Involvement of indirectly allostimulated CD4⁺CD43^{high}CD45RO⁺ T cell proliferation in the development of chronic allograft nephropathy

Yu-Mee Wee^{1,2}, Joo-Hee Jung¹, Yang-Hee Kim¹, Monica-Y Choi¹, Young-Hoon Kim¹, Do-Sook Choi¹, Myung-Hwan Cho² and Duck-Jong Han¹

¹Department of Surgery, Ulsan University College of Medicine & Asan Medical Center, Songpa-gu, Seoul 138-736, Korea; ²Department of Biological Science, Konkuk University, Kwangjin-gu, Seoul 143-701, Korea

Corresponding author: Duck-Jong Han, Email: djhan@amc.seoul.kr; Myung-Hwan Cho. Email: mcho@konkuk.ac.kr

Abstract

The goal of this study was to identify immunological markers for use in antigen-specific assays that predict long-term survival after renal allograft and distinguish stable-functioning (SP) patients from poorly functioning (PP) patients. For this prospective study, 20 patients were enrolled. Eight SP and six PP patients were enrolled in this study. Serum cytokine/chemokine levels were analyzed by the Luminex multiplex assay. To detect indirect alloreactive T cells, we performed indirect mixed lymphocyte reaction using donor-antigen-pulsed autologous dendritic cells as stimulators. Serum induced protein-10 levels were significantly higher in the serum of PP patients, whereas sCD40L levels were higher in SP patients. The PP patients had significantly higher numbers of donor-specific CD4⁺CD43^{high}CD45RO⁺ T cells after indirect allostimulation, whereas this cell population was unchanged in SP patients. The donor-specific CD4⁺CD43^{high}CD45RO⁺ T cells had the effector memory T cell phenotype. Prospectively, we studied whether these cells influence graft outcome and found that their strong proliferation in pre-transplant patients is related to a poorly functioning graft. Indirectly allostimulated CD4⁺CD43^{high}CD45RO⁺ T cells may not only contribute to chronic allograft nephropathy development but may also have a role in the progression of acute rejection. Thus, these cells may have potential use as immune-monitoring markers in a noninvasive *in vitro* assay that predicts graft outcome.

Keywords: CD4⁺CD43^{high}CD45RO⁺ T cell, indirect allostimulation, effector memory T cells, induced protein-10, chronic allograft nephropathy, progression of acute rejection

Experimental Biology and Medicine 2016; 241: 1217–1228. DOI: 10.1177/1535370215601522

Introduction

Advances in post-transplant care during the last decade, including the introduction of new potent immunosuppressive drugs, have dramatically decreased the rate of acute rejection after transplantation. However, achieving long-term, drug-free graft acceptance remains an unsolved issue in clinical transplantation. The availability of clinical parameters of graft function could help guide the choice and dosage of immunosuppressive therapy. However, significant organ damage can occur before the ongoing pathology is recognized.^{1,2} It is possible that rejection-related biomarkers are released into the peripheral blood from graft tissues or cells. If this is the case, their detection could provide the basis for making noninvasive or minimally invasive tools for the detection of alloimmune reactions. Since cellular immunity is thought to play a central role in immunological responses, especially alloimmune reactions,

the transplant community has been searching intensely for many years for reliable cellular immunity markers that allow the early prediction of alloimmune reactions, alloimmune quiescence, and hypo-responsiveness.³

T cells are key initiators and mediators of alloimmune responses, especially alloreactive T cells from a substantial proportion of the naïve T cell repertoire.^{4,5} Alloreactive T cells recognize human leukocyte antigen (HLA)-mismatched tissue.⁶ Alloantigens are conventionally processed by autologous antigen-presenting cells (APCs) and then presented to CD4⁺ T cells as peptides in the context of self-MHC molecules.^{7–11} These APCs include dendritic cells (DCs), which are referred to as professional APCs. Mature autologous antigen-presenting DCs can be generated *in vitro* from peripheral blood monocytes¹² and can trigger allospecific T cells indirectly when they are pulsed with cellular fragments.^{13,14}

A single center study that sought specific immunologic cells characteristics predicting the development of chronic allograft nephropathy (CAN) is reported here. Antigen-specific bioassays were performed with peripheral blood from patients who had stable-functioning grafts (SP) or poor-functioning grafts (PP) to identify specific immunological characteristics that distinguish the two groups. The *in vitro* indirect mixed lymphocyte reaction (MLR) assay was used to identify early cellular biomarkers of chronic rejection. Several candidate biomarkers in the circulation, including interferon gamma (IFN- γ)-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), donor-specific antibodies (DSA), and T cell subsets, were analyzed. CAN signatures, such as serum IP-10, MCP-1, DSA specific for MHC class I, and donor-specific CD4⁺CD43^{high}CD45RO⁺ T cells after indirect allostimulation, were detected at higher levels in the PP group than in the SP group.

In this prospective analysis, the higher number of donor-antigen-specific CD4⁺CD43^{high}CD45RO⁺ T cells, which were harvested from pre-transplant PBMC after indirect allostimulation, were the most effective biomarker predicting graft outcome.

