Higher infiltration by Th17 cells compared with regulatory T cells is associated with severe acute T-cell-mediated graft rejection

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Abbreviations: AAMR, acute antibody mediated rejection; ah, arteriolar hyaline thickening; ATCMR, acute T cell mediated rejection; cg, glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, chronic vascular change; eGFR, estimated glomerular filtration rate; g, glomerulitis; i, interstitial inflammation; IF/TA, interstitial fibrosis/tubular atrophy; IHC, immunohistochemistry; MDRD, modified diet renal disease; Scr, serum creatinine; t, tubulitis; Treg, FOXP3 positive regulatory T cell; v, vasculopathy

Abstract

The aim of this study was to evaluate whether the Th17 and Treg cell infiltration into allograft tissue is associated with the severity of allograft dysfunction and tissue injury in acute T cell-mediated rejection (ATCMR). Seventy-one allograft tissues with biopsy-proven ATCMR were included. The biopsy specimens were immunostained for FOXP3 and IL-17. The allograft func-

tion was assessed at biopsy by measuring serum creatinine (Scr) concentration, and by applying the modified diet in renal disease (MDRD) formula, which provides the estimated glomerular filtration rate (eGFR). The severity of allograft tissue injury was assessed by calculating tissue injury scores using the Banff classification. The average numbers of infiltrating Treg and Th17 cells were 11.6 ± 12.2 cells/mm² and 5.6 ± 8.0 cells/mm², respectively. The average Treg/Th17 ratio was 5.6 ± 8.2 . The Treg/Th17 ratio was significantly associated with allograft function (Scr and MDRD eGFR) and with the severity of interstitial injury and tubular injury ($P < 0.05$, all parameters). In separate analyses of the number of infiltrating Treg and Th17 cells, Th17 cell infiltration was significantly associated with allograft function and the severity of tissue injury. By contrast, Treg cell infiltration was not significantly associated with allograft dysfunction or the severity of tissue injury. The results of this study show that higher infiltration of Th17 cell compared with Treg cell is significantly associated with the severity of allograft dysfunction and tissue injury.

Keywords: FOXP3 protein, human; graft rejection; interleukin-17; Th17 cells; T-lymphocytes, regulatory; transplantation, homologous

Introduction

FOXP3⁺ regulatory T (Treg) cells play an important role in suppressing immune responses in various immune disorders (Hori *et al.*, 2003; Allan *et al.*, 2005) and are therefore expected to have beneficial effects by suppressing alloimmune responses in kidney transplantation. In one study, a high number of FOXP3⁺ Treg cells was associated with preserved allograft function or less severe interstitial inflammation (Taflin *et al.*, 2010). By contrast, FOXP3 expression correlated with the severity of allograft tissue injury in another study (Bunnag *et al.*, 2008). The association between Treg cell infiltration and the severity of allograft tissue injury in acute T cell-mediated rejection (ATCMR) is controversial.

Th17 cells have been discovered recently as the third subset of effector T cells (Steinman, 2007; Korn

et al., 2009). Although they belong to the effector T cell lineage, they are related more closely to FOXP3⁺ Treg cells than to Th1 or Th2 cells (Dong, 2011). Of note, the Th17 cell developed from a common precursor with the FOXP3⁺ Treg (Awasthi *et al.*, 2008; Weaver and Hatton, 2009). In addition, Th17 cells can interconvert with Treg cells depending on the microenvironment (Lee *et al.*, 2009). The imbalance between Th17 and Treg cells has been proposed as an important mechanism that modulates immune responses in various autoimmune disorders (Lee *et al.*, 2009; Nistala and Wedderburn, 2009; Ochs *et al.*, 2009; Eastaff-Leung *et al.*, 2010; Shin *et al.*, 2010).

As their clinical significance in autoimmune disorder has been revealed, the interplay between Treg and Th17 cells is now of interest in transplantation (Loong *et al.*, 2000; Hsieh *et al.*, 2001; San Segundo *et al.*, 2008; Crispim *et al.*, 2009; Mitchell *et al.*, 2009). However, the significance of their interplay has not been investigated in acute T cell-mediated rejection (ATCMR). In this report, we

investigated Th17 and Treg cell infiltration into allograft tissue from biopsy-proven ATCMR. By analyzing the results with clinical information, we intended to evaluate whether the Th17 and Treg cell infiltration into allograft tissue is associated with the severity of allograft dysfunction and tissue injury.

