence of tertiary lymphoid tissues in transplanted kidney

Relative frequency of patients with no TLT (gray), stage I TLTs (green), and stage II TLTs (orange) at various time points after kidney transplantation. The overall prevalence of TLTs increased from 3.8% at 0-hour baseline to 46.9% at 1 month after kidney transplantation, and then slightly further during 12 months. By contrast, stage II TLTs exhibited a more gradual increase in prevalence, reaching 18.9% at 12 months post-transplantation. * $p < 0.001$ by trends analysis

TLTs, tertiary lymphoid tissues.

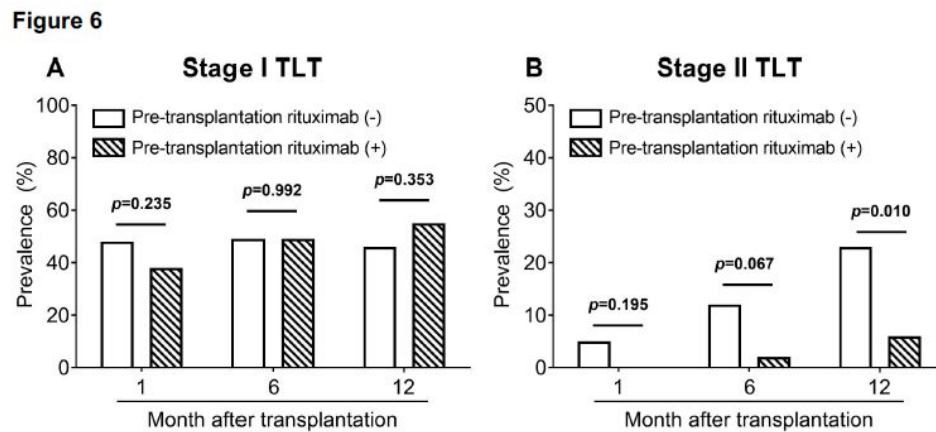
Figure 5. Renal allograft outcomes according to the staging of tertiary lymphoid tissues



(A-C) The cumulative incidence rate of death-censored renal function decline and (D-F) the longitudinal trends of eGFR after kidney transplantation according to the stages of TLTs at given time points. One-month, 6-month, and 12-month refer to biopsy time points after kidney transplantation. Patients with stage II TLTs at 6- or 12-month experienced significantly accelerated graft dysfunction compared to those without TLT. The statistical comparisons between groups are performed by (A-C) Cox regression analysis and (D-F) linear mixed effect models with multiple adjustments, and their results are shown in **Table 2 and 3**, respectively. (D-F) Data are expressed as mean $\pm$ standard error for each time point of follow-up. ****** $p < 0.005$, vs. No TLT.

eGFR, estimated glomerular filtration rate; TLTs, tertiary lymphoid tissues.

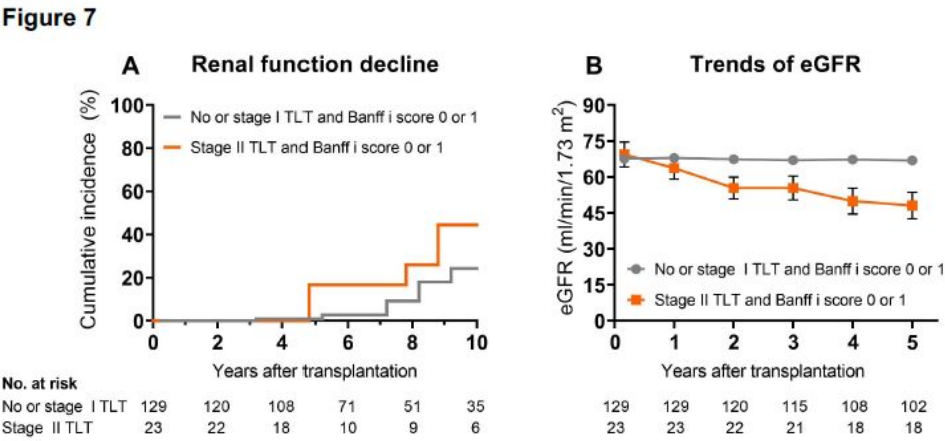
Figure 6. The effects of pre-transplantation rituximab on the prevalence of tertiary lymphoid tissues



Prevalence of (A) stage I and (B) stage II TLTs at each time point of follow-up according to the use of pre-transplantation rituximab. The administration of rituximab before kidney transplantation was associated with a lower prevalence of stage II TLTs, but not with the change in stage I TLTs regardless of biopsy time point.

TLT, tertiary lymphoid tissues.

Figure 7. Renal allograft outcomes according to the staging of tertiary lymphoid tissues among patients with mild interstitial inflammation in the 12-month biopsies



(A) The cumulative incidence rate of death-censored renal function decline and (B) eGFR over a period of 5 years after kidney transplantation according to the stages of TLTs in recipients with mild interstitial inflammation (Banff i score of 0 or 1). Patients with stage II TLTs in the 12-month biopsies experienced progressive declines in renal function even though the degree of overall interstitial inflammation was trivial. The statistical comparisons between groups are performed by (A) Cox regression analysis and (B) linear mixed effect models with multiple adjustments, and their results are shown in **Table 6**. (B) Data are expressed as mean $\pm$ standard error for each time point of follow-up.

eGFR, estimated glomerular filtration rate; TLT, tertiary lymphoid tissues.

Advanced Tertiary Lymphoid Tissues in Protocol Biopsies are Associated with Progressive Graft Dysfunction in Kidney Transplant Recipients

JASN
JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY

METHODS

Longitudinal retrospective cohort
Recruited in 2004–2016

Study participants (N=214)

- ✓ Living donor-related kidney transplant
- ✓ No clinical or subclinical rejection within first year after transplantation

Data acquisitions

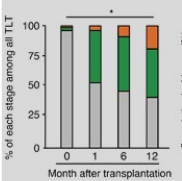
- ✓ Protocol biopsy samples (obtained at 0-, 1-, 6, and 12-month post-transplantation)
- ✓ 5-year trends of renal allograft function

Tertiary lymphoid tissue (TLT) staging

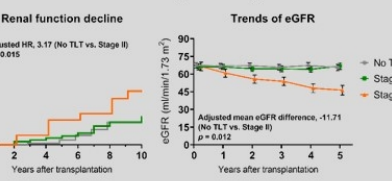
- ✓ Stage I: presence of lymphocyte aggregates with signs of proliferation
- ✓ Stage II: presence of follicular dendritic cells