Patients and materials and methods

Patient characteristics

Out of 2000 eligible transplant patients, the study population was composed of live-donor renal transplant patients for whom the donor was available for the donor-specific assay. The subjects were categorized into the SP group if they had maintained stable creatinine levels (<1.4 mg/dL) for more than 10 years, exhibited changes in creatinine (Δ Cr) of <0.5 during the previous one year, and had not experienced calcineurin inhibitor (CNI) toxicity, cytomegalovirus (CMV) infection, or BK virus infection. As protocol biopsies are not routine in our center, biopsies were not available for these stable patients. The PP group consisted of patients with poor-functioning renal allografts. These patients had biopsy-confirmed acute rejection and were given steroid-pulse treatment after rejection. They had also experienced tubular atrophy and interstitial fibrosis (TA/IF), and had exhibited serum creatinine elevation (>3 mg/dL) for at least one year or were undergoing dialysis. All patients with CAN caused by CNI toxicity, disease recurrence, or BK virus nephropathy were excluded from the PP group. Ultimately, eight and six representative patients satisfied these criteria and were labeled SP and PP, respectively. Both groups were maintained on standard CNI immunosuppression based on cyclosporine A (CsA) or FK506 administration, and low-dose glucocorticoid and azathioprine or mycophenolate mofetil. The clinical characteristics of the patients are described in Table 1.

For the prospective study, 20 patients were enrolled according to the plan described in a former study. The population was composed of live-donor renal transplantation patients. At the state of pre-transplantation, the patients were categorized according to the donor-specific CD4⁺CD43^{high}CD45RO⁺ T cell proliferation compared with their third party control in the indirect MLR, strong proliferation *versus* low proliferation (donor/3rd party <1.0 vs.

Table 1 Demographics and transplant characteristics of the SP and PP patients

	SP	PP
Donor age, year	36.3 $\pm$ 9.8	35.5 $\pm$ 8.0
Donor type, <i>n</i>		
Living-related	6	6
Living-unrelated	2	0
Donor gender, <i>n</i>		
Female	5	4
Male	3	2
Recipient age, year	48.6 $\pm$ 11.1	50.3 $\pm$ 15.4
Recipient gender, <i>n</i>		
Female	2	4
Male	6	2
Creatinine level, mg/dL	1.1 $\pm$ 0.2	5.8 $\pm$ 2.7
Interval between transplant and enrollment, year	11.3 $\pm$ 1.4	7.2 $\pm$ 2.5
HLA mismatch ^a	3.3 $\pm$ 1.0	3.7 $\pm$ 1.2
Type of immunosuppression		
CsA-based	7	2
FK-based	1	4
Retuximab therapy	0	3
Rejection history, <i>n</i>		
AR/ACR	1 ^b	5
Chronic nephropathy(incl. AMR)	0	6
AMR	0	3
Donor-specific antibodies (DSA), <i>n</i>		
DSA against HLA class I	0	3
DSA against HLA class II	3	2
Missing data	2	2

SP, stable-functioning patient; PP, poor-functioning patient; AR, acute rejection; ACR, acute cellular rejection; AMR, antibody-mediated rejection.

Ages, time intervals, creatinine levels, and HLA data are expressed as means $\pm$ SD.

^aNumber of HLA-A, -B, and -DR mismatches between the donor and recipient (maximum, 6).

^bOne episode of acute rejection within 1 month after transplantation.

donor/3rd party >1.0, respectively). Both groups were maintained on standard CNI immunosuppression based on CsA or FK506 and were additionally treated with Rituximab monotherapy in the case of ABO mismatched transplantation (4 of 20 in all). All patients had not experienced CNI toxicity, CMV infection, or BK virus infection. Graft stability was confirmed at 60 days. The clinical characteristics of the patients are described in Table 2.

This study protocol was approved by the Institutional Review Board (IRB) of Asan Medical Center (application: 2008-0502 and 2011-0261). All participants in this study were provided with informed written consent.

Measurement of DSA in serum

Human blood from recipient patients was centrifuged (500 g for 10 min), and the serum fraction was removed and stored at -70°C until use. All SP and PP patients' sera were screened for DSA by using HLA class I and II single-antigen beads (Gen-Probe, CT) according to the

Table 2 Demographics and transplant characteristics of patients in the prospective study

	All patients	Strong proliferation (donor/3 rd party <1.0)	Low proliferation (donor/3 rd party >1.0)
Donor age, year	39.5 ± 11.7	38.3 ± 11.7	40.9 ± 10.0
Donor type, <i>n</i>			
Living-related	13	8	5
Living-unrelated	7	3	4
Donor gender, <i>n</i>			
Female	8	6	2
Male	12	5	7
Recipient age, year	44.8 ± 11.2	47.2 ± 10.7	41.8 ± 11.7
Recipient gender, <i>n</i>			
Female	8	4	4
Male	12	7	5
HLA mismatch ^a	3.4 ± 1.3	3.4 ± 1.4	3.3 ± 1.3
HLA class I PRA (%)	11.3 ± 24.4	8.4 ± 18.7	17.9 ± 30.7
HLA class II PRA (%)	17.0 ± 26.6	10.6 ± 21.0	24.8 ± 31.6
Type of immunosuppression			
CsA-based	5	3	3
FK-based	15	8	7
Retuximab therapy	4	3	1
Rejection history, <i>n</i>	3	0	3

Age, HLA mismatch, and PRA data are expressed as means ± SD.