Results

Baseline characteristics of the biopsy tissues

The interval between kidney transplantation to allograft biopsy was 9.4 ± 14.0 months. Scr concentration at allograft biopsy was 2.7 ± 1.6 mg/dl, and the MDRD eGFR was 36.6 ± 16.5 ml/min. Forty-six cases (65%) were the first rejection and 25 cases (35%) were repeat rejection

Table 1. Clinical and histological characteristics of renal allograft biopsy tissue

Characteristics of biopsy	n = 71
Time from KT (month)	9.4 ± 14.0
Rejection frequency	
First ATCMR (%)	46 (65)
Repeated ATCMR (%)	25 (35)
Serum creatinine (mg/dl)	2.7 ± 1.6
MDRD eGFR (ml/min)	36.6 ± 16.5
ATCMR stage	
ATCMR I (%)	53 (75)
ATCMR II (%)	18 (25)
IF/TA grade	
No IF/TA	32 (45)
IF/TA I (%)	34 (48)
IF/TA II (%)	5 (7)
Banff score	
t	1.94 ± 0.84
ct	0.75 ± 0.66
i	1.86 ± 0.80
ci	0.71 ± 0.67
v	0.35 ± 0.72
cv	0.08 ± 0.36
g	0.63 ± 0.96
cg	0
ah	0.21 ± 0.59
Positive C4d (%)	16 (23%)

KT, kidney transplantation; ATCMR, acute T cell mediated rejection; MDRD, modified diet renal disease; eGFR, estimated glomerular filtration rate; IF/TA, interstitial fibrosis/tubular atrophy; t, tubulitis; ct, tubular atrophy; i, interstitial inflammation; ci, interstitial fibrosis; v, vasculopathy; cv, chronic vascular change; g, glomerulitis; cg, glomerulopathy; ah, arteriolar hyaline thickening.

Figure 1. Detection of IL-17⁺ and FOXP3⁺ cells in renal allograft tissue with ATCMR. (A, C) Representative staining of IL-17⁺ and FOXP3⁺ cells in renal allograft tissue with ATCMR. Both cell types were found mostly within interstitial lymphocyte infiltration (A, B: Original magnification $\times 400$). (B, D) Representative staining for isotype control. (E, F) Cell count results of IL-17⁺ and FOXP3⁺ cells in renal allograft tissue with ATCMR. The total number of Th17 (E) and Treg cells (F) were measured by defining regions of interest (ROI, cell counts/mm²) using automated cell acquisition and quantification software for immunohistochemistry (Histoquest). Blue mean intensity for total cells and brown mean intensity for IL-17 (E) or Foxp3 (F) positive cells.

Figure 2. Relationships between renal allograft function and infiltrating Treg and Th17 cell numbers, and the Treg/Th17 ratio. (A, B) Log Treg/Th17 ratio correlated inversely with Scr concentration ($r = -0.24$, $P = 0.037$) and positively eGFR ($r = 0.24$, $P = 0.046$). (C, D) Log Th17 cell number correlated positively with Scr concentration ($r = 0.38$, $P = 0.01$) and inversely with eGFR ($r = -0.45$, $P = 0.00$). (E, F) Log Treg cell number did not correlate significantly with Scr concentration ($r = 0.04$, $P = 0.687$) or with eGFR ($r = -0.145$, $P = 0.228$). * $P < 0.05$.

(Table 1). Fifty-three cases (75%) were ATCMR I, and 18 were ATCMR II (25%). IF/TA was found in 39 cases (55%). C4d staining was positive in 16 cases (23%) (Table 1).