OUTCOME

Prevalence of TLT



12-month TLT stages and graft functions



Pre-transplant rituximab



Stage II TLT ↓
Odd ratio: 0.17
(95% CI 0.04 – 0.72)

TLT stages and Banff scores

12-month Banff score	12-month biopsies			p value (No TLT vs. stage II)
	No TLT	Stage I	Stage II	
t	0.12 ± 0.36	0.38 ± 0.68	0.60 ± 0.91	<0.001
ct	0.77 ± 0.72	1.01 ± 0.62	1.29 ± 0.75	<0.001
ci	0.70 ± 0.63	0.99 ± 0.68	1.17 ± 0.92	0.008

Conclusion

TLTs are commonly found in clinically stable transplanted kidneys and advanced stage II TLTs are associated with progressive graft dysfunction after kidney transplantation.

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SUPPLEMENTAL APPENDIX FOR THE STUDY:

Advanced tertiary lymphoid tissues in protocol biopsies are associated with progressive

graft dysfunction in kidney transplant recipients

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Supplemental Figure 8. Association between the stages of tertiary lymphoid tissues and interstitial inflammation scores

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Supplemental Table 1. Baseline characteristics and clinical parameters of patients according to ABO incompatibility

	ABO-compatible	ABO-incompatible	<i>p</i> value
Number of patients	161	53	
Age (year)	47.0 ± 12.7	54.4 ± 10.1	<0.001
Gender (Male, %)	106 (65.8)	33 (62.3)	0.636
Body mass index (kg/m ²)	22.5± 3.7	22.1 ± 3.8	0.487
Etiology of end-stage renal disease (n, %)			
Chronic glomerulonephritis	91 (56.5)	31 (58.5)	0.902
Diabetes mellitus	28 (17.4)	8 (15.1)	
Hypertension	11 (6.8)	5 (9.4)	
Polycystic kidney disease	10 (6.2)	4 (7.5)	
Others ^a	21 (13.0)	5 (9.5)	
Time on dialysis (month)	17.0 [4.0, 49.0]	21.0 [5.5, 51.5]	0.346
Preemptive kidney transplantation (n, %)	33 (20.5)	7 (13.2)	0.238
Number of HLA mismatching (n)	3.2 ± 1.5	3.5 ± 1.4	0.139
Positive crossmatch (n, %)	10 (6.2)	4 (7.5)	0.752
Pre-transplantation rituximab (n, %)	7 (4.3)	50 (94.3)	<0.001
Cold ischemic time (minute)	142.7 ± 33.0	147.9 ± 49.2	0.388
Warm ischemic time (minute)	5.1 ± 4.2	4.8 ± 1.4	0.600
Induction immunosuppressant (n, %)			
Basiliximab	161 (100)	53 (100)	1.000
Maintenance immunosuppressant ^b (n, %)			
Prednisolone	127 (78.9)	44 (83.0)	0.514
Tacrolimus	161 (100)	53 (100)	1.000
Mycophenolate mofetil	161 (100)	53 (100)	1.000
Borderline T cell-mediated rejection ^c (n, %)	48 (29.8)	18 (34.0)	0.571
Donor specific antibody at 1-year post-transplantation (n, %) ^d	10/158 (6.3)	3/49 (6.1)	1.000
Class I	5 (50.0)	2 (66.7)	1.000
Class II	5 (50.0)	2 (66.7)	1.000

Posttransplant eGFR (ml/min/1.73 m ²)			
1-month	65.1 ± 20.7	69.7 ± 20.0	0.155
1-year	65.1 ± 18.9	65.4 ± 18.6	0.918
5-year	61.2 ± 22.3	65.9 ± 18.7	0.262
Donor age (year)	58.1 ± 9.9	59.2 ± 9.7	0.453
Donor gender (Male, %)	62 (38.5)	20 (37.7)	0.920
Pre-transplantation donor eGFR (ml/min/1.73 m ²)	103.1 ± 10.8	102.6 ± 10.8	0.743

Data were expressed as mean ± standard deviation or the number of patients (percentage), and were compared by independent t-tests, chi-square tests, or Fisher's exact tests. Time on dialysis was described as median [1st and 3rd interquartile range] and was compared by Mann–Whitney test because it was non-normally distributed.

^a Others include chronic tubulointerstitial nephritis, gestational hypertension, vesicoureteral reflux disease, sepsis, cystinuria, and bone marrow transplant nephropathy.

^b Data obtained at the time of outpatient visit 1 year after kidney transplantation.

^c At least one episode during the first year after transplantation.

^d Not assessed in 7 recipients.

Abbreviation: HLA, human leukocyte antigen; eGFR, estimated glomerular filtration rate.

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Supplemental Table 2. Baseline characteristics, clinical parameters, and pathologic classifications of patients who experienced acute rejection within the first year post-transplantation

Number of patients	17
Age (year)	50.6 ± 8.2
Gender (Male, %)	8 (47.1)
Body mass index (kg/m ²)	23.2 ± 5.0
Etiology of end-stage renal disease (n, %)	
Chronic glomerulonephritis	14 (82.4)
Diabetes mellitus	2 (11.8)
Polycystic kidney disease	1 (5.9)
Time on dialysis (month)	26.0 [12.0, 54.0]
Preemptive kidney transplantation (n, %)	3 (17.6)
Number of HLA mismatching (n)	3.3 ± 1.5
Positive crossmatch (n, %)	4 (23.5)
ABO-incompatible kidney transplantation (n, %)	6 (35.3)
Pre-transplantation rituximab (n, %)	9 (52.9)
Cold ischemic time (minute)	171.6 ± 40.6
Warm ischemic time (minute)	4.5 ± 1.7
Induction immunosuppressant (n, %)	
Basiliximab	17 (100)
Maintenance immunosuppressant ^a (n, %)	
Prednisolone	15 (88.2)
Tacrolimus	17 (100)
Mycophenolate mofetil	17 (100)
Donor specific antibody at 1-year post-transplantation (n, %)	5 (29.4)
Class I	4 (23.5)
Class II	2 (11.8)
Posttransplant eGFR (ml/min/1.73 m ²)	
1-month	58.0 ± 17.1
1-year	54.3 ± 24.0
5-year	55.5 ± 18.3

Donor age (year)	56.2 ± 15.0
Donor gender (Male, %)	9 (52.9)
Pre-transplantation donor eGFR (ml/min/1.73 m ²)	101.1 ± 17.2
Rejection type (n, %)	
Acute T cell-mediated rejection, IA	3 (17.6)
Acute T cell-mediated rejection, IB	1 (5.9)
Acute antibody-mediated rejection	13 (76.5)

Data were expressed as mean ± standard deviation or the number of patients (percentage). Time on dialysis was described as median [1st and 3rd interquartile range].