^aNumber of HLA-A, -B, and -DR mismatches between the donor and recipient (maximum, 6).

manufacturer's protocol.¹⁵ Briefly, 10 µL of sera was added to 40 µL of antigen beads and incubated in the dark for 30 min at room temperature (RT). PE-conjugated secondary antibody was then added to the beads and incubated for 30 min. Fluorescence was detected on a LABScreen 100 Luminex system (PerkinElmer, CA) and expressed as the mean fluorescence intensity (MFI) of each single-antigen bead. Raw MFI values were then normalized to the results obtained by using negative control serum. Normalized MFI values of >1000 were defined as a positive reaction.

Generation of DCs from recipient peripheral blood mononuclear cells (PBMCs) and donor antigen loading

Heparin-anticoagulated peripheral blood samples were collected from all recipients and donors. PBMCs were isolated by Ficoll-Histopaque (1.077, Sigma-Aldrich, MO) gradient centrifugation and then cryopreserved in liquid nitrogen until use. The two-stage *in vitro* DC differentiation procedure was then performed. Briefly, recipient monocytes were cultured at 37°C in a humidified 5% CO₂ atmosphere for five days in AIM-V medium (Gibco-BRL, CA) supplemented with 1000 IU/mL granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (R&D Systems, MN). The immature recipient DCs were

then pulsed with donor antigens by mixing fragmented donor PBMCs. At this time, donor PBMCs were subject to three freeze-thaw cycles and then sonicated three times for 10 s each. In all experiments using cell-free donor antigens, intact cells were not evident in microscopic examinations.^{14,16,17} Two hours later, tumor necrosis factor (TNF)-α (R&D Systems) and prostaglandin E₂ (PGE₂, Sigma-Aldrich) were added to the culture medium.^{12,18,19} After two days, the antigen-loaded DCs were used to stimulate recipient T cells.

Phenotyping of mature DCs

DCs were labeled with the following fluorescence-conjugated mouse monoclonal antibodies along with appropriate isotype controls: allophycocyanin (APC)-conjugated anti-human CD14 (clone HCD114), fluorescein isothiocyanate (FITC)-conjugated anti-human CD83 (clone HB15e), and phycoerythrin (PE)-conjugated anti-human CD86 (clone IT2.2). The antibodies were all purchased from BioLegend (San Diego, CA). The antibodies were added to mature DCs and incubated for 30 min on ice. Flow cytometry was performed on a FACSCalibur (BD Bioscience, NJ). The data were analyzed by using FlowJo software (Tree Star; Ashland, OR).

T cell purification and direct and indirect MLRs

Recipient T cells were purified from PBMCs by negative selection using magnetic beads coupled to antibodies against human CD11b, CD16, CD19, CD36, and CD56 (Miltenyi Biotec, Germany). The purified T cells (1×10^5 cells/well) served as the responder cells in direct and indirect MLRs, where the stimulators were 2.5×10^5 donor PBMCs (direct) or 1×10^4 donor-antigen-pulsed autologous DCs (indirect). The stimulators were rendered unable to proliferate by cesium irradiation (20 Gy; Cs irradiator, Cis-Bio International, MA). The responder and stimulator cells were cultured in 96-well plates for two days. On day 1, 1 µCi of [³H] thymidine was added and the cells were incubated for an additional 24 h. After harvesting the cells on filter plates, ³H-thymidine incorporation was measured by using a β-counter (TopCount NXT; PerkinElmer). At this time, the wells with responder cells cultured without stimulators were used as a negative control for background proliferation.

Other characteristics of the alloreactive T cells, namely, allostimulated cytokine/chemokine production and changes in T cell subset frequencies, were analyzed by harvesting the supernatants and T cells from four direct and indirect MLR wells 24 h after commencing allostimulation.

Analysis of T cell subsets

To detect alloreactive T cell after allogeneic culture, the cultured T cells were simultaneously stained with PE-conjugated CD43 and APC-conjugated CD45RO monoclonal antibodies (mAb, eBioscience, CA), and FITC-conjugated CD4 or CD8. The proportions of CD4⁺CD45RO⁺ cells that expressed high, intermediate, and low levels of CD43 were measured. To determine whether the

CD4⁺CD43^{high}CD45RO⁺ T cells had an effector memory T cell (T_{EM}) or a central memory T cell (T_{CM}) phenotype, CD4⁺ lymphocytes were gated for CD43^{high}CD45RO⁺ and CD43^{low}CD45RO⁺, and analyzed with FITC-conjugated anti-CCR7, anti-CD62L, and anti-CD95(Fas) mAbs.

The Treg subset was examined by simultaneously staining naïve and alloantigen-stimulated T cells with FITC-CD4 in combination with PE-CD25 and APC-FoxP3 (eBioscience). The FoxP3-expressing CD4⁺CD25^{high} cells were measured by flow cytometry. The data were analyzed by using FlowJo software.