Treg and Th17 infiltration in renal allograft tissue with ATCMR

Figure 1 shows representative staining of IL-17⁺ and FOXP3⁺ cells in renal allograft tissue with ATCMR. Both cell types were found mostly within interstitial lymphocyte infiltration. The average numbers of infiltrating Treg and Th17 cells were 11.6 ± 12.2 cells/mm² and 5.6 ± 8.0 cells/mm²,

Figure 3. Relationship between the severity of acute tissue injury (interstitial inflammation and tubulitis) and the number of Th17 or Treg cells, or their ratio (Treg/Th17). (A) The i score was inversely related to Log Treg/Th17. Biopsies with an i score of 3 had a significantly lower Log Treg/Th17 than did those with an i score of 1 ($P = 0.005$). (B) Similarly, biopsies with a t score of 3 had a significantly lower Log Treg/Th17 than did those with a t score of 1 ($P = 0.005$). (C) The i score was positively related to Th17 cell infiltration. Biopsies with an i score of 3 had a significantly higher Log Th17 than did those with an i score of 1 ($P = 0.005$). (D) Similarly, biopsies with a t score of 3 had a significantly higher Log Th17 than did those with a t score of 1 ($P = 0.005$). (E, F) Log Treg did not correlate significantly with the i score or t score. * $P < 0.05$.

respectively. The average Treg/Th17 ratio was 5.6 ± 8.2 . We used log transformation to correct for skewed data for Treg and Th17 cell infiltration and for the Treg/Th17 ratio. The median value of the log-transformed Th17 cell infiltration (Log Th17) was 0.3 (range -0.52 to 1.64) and for Treg (Log Treg) was 0.7 (range -0.52 to 2.09). The median value for the log transformed Treg/Th17 ratio (Log Treg/Th17) was 0.45 (range -1.12 to 2.08).

Figure 4. Relationship between the severity of chronic tissue injury (interstitial fibrosis and tubular atrophy) and the number of Th17 or Treg cells, or their ratio (Treg/Th17). (A) The ci score inversely related to Log Treg/Th17. Biopsies with a ci score of 3 had a significantly lower Log Treg/Th17 than did those with a ci score of 1 ($P = 0.02$). (B) Similarly, biopsies with a ct score of 3 had a significantly lower Log Treg/Th17 than did those with a ct score of 1 ($P = 0.03$). (C) The ci score positively related to Th17 cell infiltration. Biopsies with a ci score of 3 had a significantly higher Log Th17 than did those with a ci score of 1 ($P = 0.01$). (D) Similarly, biopsies with a ct score of 3 had a significantly higher Log Th17 than did those with a ct score of 1 ($P = 0.01$). (E, F) Log Treg did not correlate significantly with the ci score or ct score. * $P < 0.05$.

Association between allograft function and Treg or Th17 cell infiltration into renal allograft tissue

The Treg/Th17 ratio correlated significantly with Scr level ($r = -0.24$, $P = 0.037$) and calculated MDRD eGFR ($r = 0.24$, $P = 0.046$) (Figures 2A and 2B). The number of infiltrating Th17 cells correlated significantly with Scr level ($r = 0.38$, $P = 0.01$) and with calculated MDRD eGFR ($r = -0.45$, $P = 0.00$) (Figures 2C and 2D). By contrast, the number of infiltrating Treg cells did not correlate significantly with Scr level ($r = 0.04$, $P = 0.687$) or with MDRD eGFR ($r = -0.145$, $P = 0.228$) (Figures 2E and 2F).

Figure 5. The relationship between the interval between transplantation and biopsy. (A) It correlated significantly with the number of infiltrating Treg cells ($r = 0.26$, $P = 0.029$). But it did not show significant correlation with (B) the number of infiltrating Th17 cells ($r = 0.17$, $P = 0.07$) or (C) the Treg/Th17 ratio ($r = 0.2$, $P = 0.616$).

Association between the severity of allograft tissue injury and Treg or Th17 cell infiltration into renal allograft tissue

The Treg/Th17 ratio correlated significantly with interstitial inflammation (i score) and tubulitis (t score). Biopsies with an i score or t score of 3 had a significantly lower Log Treg/Th17 than did biopsies with an i score of 1 ($P = 0.005$ and 0.006 , respectively) (Figures 3A and 3B). Th17 cell infiltration correlated significantly with the i score and the t score (Figures 3C and 3D). The Treg/Th17 ratio and Th17 cell infiltration showed a similar association with chronic injury scores such as interstitial fibrosis (ci) and tubular atrophy (ct) (all $P < 0.05$) (Figures 4A-4D). However, Treg infiltration did not correlate significantly with interstitial (i, ci) or tubular (t, ct) injury scores (Figures 3E and 3F, 4E and 4F). Vascular (v and cv scores) and glomerular (g and cg scores) injury scores were not significantly associated with Treg or Th17 cell infiltration or the Treg/Th17 ratio. The C4d status of the biopsies (positive or negative) was also not related to Treg or Th17 cell infiltration, or the Treg/Th17 ratio.