^aData obtained at the time of outpatient visit 1 year after transplantation.

Abbreviation: HLA, human leukocyte antigen; eGFR, estimated glomerular filtration rate.

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Supplemental Table 3. Hazard ratios of the stages of tertiary lymphoid tissues for death-censored renal function decline in subgroup of recipients who underwent ABO-compatible kidney transplantation

Biopsy time point	TLT stages	No. of events ^a (%)	Adjusted HR ^b (95% CI)	<i>p</i> value
1-month	No TLT (n=72)	13 (18.1)	Reference	-
	Stage I (n=62)	14 (22.6)	1.24 (0.57 to 2.71)	0.594
	Stage II (n=7)	3 (42.9)	3.15 (0.82 to 12.09)	0.094
6-month	No TLT (n=55)	7 (12.7)	Reference	-
	Stage I (n=51)	10 (19.6)	1.12 (0.40 to 3.16)	0.827
	Stage II (n=13)	6 (46.2)	3.97 (1.15 to 13.76)	0.030
12-month	No TLT (n=60)	10 (16.7)	Reference	-
	Stage I (n=49)	7 (14.3)	0.72 (0.25 to 2.11)	0.723
	Stage II (n=33)	14 (42.4)	2.81 (1.05 to 7.51)	0.039

^a Renal outcome was defined as a decline of at least 30% in the eGFR from 1-year post-transplant graft function.

^b The comparisons between groups are performed by Cox regression analysis with multiple adjustments for confounders including age, sex, the presence of diabetes after transplantation, positive crossmatch, the presence of donor specific antibody at 1-year post-transplantation, and pre-transplantation donor eGFR.

Abbreviation: TLT, tertiary lymphoid tissue; HR, hazard ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate.

Supplemental Table 4. Association between the stages of tertiary lymphoid tissues and graft function in subgroup of recipients who underwent ABO-compatible kidney transplantation

Biopsy time point	TLT stages	Adjusted difference in eGFR ^a (95% CI)	<i>p</i> value
1-month	No TLT (n=72)	Reference	-
	Stage I (n=62)	3.03 (-3.45 to 9.50)	0.359
	Stage II (n=7)	-12.04 (-31.11 to 7.04)	0.216
6-month	No TLT (n=55)	Reference	-
	Stage I (n=51)	-2.92 (-9.89 to 4.05)	0.412
	Stage II (n=13)	-14.18 (-25.67 to -2.69)	0.016
12-month	No TLT (n=60)	Reference	-
	Stage I (n=49)	-1.75 (-7.12 to 3.61)	0.522
	Stage II (n=33)	-13.26 (-23.26 to -3.25)	0.009

^a The comparisons between groups are performed by linear mixed effect models with multiple adjustments for confounders including recipient age and gender, donor age and gender, recipients body mass index, preemptive kidney transplantation, the presence of diabetes after transplantation, the number of HLA mismatching, positive crossmatch, the use of immunosuppressant, baseline graft function, the presence of donor specific antibody at 1-year post-transplantation, and pre-transplantation donor eGFR. Baseline graft function was set as eGFR levels for each time point. The differences in eGFR are calculated by comparing eGFR at baseline and eGFR at 4-5 years after kidney transplantation.

Abbreviation: TLT, tertiary lymphoid tissue; eGFR, estimated glomerular filtration rate; CI, confidence interval; HLA, human leukocyte antigen.

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Supplemental Table 5. Multivariable analyses of risk factors for the development of stage I tertiary lymphoid tissues in 12-month biopsies

Variables	OR (95% CI)	<i>p</i> value
Recipient age (per 10-year increase)	1.08 (0.79 – 1.48)	0.631
Donor age (per 10-year increase)	0.69 (0.57 – 1.46)	0.688
Recipient gender (male)	2.09 (0.86 – 5.06)	0.103
Donor gender (male)	0.82 (0.37 – 1.82)	0.622
Body mass index (per 1 kg/m ² increase)	1.07 (0.97 – 1.19)	0.191
Diabetes mellitus	1.20 (0.53 – 2.68)	0.665
Number of HLA mismatching (per one mismatch increase)	0.96 (0.74 – 1.25)	0.767
Positive crossmatch	0.10 (0.01 – 1.01)	0.051
Pre-transplantation rituximab	1.76 (0.81 – 3.80)	0.151
Steroid maintenance therapy at 1-year post-transplantation	2.62 (1.01 – 6.82)	0.048
Cold ischemic time (per ten-minute increase)	1.08 (1.01 – 1.15)	0.031
Warm ischemic time (per an minute increase)	1.08 (0.97 – 1.20)	0.173
Donor specific antibody at 1-year post-transplantation	0.34 (0.06 – 2.10)	0.246
12-month eGFR (per 10 ml/min/1.73 m ² increase)	0.96 (0.77 – 1.21)	0.729
Donor eGFR (per 10 ml/min/1.73 m ² increase)	1.33 (0.86 – 2.08)	0.203
Borderline T cell-mediated rejection	2.05 (0.97 – 4.34)	0.060

ABO incompatibility was not used as variables because of its significant correlation with the use of pre-transplantation rituximab.

Abbreviation: OR, odds ratio; CI, confidence interval; HLA, human leukocyte antigen; eGFR, estimated glomerular filtration rate.

Supplemental Table 6. The stages of tertiary lymphoid tissues according to ABO compatibility

	TLT stages	ABO-compatible	ABO-incompatible	<i>p</i> value
1-month (n=192)	No TLT	72 (51.1)	30 (58.8)	0.172
	Stage I	62 (44.0)	21 (41.2)	
	Stage II	7 (5.0)	0 (0)	
6-month (n=163)	No TLT	55 (46.2)	21 (47.7)	0.368
	Stage I	51 (42.9)	22 (50.0)	
	Stage II	13 (10.9)	1 (2.3)	
12-month (n=185)	No TLT	60 (42.3)	17 (39.5)	0.222
	Stage I	49 (34.5)	24 (55.8)	
	Stage II	33 (23.2)	2 (4.7)	

Data were expressed as the number of patients (percentage), and were compared by trend analysis.