Luminex detection of cytokine/chemokine in MLR supernatants and serum

The culture supernatants were harvested and stored at -20°C until use. The levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17 A, IL-23, IFN-γ, TNF-α, and transforming growth factor-β (TGF-β) were measured with Luminex technology by using a human cytokine/chemokine kit (Affymetrix, CA, USA), according to the manufacturer's protocol. Briefly, antibody-coated, cytokine-specific beads were added to 96-well filter plates. Thereafter, 50 μL of supernatant was added and the plates were incubated for 1 h at RT in the dark with shaking to allow the cytokines to bind to the cytokine-specific beads. Subsequently, plates were incubated with the detection antibody for 30 min. After washing, streptavidin-PE was added and the plates were incubated for 30 min. The fluorescence labeling of cytokine-specific beads was analyzed by using Luminex acquisition software (ver. 3.1; xPONENT Software Solutions, TX). An additional cytokine, macrophage colony-stimulating factor (M-CSF), and TGF-β were screened for in the culture supernatants in a similar manner.

Statistical analysis

Statistical analyses were performed by using the SPSS software package (version 18.0; SPSS Inc., Chicago, IL). Comparisons of the groups in terms of biomarker levels were performed by using the Mann-Whitney U-test. The results are expressed in the text as median ± standard error. Kaplan-Meier survival analysis was used to compare the incidence of rejection-free grafts with the incidence of graft dysfunction after two months, by measuring donor specific T cells ratios to a third party control. A *P* value of <0.05 was considered to be statistically significant.

Results

Transplant patients demographics

Patients in the SP group (*n* = 8) had a high percentage of living-related donors (6/8), HLA mismatching (mean mismatches, 3.4), and CsA-based immunosuppression (7/8), and were predominantly male (6/8). These patients had relatively uneventful post-transplantation courses, with only one patient having a documented episode of acute rejection, which occurred one month after transplantation (Table 1). The patients in the PP group (*n* = 6) had a high percentage of parent or offspring donors (4/6), HLA

mismatching (mean mismatches, 3.7), and FK506-based immunosuppression (4/6), and were predominantly female (4/6). All PP patients had experienced at least one steroid-pulse treatment as a rejection therapy. One underwent plasma pheresis, and three underwent Rituximab treatment. All patients had biopsy-proven TA/IF, maintained high blood creatinine levels (>3 mg/dL) for at least one year, and exhibited a ΔCr greater than 2.0 (Table 1).

In the prospective study, the patients in the strong proliferation group (*n* = 11) had a higher percentage of living-related donors (8/11), and FK506-based immunosuppression (8/11). This group did not have eventful post-transplantation elevation of serum creatinine level. The patients in low proliferation group (*n* = 9) had a higher percentage of male donor (7/9) and FK506-based immunosuppression (7/9). Both groups exhibited similar number of HLA mismatches (mean mismatches, 3.4) and similar percentage of PRA to HLA class I and HLA class II. Four patients received Rituximab treatment due to ABO mismatch regardless of the experimental group. Three patients in the strong proliferation group had an episode of acute rejection on days 20, 35, and 40 after transplantation whereas no patient experienced such an episode in the low proliferation group within 60 days (Table 2).

DSA to class I HLAs was readily detected in the serum of PP patients

The sera from four PP and seven SP patients were screened for donor HLA-specific antibodies by Luminex single-antigen bead assays, which allow detection of antibodies specific for HLA-A, -B, and -DR.²⁰ HLA class I-specific antibodies were found in three of the four PP patients, but in none of the SP patients. This difference was statistically significant. However, the two groups did not differ significantly with respect to the presence of HLA class II-specific antibodies: 3/7 of SP and 2/4 of PP patients had antibodies (Table 1).

IP-10 and MCP-1 levels were higher in PP patients, whereas sCD40L levels were higher in SP patients

Following transplantation, peripheral blood is an easily accessible sample for biomarker assays. Several cytokine, chemokine, and growth factors in the serum that are known to associate with graft rejection or graft failure were analyzed by the Luminex bead assay.^{4,21-25} The SP patients tended to have higher sCD40L levels, while the PP patients had higher IP-10 and MCP-1 levels (*P* < 0.05) (Figure 1a-c). At this time, sCD40L and IP-10 level from healthy control (HC) and SP exhibited similar levels. And serum IP-10 level was significantly increased in PP compared with HC and SP whereas sCD40L level was decreased (Figure 1a, b). The two groups did not differ with regard to other factors, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-17 A, IL-23, MMP-12, M-CSF, and TGF-β (data not shown).