Association between the interval after transplantation and Treg or Th17 cell infiltration into renal allograft tissue

The interval between transplantation and biopsy correlated significantly with the number of infiltrating Treg cells ($r = 0.26$, $P = 0.029$), but not with the number of infiltrating Th17 cells ($r = 0.17$, $P = 0.07$) or the Treg/Th17 ratio ($r = 0.2$, $P = 0.616$) (Figure 5). The interval between transplantation and biopsy did not correlate significantly with any of the Banff scores or indicators of allograft function (Scr and MDRD eGFR) (all $P < 0.05$).

Association between the rejection frequency and Treg or Th17 infiltration in renal allograft tissue

In comparison between the first rejection and the repeat rejection, Log Treg/Th17 was significantly lower in the repeat rejection ($P = 0.04$) (Figure 6). However, the Treg and Th17 infiltration did not show significant differences between the first ATCMR and repeat ATCMR ($P = 0.615$, $P = 0.242$, respectively). The severity of interstitial fibrosis (ci) and tubular atrophy (ct) was significantly higher in the repeat rejection. Another assessment of Banff scores and allograft function at biopsy (Scr, MDRD eGFR) did not differ significantly between the first rejection and the repeat rejection (All indicators, $P < 0.05$).

Discussion

We investigated the infiltration by Treg and Th17 cells, and the Treg/Th17 ratio in renal transplant biopsies for cause, focusing on their association with the severity of allograft rejection. Our results demonstrate clearly that the Treg/Th17 ratio and Th17 cell infiltration were closely associated with the severity of allograft dysfunction and tissue injury in ATCMR. Our data suggest that the Th17 cell may play a role in the progression of allograft dysfunction or tissue injury in ATCMR.

The Th17 cell numbers and Treg/Th17 ratio correlated with the severity of allograft dysfunction at biopsy and with the severity of tissue injury. The Treg-Th17 axis is significantly affected by the surrounding microenvironment (Afzali *et al.*, 2007; Cheng *et al.*, 2008). For example, inflammatory conditions can drive the Th17-dominant state, and when inflammation disappears, the axis favors the development of Treg cells. Allograft rejection is an alloimmune response to a grafted kidney and is usually accompanied by localized or systemic inflammation. In this study, acute inflammatory markers in the allograft tissue, such as the i and t scores showed an increasing pattern with a

Figure 6. Comparison of the Treg/Th17 ratio between the first rejection and the repeat rejection. Note that the Treg/Th17 ratio was significantly lower in the repeat rejection compared with the first rejection ($P = 0.04$). * $P < 0.05$.

decrease in the Treg/Th17 ratio. Together with data from previous reports, our data suggest that acute inflammation induced by acute rejection drives the Th17-dominant state.

The Th17 cell number and Treg/Th17 ratio also showed significant relation with the indicators of chronic allograft injury such as interstitial fibrosis (ci score) and tubular atrophy (ct score). Chronic injury is the most common cause of allograft failure after one-year from transplantation and is a significant determinant of long-term allograft outcome (Arndt *et al.*, 2010; Nankivell *et al.*, 2001). Accumulating evidence shows the role of Th17 cells in the progression of chronic injury in various disorders (Decraene *et al.*, 2010; Syrjälä *et al.*, 2010; Hammerich *et al.*, 2011). In kidney transplantation, Th17 cell infiltration hastens clinical chronic rejection in allograft tissue as well (Deteix *et al.*, 2010). It is possible that Th17 cell dominant state induced by acute inflammation is involved in the progression of chronic inflammation.