Abbreviation: TLT, tertiary lymphoid tissue.

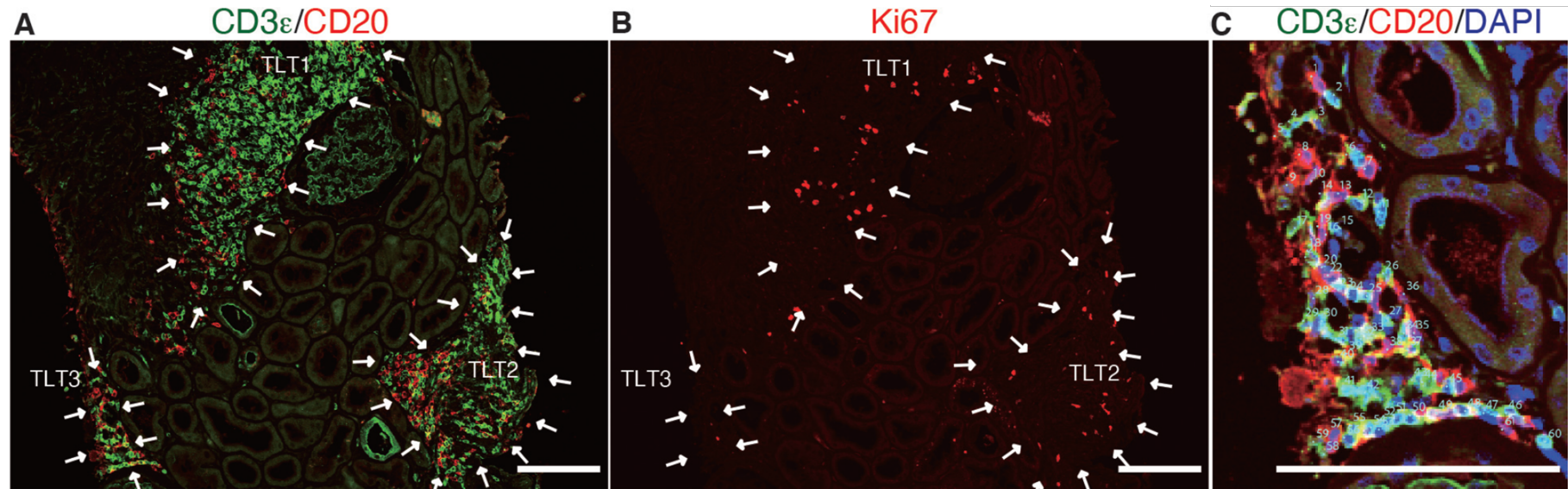
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Supplemental Table 7. Banff pathologic score at 12-month post-transplantation according to ABO compatibility

12-month Banff scores	ABO-compatible	ABO-incompatible	<i>p</i> value
i	0.68 ± 0.83	0.86 ± 0.81	0.104
t	0.27 ± 0.61	0.40 ± 0.67	0.091
v	0 ± 0	0 ± 0	1.000
g	0.01 ± 0.11	0.02 ± 0.14	0.697
ptc	0.11 ± 0.35	0.10 ± 0.36	0.782
ct	0.93 ± 0.69	1.04 ± 0.70	0.282
ci	0.81 ± 0.74	0.96 ± 0.70	0.150
cv	0.02 ± 0.24	0 ± 0	0.576
cg	0 ± 0	0 ± 0	1.000
C4d	0.27 ± 0.65	1.93 ± 1.09	<0.001

Data were expressed as mean ± standard deviation, and were compared by Kruskal-Wallis test. Abbreviation: i, interstitial inflammation; t, tubulitis; v, intimal arteritis; g, glomerulitis; ptc, peritubular capillaritis; ct, tubular atrophy; ci, interstitial fibrosis; cv, chronic fibrous intimal thickening; cg, transplant glomerulopathy.

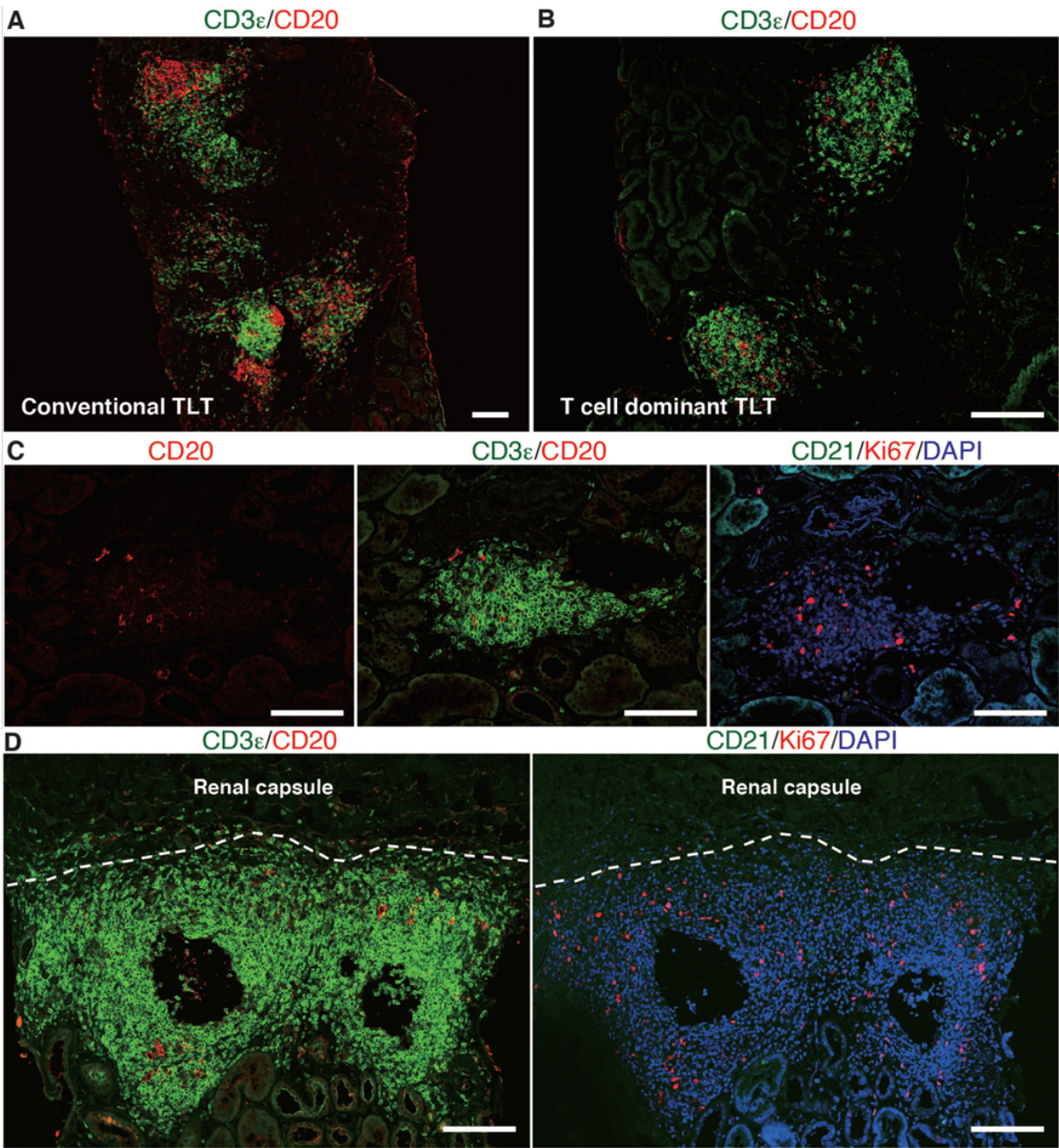
Supplemental Figure 1. Variations in the sizes of tertiary lymphoid tissues in transplanted kidneys