Figure 1 Serum cytokine/chemokine levels in kidney transplant recipients. The serum levels of sCD40L (a) and IP-10 (b) in HC, SP and PP, and MCP-1 (c) in SP and PP were measured by using a cytometric bead array with Luminex technology. The box plots show the medians and interquartile ranges. Two-sided *P* values when the HC, SP, and PP were compared by using the Mann–Whitney U-test. Hatched boxes, HC; white boxes, SP; gray boxes, PP. (**P* < 0.05, ***P* < 0.005)

The SP and PP groups did not differ in T cell proliferation or cytokine production after indirect or direct MLRs

To mimic the *in vivo* mechanism of indirect allostimulation, indirect MLR assays were performed. In the indirect MLR, recipient T cells were stimulated by recipient DCs that present donor peptides. For direct MLR assays, irradiated donor PBMCs served as stimulators. Recipient T cells were the responder cells in both MLR assays. As a control, PBMCs/DCs from an unrelated healthy volunteer

(designated as the third party) were the stimulators. In the indirect MLR, the SP group tended to have slightly higher T cell proliferation than the PP group and the third party, but there was no statistical difference between SP and PP groups (2401 ± 795 cpm vs. 1811 ± 424 cpm, respectively) (Figure 2a). Also, the direct MLR demonstrated no difference between the SP and PP groups (851 ± 228 cpm vs. 580 ± 148 cpm, respectively) (Figure 2b). Accordingly, in direct and indirect MLR assays, the two groups could not be distinguished on the basis of T cell proliferation.

To characterize the alloreactive T cells further and to obtain a proper overview of actual allo-immune activity, the same T cells used in the MLRs described above were harvested after 24 h of culture and the cytokine production patterns in their supernatants were assessed. In the indirect MLR cultures, the PP group tended to have higher IL-1 β , IL-6, TNF- α , and IFN- γ production than the SP group, although these differences did not achieve statistical significance (Figure 2c–f). By contrast, in the direct MLR cultures, the SP group tended to have higher IL-2, IFN- γ , and TNF- α production than the PP group. The two groups did not differ in other cytokines, including IL-1 β , IL-4, IL-5, IL-10, IL-12(p70), IL-17A, IL-23, and TGF- β , regardless of the allostimulation conditions (data not shown). Overall, direct MLR was associated with a lower cytokine level than indirect MLR.

PP patients showed increased CD4⁺CD43^{high}CD45RO⁺ T cell and regulatory T cell frequencies after indirect allostimulation

Before antigen stimulation, the HC, SP, and PP groups had similar proportions of CD4⁺CD43^{high}CD45RO⁺ T cells ($7.16 \pm 2.78\%$, $9.0 \pm 5.8\%$ vs. $7.5 \pm 6.2\%$, respectively). After allogeneic stimulation for 24 h under indirect MLR conditions, the PP group showed a significantly increased (by 2.7-fold) frequency of CD4⁺CD43^{high}CD45RO⁺ T cells relative to the unstimulated cell frequency (*P* < 0.05) (Figure 3a, b). By contrast, this subset did not change in the SP group. Neither group showed changes of CD8⁺CD43^{high}CD45RO⁺ T cell subsets frequencies after allostimulation (data not shown). The CD4⁺CD43^{high}CD45RO⁺ T cell subsets did not change significantly in either group after direct MLR assay; the values before and after stimulation were $9.0 \pm 5.8\%$ and $7.8 \pm 5.9\%$ for SP patients, and $7.5 \pm 6.2\%$ and $8.8 \pm 6.5\%$ for PP patients, respectively. In addition, SP and PP groups had similar proportions of CD4⁺CD43^{low}CD45RO⁺ T cells ($25.6 \pm 7.4\%$ vs. $19.9 \pm 10.6\%$, respectively). At this time, CD4⁺CD43^{low}CD45RO⁺ T cells in HC exhibited a lower proportion than experimental groups ($6.66 \pm 2.91\%$, data not shown). After indirect allogeneic stimulation, the PP group showed a decreased frequency of CD4⁺CD43^{low}CD45RO⁺ T cells relative to the unstimulated cell frequency. Since CD4⁺CD43^{low}CD45RO⁺ T cells did not change in the SP group after indirect allogeneic stimulation, the frequency of these cells was increased in the SP group compared with the PP group (*P* < 0.05) (data not shown).

Meanwhile, the HC and PP groups had similar proportions of the CD4⁺CD25^{hi}FoxP3⁺ regulatory T cell (Treg) frequency whereas the SP group had lower than others

Figure 2 T cell proliferation and cytokine production after indirect or direct allostimulation in kidney transplant recipients. Purified T cells were used as responder cells in indirect or direct MLRs at a density of 10^5 cells/well with 1×10^4 donor-antigen-pulsed autologous DCs (indirect, a) or 2.5×10^5 donor PBMCs (direct, b) as stimulators. The stimulators were rendered unresponsive to proliferation by cesium irradiation. For third-party allostimulation, PBMCs/DCs from an unrelated healthy volunteer were used. (c–f) Wells from the preceding MLR assays were harvested 24 h after starting the assay and the cytokines in the supernatants were determined. The cytokines IL-2 (c), IL-6 (d), TNF- α (e), and IFN- γ (f) were measured by using Luminex technology. The box plots show medians and interquartile ranges. Mann–Whitney U-tests revealed no significant differences between SP and PP patients

($1.7 \pm 0.6\%$ and $1.8 \pm 0.1\%$, $0.9 \pm 0.8\%$, respectively) (data not shown). Treg cell frequencies in SP patients rose after indirect MLR (from $0.9 \pm 0.8\%$ to $1.4 \pm 0.4\%$), whereas there was no change in PP patients (from $1.8 \pm 0.1\%$ to $1.9 \pm 0.5\%$), although this difference did not achieve statistical significance. Direct MLR conditions yielded similar results (Figure 3c).