Previous reports identified recurrence of acute rejection as a strong predictor of graft loss (Ferguson, 1994; Gaber *et al.*, 1996). Our study showed that repeat rejection is characterized by more severe chronic tissue injury compared with the first rejection. The Treg/Th17 ratio was significantly lower in repeat rejection compared with the first rejection, and this Th17-dominant condition may be associated with advanced chronic injury in repeat rejection. Of note, the numbers of Th17 and Treg cells did not change significantly from the first to repeat rejection, and only their ratio differed significantly between the first rejection and repeat rejection. This suggests that the relative dominance of Th17 cells compared with Treg cells is more important than the increase in the absolute Th17 cell count in repeat rejection.

The mechanism underlying the development of severe tissue injury in allograft tissue with high infiltration of Th17 cell is unclear and we speculate

that there are several possibilities. First, renal epithelial cells exposed to IL-17 can produce inflammatory mediators with the potential to stimulate early alloimmune responses (Loong *et al.*, 2002). Second, IL-17 might rapidly recruit neutrophils, which are frequently observed in biopsies with more severe rejection (Healy *et al.*, 2006). Third, Th17 might drive alloimmune responses by promoting lymphoid neogenesis. (Deteix *et al.*, 2010). After all, exposure to a higher level of Th17 induces stronger and sustained alloimmune responses, which cause severe tissue injury.

The timing of ATCMR development is often associated with allograft outcome. ATCMR occurring after 6 months or 1 yr is called late-onset rejection and is often associated with an adverse graft outcome because it confers a high risk of chronic rejection (Humar *et al.*, 1999; Sijpkens *et al.*, 2003). In this study, Treg cell infiltration was associated with the timing of the ATCMR episode. The increase in FOXP3⁺ Treg cell infiltration is time dependent in most renal allograft tissue. The increased entry of Treg cells represents a natural mechanism for stabilizing inflammatory sites (Bunnag *et al.*, 2008). Th17 cell infiltration also increased with time from transplantation, even though it did not reach statistical significance. The Treg/Th17 ratio did not correlate significantly with the interval from transplantation, possibly because of the simultaneous time-dependent increase in Treg and Th17 cell infiltration.

We evaluated C4d positivity and whether the Treg/Th17 axis is associated with humoral immunity. Our results showed that the Treg/Th17 axis is not significantly associated with C4d positivity. In addition, glomerulopathy or vasculopathy, which is often associated with humoral immunity, was not associated with the Treg and Th17 cell and the ratio between them (Sis *et al.*, 2007; Steinmetz *et al.*, 2007; Cosio *et al.*, 2008). These findings suggest that the impact of Th17 and Treg cells on humoral immunity is not as strong as its effect on T cell-mediated immunity.

In this study, we aimed to evaluate the impact of the Treg/Th17 ratio in acute rejection. However most of the ratio's impact was dependent upon Th17 cell infiltration alone and detection of FOXP3⁺ Treg cell did not show any significant association with allograft function or tissue injury. The reason for these is unclear but it seems to be related to the inflammatory environment in ATCMR. Under active inflammation, FOXP3⁺ T cell could differentiate into IL-17 producing cell (Afzali *et al.*, 2007; Beriou *et al.*, 2009; Voo *et al.*, 2009). In addition, during a development or transition state, effector T cells could express FOXP3. Our results suggest that

detection of Th17 cell alone could be useful marker for the severity of allograft injury and dysfunction in ATCMR. By contrast, quantification of the FOXP3 expression is not suitable as a marker of suppression in the inflammatory condition.

In summary, high infiltration of Th17 cells into renal allograft tissue was associated with deteriorated allograft function and the severity of acute and chronic allograft tissue injury. This suggests that the Th17 cell have a important role in the progression of allograft dysfunction or tissue injury in ATCMR. Unveiling the mechanism of the development of a Th17-dominant condition may help prevent or limit the progression of tissue injury and may help improve allograft survival.

Methods

Patients and clinical information

The study population comprised 71 clinically indicated renal allograft biopsies performed in our transplant center from January 2001 to August 2008. Clinical information was collected by retrospective chart review. The indication for the allograft biopsy was graft dysfunction defined as an increment in the serum creatinine (Scr) concentration $\geq$ 10% from the baseline value. Allograft function was assessed using the modified diet in renal disease (MDRD) formula, which provides the estimated glomerular filtration rate (eGFR) (Levey *et al.*, 1999).