Immunofluorescence of (A, C) CD3ε and CD20 and (B) Ki67 in transplanted kidneys. **Supplementary Figure 1C** shows a magnified view of TLT3 in **Supplementary Figure 1A**. Scale bars: 100 μm.

Abbreviation: TLT, tertiary lymphoid tissues.

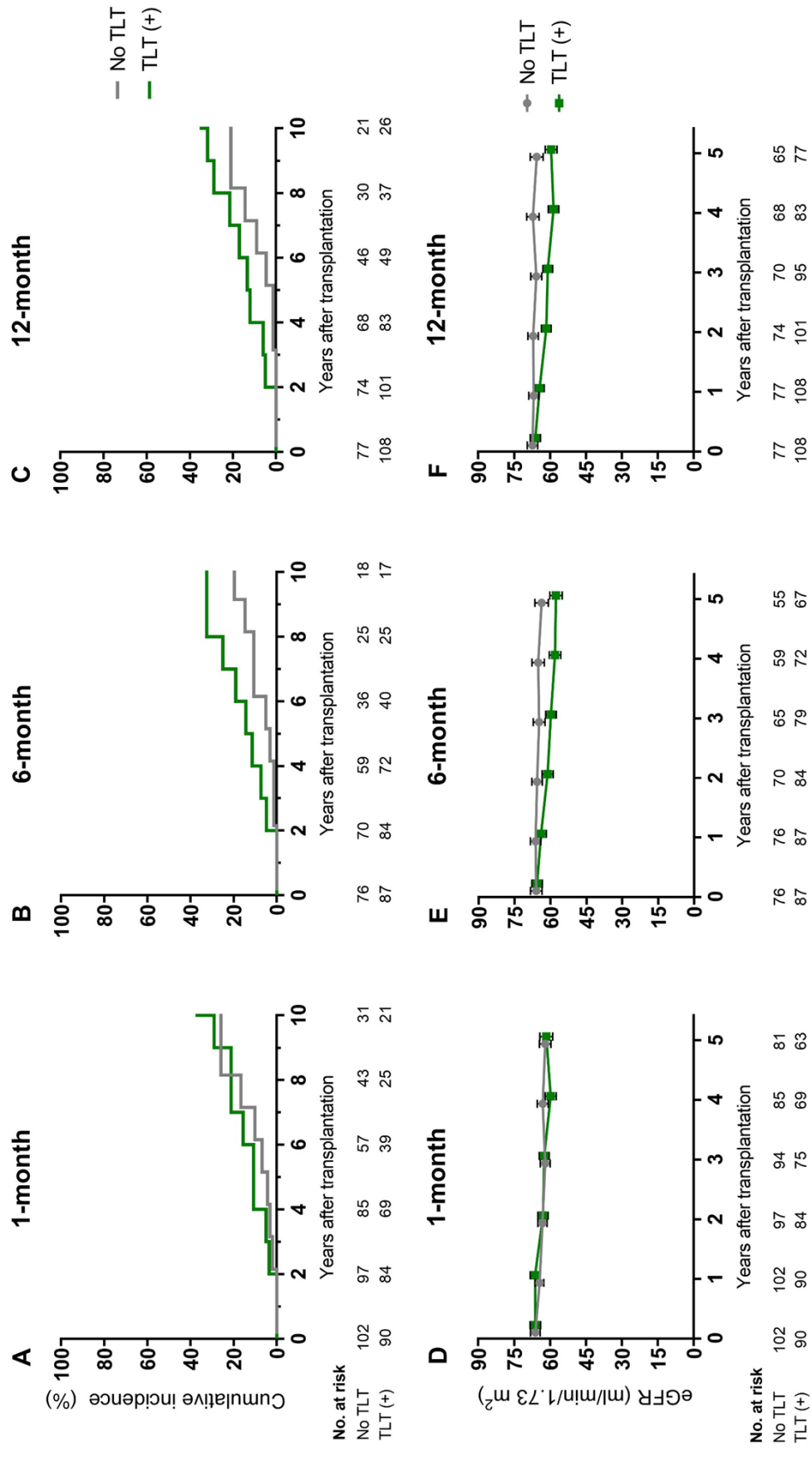
Supplemental Figure 2. T cell-dominant tertiary lymphoid tissues in transplanted kidneys treated with pre-transplantation rituximab



(A-D) Immunofluorescence of (A-B) CD3ε and CD20 and (C-D) CD3ε and CD20, CD21 and Ki67 in transplanted kidneys. Serial sections were stained in (C-D). Scale bars: (A, B, D) 100 μm, (C) 50 μm.

Abbreviation: TLT, tertiary lymphoid tissues.