The CD4⁺CD43^{high}CD45RO⁺ T cells had effector memory T cell phenotypes

Memory T cells consist of two distinct populations, namely, central memory (T_{CM}) and effector memory (T_{EM}) T cells.²⁶ We examined whether CD4⁺CD43^{high}CD45RO⁺ T cells were T_{EM} cells. Indeed, flow cytometric analysis with antibodies specific for CCR7, CD62L, and CD95 revealed that they were CCR7[−], CD62L[−], and CD95⁺, indicating that they had the T_{EM} phenotype. By contrast, the CD4⁺CD43^{low}CD45RO⁺ T cells were CCR7⁺, CD62⁺, and CD95⁺, indicating that they had the T_{CM} phenotype (Figure 4a).

Closer analysis of the effect of indirect allostimulation of both the T_{EM} and T_{CM} populations in SP and PP groups revealed that the two groups had similar T_{EM} and T_{CM} cell frequencies before allostimulation. When the frequency of allostimulated cells were calculated in ratio to the frequency of unstimulated cells, after indirect allostimulation, the increasing ratio of T_{EM} cell rose more significantly compared with that of the T_{CM} in the PP group ($P < 0.05$), whereas in the SP group, increasing ratio of T_{EM} cell tended to increase compared with that of the T_{CM} . When this parameter was compared between the SP and PP groups, the increasing ratio of T_{EM} tended to increase in the PP group whereas the increasing ratio of T_{CM} did not rise in the SP group (Figure 4c).

One SP patient who manifested elevated creatinine levels displayed an immunological profile similar to that of PP patients

During the experimental period, one patient exhibited an increase in serum creatinine from 1.4 mg/dL to 1.6 mg/dL

Figure 3 Frequencies of CD4⁺CD43^{high}CD45RO⁺ T cells and Tregs in PP and SP patients before and after allostimulation. The naïve and alloantigen-stimulated T cells were simultaneously stained with FITC-CD4, APC-CD45RO, and PE-CD43 to identify memory T cells, and with FITC-CD4, PE-CD25, and APC-FoxP3 to identify Tregs. (a) The frequencies of CD43^{high}CD45RO⁺ cells in the CD4⁺ T cell population of one SP patient (upper panels) and one PP patient (lower panels) after each stimulus regimen are shown. Left panel, before antigen stimulation; middle panels, after indirect allostimulation; right panels, after direct allostimulation. The frequencies of CD4⁺CD43^{high}CD45RO⁺ memory T cells (b) and CD4⁺CD25^{high}FoxP3⁺ Tregs (c) in the eight SP and six PP patients were analyzed by flow cytometry. White boxes, before MLR; gray boxes, after indirect MLR; hatched boxes, after direct MLR. Box plots show medians and interquartile ranges.

Figure 4 Phenotypic analysis of the CD4⁺ CD43^{high} CD45RO⁺ T cells. The CD4⁺ lymphocytes of each patient were gated for CD43^{high}CD45RO⁺ (a) and CD43^{low}CD45RO⁺ (b), and analyzed by multicolor flow cytometry with anti-CCR7, anti-CD62L, and anti-Fas monoclonal antibodies. The results of a representative analysis in one PP patient are shown. (c) After indirect allostimulation, the increasing ratio of post to pre allostimulation of T_{EM} and T_{CM} cells in the SP and PP groups were analyzed (%). PP patients had significantly higher T_{EM} cell, whereas SP patients tended to have higher T_{CM} cell frequencies than PP patients. White box, T_{CM} cells; gray box, T_{EM} cells. The box plots show medians and interquartile ranges. Two-sided *P* values for Mann-Whitney U-tests comparing SP and PP groups are shown (**P* < 0.05).

and showed evidence of chronic changes in a kidney biopsy performed about nine months after the initial blood samplings at 10 years and 6 months after transplantation. This patient was the recipient of a kidney transplant from her husband and was under FK506-based immunosuppression. No acute rejection episodes, CNI toxicity, or donor-specific HLA class I or II antibodies were reported. The serum cytokine/chemokine levels showed the same trend as those in the SP group; IP-10 and MCP-1 levels were lower, whereas sCD40L levels were higher than those in PP patients (data not shown). However, the frequencies of effector memory CD4⁺ T cells increased after indirect allostimulation (data

not shown). Furthermore, in indirect MLR supernatants, IL-2 and TNF- α level were higher than those in SP patients, although IL-6 levels were not (data not shown). Thus, this patient displayed an immunological profile similar to that of PP patients with indirect allostimulation, but lost this profile after direct allostimulation (data not shown).