Inclusion of allograft biopsy tissue

Biopsy samples were selected only from tissues with a diagnosis of ATCMR type I or II according to the Banff working classification, a sufficient amount of paraffin-embedded tissue, and an adequate number of glomeruli (> 7) in the allograft tissue (Solez *et al.*, 2007). BK virus or cytomegalovirus nephropathy, interstitial fibrosis or tubular atrophy (IFTA) III, and lymphoproliferative disorders were not present in these patients or biopsies.

Allograft biopsy procedure

Allograft biopsies were performed with ultrasound guidance using an automatic spring-loaded core biopsy system. Standard procedures were used to process 10% formalin-fixed tissues for light microscopy. The paraffin-embedded tissue was serially sectioned at 3 μ m in thickness. Slides were sequentially stained with hematoxylin and eosin, periodic acid-Schiff, silver methenamine, and Masson trichrome stains.

Evaluation of the severity of allograft tissue injury

All biopsies were evaluated by a pathologist blinded to the immunohistochemical (IHC) results who assessed the severity of tissue injury using the Banff criteria (Solez *et al.*, 2007). The following were assessed: tubulitis (t score), tub-

ular atrophy (ct score), interstitial inflammation (i score), interstitial fibrosis (ci score), vasculopathy (v score), chronic vascular change (cv score), glomerulitis (g score), glomerulopathy (cg score), and arteriolar hyaline thickening (ah score). Indirect immunofluorescence staining was performed using monoclonal antibodies against complement protein C4d (Biogenesis, Poole, UK) in 48 (68%) biopsies. In 23 (33%) biopsies where no C4d staining was performed on frozen sections, sections were obtained from paraffin blocks and subjected to IHC staining with C4d using a rabbit polyclonal antibody (Biogenesis). C4d positivity was defined as diffuse (> 50%) and linear staining of peritubular capillaries.

IHC staining of renal allograft tissue

Paraffin sections were immersed in three changes of xylene and dehydrated in a graded series of alcohols. Antigen retrieval was performed routinely by immersing the sections in sodium citrate buffer (pH 6.0) in a microwave for 15 min. The sections were depleted of endogenous peroxidase activity by adding methanolic hydrogen peroxide and were then blocked with normal serum for 30 min. After overnight incubation with polyclonal antibody against FOXP3 (sc-21072, Santa Cruz Biotechnology, Santa Cruz, CA) and antihuman IL-17 monoclonal antibody (R&D Systems Inc., Minneapolis, MN), the samples were incubated with the secondary antibody, biotinylated anti-IgG, for 20 min and then incubated with a streptavidin-peroxidase complex (Vector, Peterborough, UK) for 1 h. This was followed by incubation with 3,3'-diaminobenzidine (Dako, Glostrup, Denmark). The sections were counterstained with hematoxylin and then photographed with an Olympus photomicroscope (Tokyo, Japan).

Evaluation of Treg and Th17 infiltration in renal allograft tissue

Figure 1 (A and C) shows representative staining for FOXP3 and IL-17. Both Treg and Th17 cells were found in the interstitial lymphocyte infiltration. The total number of Th17 and Treg cells were measured by defining regions of interest (ROI) using automated cell acquisition and quantification software for immunohistochemistry (TissueQuest Software, TissueGnostics, Vienna, Austria) (Figures 1E and 1F). The area of cortex was measured using a loupe, and the data are expressed as the number of cells/mm².

Statistical analysis

The data were analyzed using SPSS software version 16.0 (SPSS Inc., Chicago, IL). Data are presented as mean $\pm$ SD or as counts and percentages, depending on the data type. For continuous variables, the means were compared using Student's *t* test. For categorical variables, Pearson's chi-square test and Fisher's exact test were used. The associations between the Treg and Th17 cell infiltration or the Treg/Th17 ratio and the clinical information (severity of allograft dysfunction, severity of allograft tissue injury, timing of allograft biopsy) were analyzed using bivariate correlational analysis. *A* *P* value < 0.05 was considered significant.

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