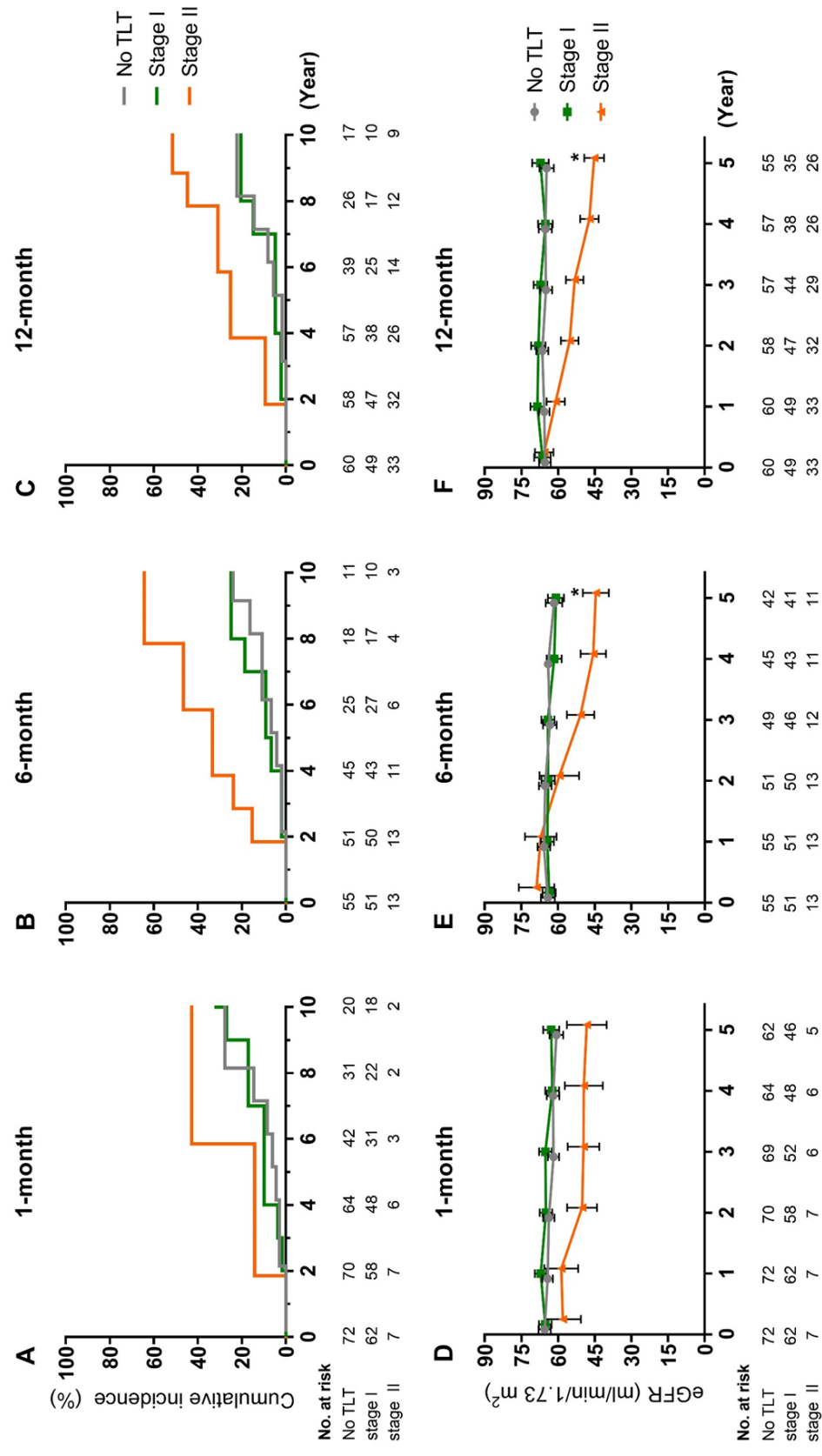
Supplemental Figure 3. Renal allograft outcomes according to the presence of tertiary lymphoid tissues



(A-C) The cumulative incidence rate of death-censored renal function decline and (D-F) the longitudinal trends of eGFR after kidney transplantation according to the presence of TLTs at given time points. One-month, 6-month, and 12-month refer to biopsy time points after kidney transplantation. Post-transplant graft function was not significantly influenced by the qualitative presence or absence of TLTs. (A-C) Adjusted hazard ratios of 1.58 (95% confidence interval [CI]: 0.82-3.06, $p = 0.175$), 1.87 (95% CI: 0.83-4.22, $p = 0.132$), and 1.79 (95% CI: 0.84-3.80, $p = 0.130$) at the 1-, 6-, 12-month biopsies by multivariate Cox regression analysis, respectively. (D-F) Data are expressed as mean $\pm$ standard error for each time point of follow-up.

Abbreviation: eGFR, estimated glomerular filtration rate; TLTs, tertiary lymphoid tissues.

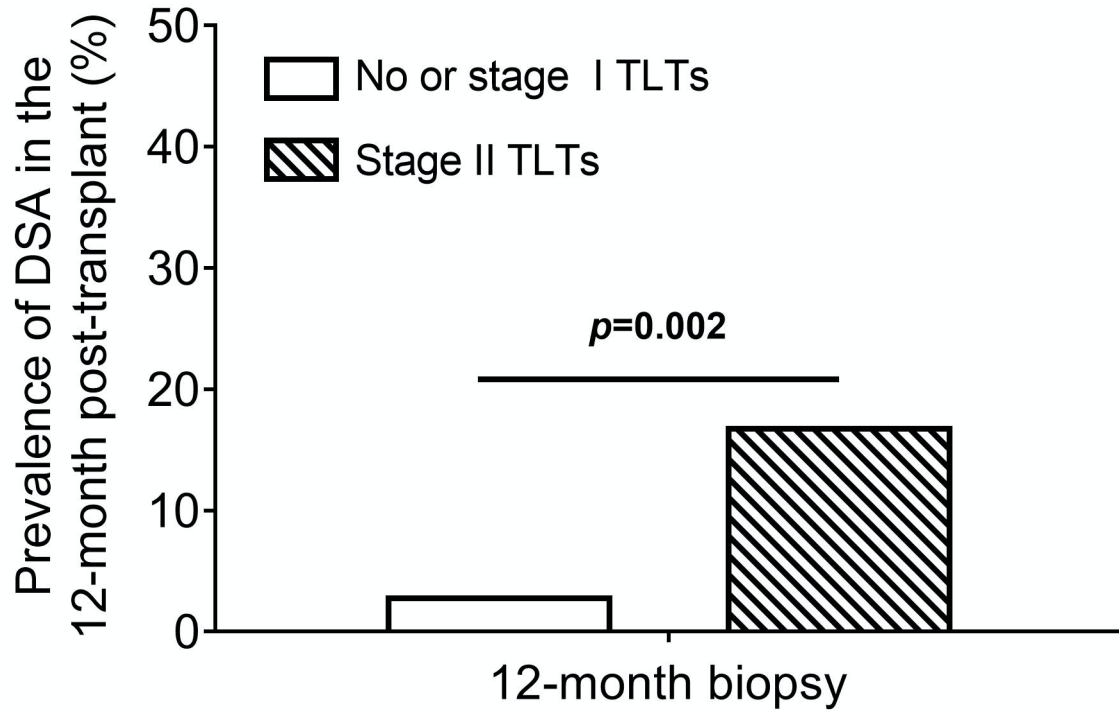
Supplemental Figure 4. Renal allograft outcomes according to the stages of tertiary lymphoid tissues in subgroup of recipients who underwent ABO-compatible kidney transplantation