The preexistence of donor-specific effector memory T cells was associated with a poorer graft outcome

To confirm the effect of preexisting donor-specific memory T cells on graft outcome, we performed the indirect MLR

Figure 5 Pre-transplant donor-specific effector memory T cells are associated with poor kidney graft survival. Graft survival rates of patients with strong proliferation of donor-specific effector memory T cells (donor/3rd party >1.0) were lower than patients with low proliferation of these cells (donor/3rd party <1.0). Kaplan-Meier survival analysis was used to compare the rate of rejection-free grafts and that of poor graft survival, by calculating the donor-specific T cell ratios of their third party controls. A *P* value of <0.05 was considered to be statistically significant

using T cells from pre-transplant recipient blood and donor or third party antigen-pulsed autologous DCs. After indirect MLR, recipients were divided into two groups following CD4⁺CD43^{hi}CD45RO⁺ T cell proliferation efficacy, donor *versus* third party ratio >1.0 and <1.0. In the former group (>1.0), three of nine patients experienced rejection episodes less than two months after transplantation, whereas all of the patients of the latter group (<1.0) did not experience rejection (Figure 5).

Discussion

Many investigators have attempted to identify markers for the immune reaction that would help to diagnose acute rejection and predict CAN or long-term graft acceptance, and distinguish these events from other disorders.^{5,27-31} This approach was also employed in the present study of patients undergoing immunosuppressant treatment with CNI to identify biomarkers that can distinguish patients with well-functioning renal transplants from those with poor renal function.

Humoral immune reactions against allografts generate antibodies specific for donor antigens that are responsible for graft rejection.^{32,33} Lee *et al.* also reported that the development of antibodies within one year after transplantation is associated with graft failure within a mean of 5.1 years. By contrast, antibody development after one year is associated with a lower failure rate, with 80% of these patients surviving for 10 years.¹⁵ De-novo DSAs are mainly class II and that DSAs against class II HLA are associated with a worse prognosis than are DSAs to class I HLA.^{34,35} In the present study, most of the PP patients had HLA class I-specific antibodies, whereas none of the SP patients had these antibodies. The two groups did not differ in terms of HLA class II-specific antibodies. Contrary to the majority of the literature, our results showed an association between graft loss due to CAN and the presence of anti-HLA class I antibodies, but not due to the presence of anti-HLA class II antibodies. Because the timing of their appearance and

significant risk factors differ among reports and the class I DSA also should not be disregarded in the graft rejection, this result requires additional further study.³⁶

Direct allorecognition occurs between the recipient T cell receptor (TCR) and intact MHC molecules expressed on the surface of donor APCs whereas indirect allorecognition occurs between the recipient TCR and peptides of allo-MHC molecules processed by autologous APCs. Vigorous proliferation of recipient T cells *in vitro* can be observed in the presence of irradiated donor APC (direct) or recipient APC (indirect) in the form of the direct and indirect MLR, respectively.¹⁰ When the donor APCs are lost from the graft, the direct alloresponse is stimulated in the early weeks after transplantation. In contrast, indirect pathway T cells are continuously stimulated by recipient APCs and the graft has no means of turning indirect-pathway T cell off.¹⁶ We have investigated the direct and indirect MLR concurrently in a cohort of longstanding renal transplants. Contrary to our expectations, the SP and PP groups could not be distinguished on the basis of T cell proliferation assays after direct or indirect allostimulation. As a result, interest has focused on the changes of activated T cell subsets. In flow cytometry analysis of the activated T cells, the SP group had lower Treg frequencies than the PP group, regardless of the antigenic stimulation method used. However, after indirect allogeneic stimulation, the CD4⁺CD43^{high}CD45RO⁺ T cell frequency of the PP group was significantly increased by 2.7-fold compared with the frequency of unstimulated cells, whereas this change did not occur after stimulation in the SP group.

Since CAN is thought to be mediated mainly by recipient T cells with indirect allospecificity, studies focus on establishing key factors for measuring indirect allorecognition *in vitro*. Autologous DCs pulsed with cellular fragments, dominant epitope on common HLA molecules or synthetic peptides, trigger T cells with indirect allospecificity. Many different approaches are reported, however, most of them lack essential controls and have notoriously poor reproducibility.¹³ Contrarily, several studies have reported that the indirect pathway Elispot positively correlated with graft outcome.^{3,17} Alloantigen-specific CD154⁺ T-cytotoxic memory cells (TcM) in 16 h live-cell MLR are highly sensitive and specific for biopsy proven rejection after renal transplantation.³⁷ Thus, immune monitoring of alloreactive effector/memory T cells primed by indirect alloantigen pathway with a sensitive *in vitro* assay may be helpful for predicting chronic rejection.

In the present study, to delineate the memory T cell population, allogeneic coculture was performed for 24 h, which eliminates non-memory T cells. The donor antigens were fragments derived from donor PBMCs. These antigens can be taken up and processed by recipient DCs. The use of donor cell fragments mimics the *in vivo* conditions in transplantation more faithfully than assays where DCs are pulsed with specific donor HLA peptides.^{12,13} In the T cell response, memory T cells are more resistant to immunosuppressants than naïve T cells and may prevent tolerance-induction by eliciting early-onset graft rejection. These cells can be divided into two general subsets based on their homing properties and effector function. T_{CM} cells