(A-C) The cumulative incidence rate of death-censored renal function decline and (D-F) the longitudinal trends of eGFR after kidney transplantation according to the stages of TLTs at given time points in subgroup of recipients who underwent ABO-compatible kidney transplantation. One-month, 6-month, and 12-month refer to biopsy time points after kidney transplantation. The overall trends are consistent with the main results (**Figure 5**); patients with stage II TLTs at 6- or 12-month had significantly higher risk of renal function decline compared to those without TLT. The comparisons between groups are performed by (A-C) Cox regression analysis and (D-F) linear mixed effect models with multiple adjustments, and their results are shown in **Supplemental Table 3 and 4**, respectively. $*p < 0.05$, vs. No TLT. (D-F) Data are expressed as mean $\pm$ standard error for each time point of follow-up.

Abbreviation: eGFR, estimated glomerular filtration rate; TLTs, tertiary lymphoid tissues.

Supplemental Figure 5. The prevalence of donor-specific antibody at 12 months after kidney transplantation according to the stages of tertiary lymphoid tissues

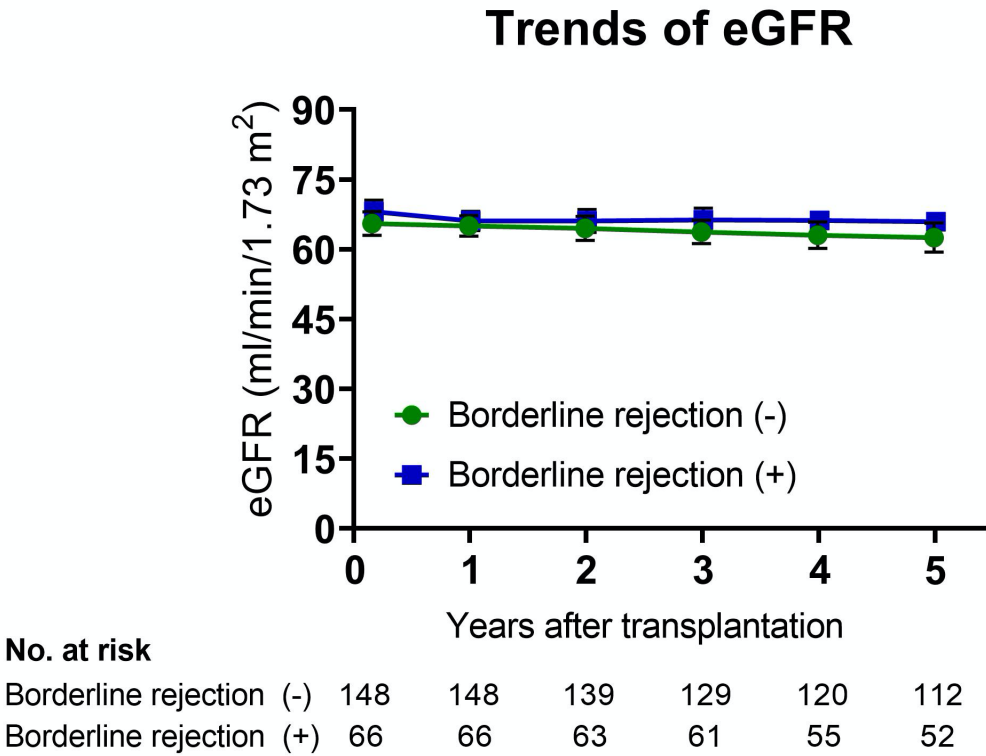


Patients with stage II TLTs at 12-month biopsies more frequently exhibited donor-specific antibodies at 12 months post-transplantation than those with no or stage I TLTs ($p = 0.002$ by chi-square test).

Abbreviation: DSA, donor-specific antibody; TLTs, tertiary lymphoid tissues.

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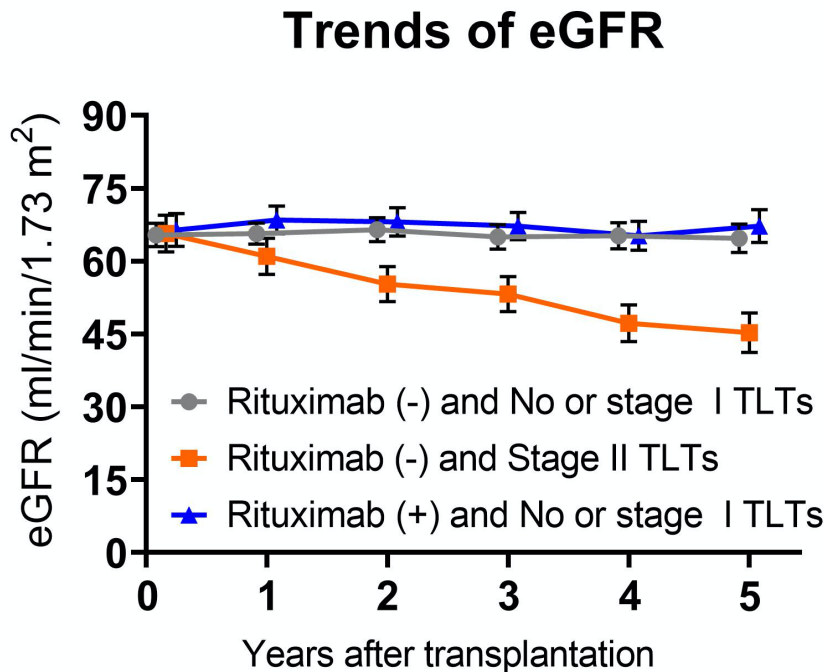
Supplemental Figure 6. Longitudinal trends of renal allograft function according to the history of borderline T cell-mediated rejection within the first year after kidney transplantation



A total of 66 kidney transplantation recipients were diagnosed with borderline T cell-mediated rejection within the first year of post-transplantation and most of them (63/66, 95.5%) were treated with steroid pulse therapy. These patients showed similar levels of graft function over the next 5 years after transplantation compared to those without borderline rejection. Data are expressed as mean ± standard error for each time point of follow-up.

Abbreviation: eGFR, estimated glomerular filtration rate.

Supplemental Figure 7. Longitudinal trends of renal allograft function according to the use of pre-transplantation rituximab and the stages of tertiary lymphoid tissues in the 12-month biopsies



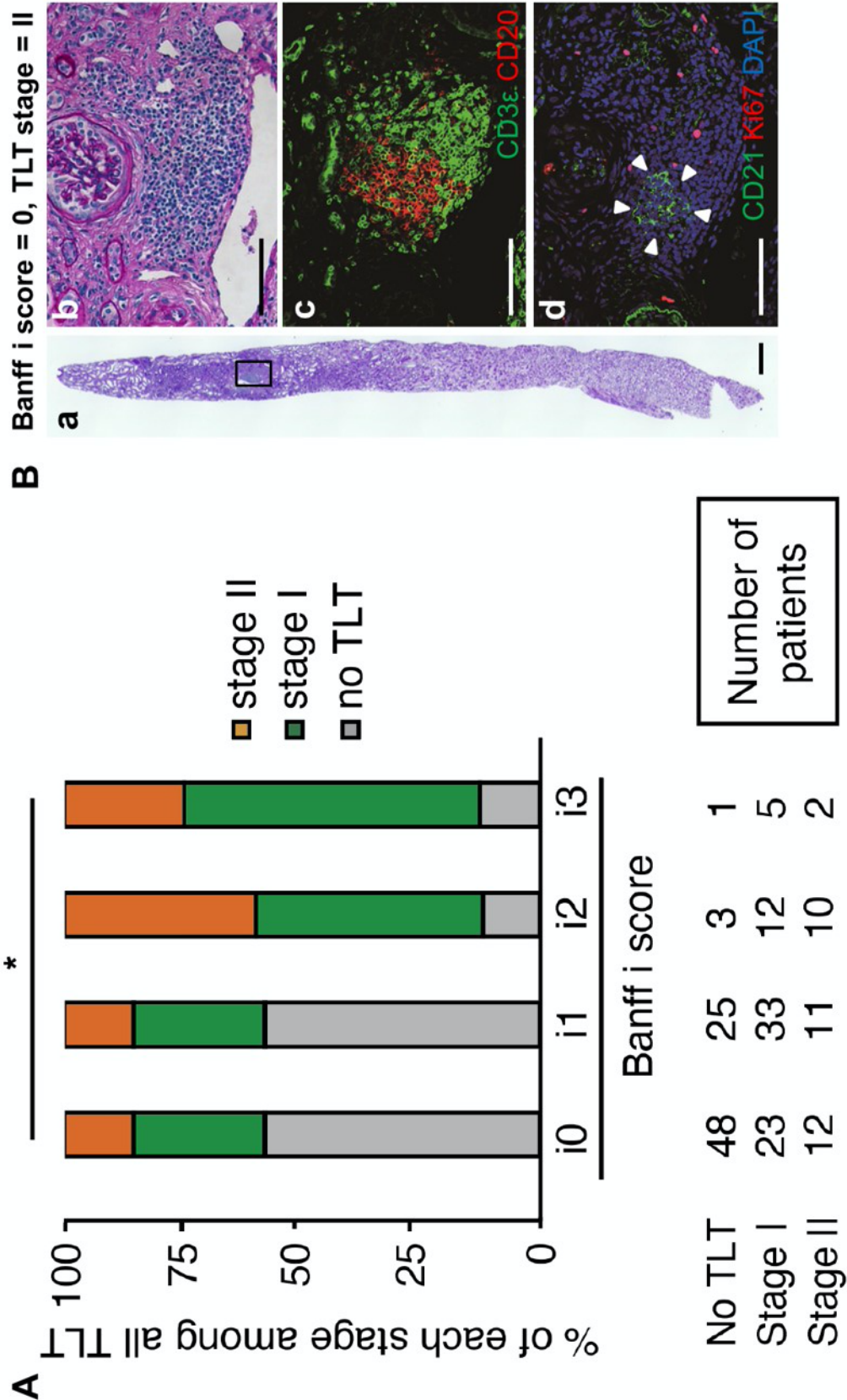
No. at risk

RTX (-), No/stage I	106	106	102	98	92	87
RTX (-), stage II	32	32	31	28	25	25
RTX (+), No/stage I	54	54	50	47	41	35

Patients treated with rituximab before transplantation showed stable graft function over 5 years after transplantation comparable to those with no or stage I TLTs not treated with rituximab. Data are expressed as mean $\pm$ standard error for each time point of follow-up. Note that the trends of eGFR of rituximab-treated patients exhibiting stage II TLTs are not shown because of their small number (n=3).

Abbreviation: eGFR, estimated glomerular filtration rate; TLT, tertiary lymphoid tissue; RTX, rituximab.

Supplemental Figure 8. Association between the stages of tertiary lymphoid tissues and interstitial inflammation scores



(A) The proportion of the stages of tertiary lymphoid tissues versus Banff i scores in 12-month biopsies. (B) Representative images of stage II tertiary lymphoid tissues in graft tissues with Banff i score of 0 (interstitial inflammation in less than 10% of parenchyma). Arrowheads indicate CD21-positive follicular dendritic cells.

* $p < 0.001$ by trends analysis.

Scale bars; (a) 500 μm ; (b, c, d) 100 μm .

Abbreviation: TLTs, tertiary lymphoid tissues.