express CCR7 and L-selectin (CD62L) and home to the lymph nodes. When they encounter antigen, they have a limited capacity to perform effector functions. By contrast, T_{EM} cells do not express CCR7, are less proliferative, and achieve their effector function by secreting IFN- γ . When these cells encounter their antigen, they preferentially enter inflamed tissue and immediately perform effector functions.^{38,39} Memory T cells can also be defined in other ways apart from T_{CM} and T_{EM} . These cells fall into three functionally distinct subsets based on their level of CD43 expression, which is a type I transmembrane protein that is heavily expressed on all hematopoietic cells except IgM⁺ B cells and erythrocytes.^{40,41} The CD43^{high} subset appears to consist of fully mature T_{EM} cells that can respond to conventional recall antigens in response to TCR stimulation. The other two subsets, CD43^{int} and CD43^{low}, exhibit marginal memory function and have an anergic phenotype.^{41,42} Thus, CD4⁺CD43^{high}CD45RO⁺ T cells were regarded as T_{EM} cells. To confirm that the CD4⁺CD43^{high}CD45RO⁺ T cells belong to T_{EM} cells, their phenotype was analyzed with antibodies specific for CD62L, CD95 (Fas), and CCR7. The CD4⁺CD43^{high}CD45RO⁺ T cells had a T_{EM} phenotype (CCR7⁻, CD62⁻, and CD95⁺), while the CD4⁺CD43^{low}CD45RO⁺ T cells had a T_{CM} phenotype (CCR7⁺, CD62⁺, and CD95⁺) (Figure 4).

Both groups were maintained on standard CNI immunosuppression consisting of CsA or FK506 administration. The SP group was more likely to have CsA-based immunosuppression (7/8 patients), while the PP group was more likely to have FK-based immunosuppression (4/6 patients). We speculate that the lower T_{EM} cell frequency in the SP group is related to CsA-based immunosuppression. FK506 and CsA, although structurally unrelated, appear to target similar signal transduction pathways in lymphoid cells by inhibiting the action of calcineurin, a serine/threonine phosphatase. Both drugs inhibit the differentiation of naïve T cells into cytokine-producing memory T cells.⁴³ However, Koenen *et al.* reported that CsA and rapamycin co-treatment results in elevated memory T cell responses, whereas FK506 and rapamycin co-treatment reduces and delays memory responses.⁴⁴ This factor does not seem to be the cause of the reduced T_{EM} cell frequency in the SP group.

During the course of this study, we excluded two SP patients from the initial group because their blood creatinine levels became elevated. About nine months after the initial blood sampling, creatinine levels increased from 1.4 mg/dL to 1.6 mg/dL in one patient and a biopsy revealed chronic changes in the kidney. In another patient, the serum creatinine level increased from 1.5 mg/dL to 1.8 mg/dL one month after the initial sampling, and mild chronic changes and severe CNI toxicity were observed in a kidney biopsy. The immunological properties of this patient, including T cell number in PBMCs and T cell proliferation after indirect MLR, were not different from those in the SP group as a whole (data not shown). Interestingly, the frequency of effector memory CD4⁺ T cells was increased after indirect allostimulation, resulting in a situation similar to that observed in PP patients (data not shown). However, the patient did not have any donor-specific HLA class I or II antibodies. The serum

cytokine levels also showed the same trend as those in the SP group, namely, high expression of sCD40L and low expression of IP-10 and MCP-1. This result suggests that effector memory CD4⁺ T cells may provide a more sensitive indicator of the patient's condition than DSA.

Further studies were performed to extend these findings and address the key events involved in the progression of rejection via effector memory CD4⁺ T cells. To analyze the effect of preexisting donor-specific memory T cells on graft outcome, we performed indirect MLR using T cells in pre-transplant recipient blood with donor or third party antigen-pulsed autologous DCs. Three of nine patients with a strong proliferation of T_{EM} cells experienced rejection episodes less than two months after transplantation, whereas all the patients with low proliferation of T_{EM} cells did not experience rejection (Figure 5). From this result, we suggest that the preexistence of donor-specific T_{EM} cells is associated with progression to acute rejection.

This single center study aimed to identify a specific immunological monitoring marker for antigen-specific assays that distinguish a long-term well-functioning graft from CAN and predict rejection episodes after a renal allograft. CAN was associated with elevated numbers of donor-specific CD4⁺CD43^{high}CD45RO⁺ T cells that had the T_{EM} phenotype (CCR7⁻, CD62⁻, and CD95⁺). Thus, it is possible that an increase in CD4⁺ T_{EM} cell frequency after indirect allostimulation is associated with the development of CAN. Moreover, in the prospective study, preexisting donor specific CD4⁺ T_{EM} cells in the pre-transplant patients was associated with progression to acute rejection. Because acute rejection contributes to the development of CAN and CD4⁺ T_{EM} cells were closely associated with CAN in our retrospective data, we suggested that indirectly allostimulated CD4⁺ T_{EM} cells may not only contribute to CAN development but also to the progression of acute rejection. Although the study was limited by the small size of the study group, monitoring this cell population could provide an immune-monitoring marker in noninvasive *in vitro* assays designed to predict graft outcome.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript: Y-MW wrote the manuscript, Y-MW, Y-HK, M-YC and D-JH conducted the experiments, J-HJ and Y-HK participated in the collection of human samples and data interpretation, D-SC participated in the data analysis, and M-HC and D-JH involved in the data and review of the manuscript.

ACKNOWLEDGMENT

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: a grant (A084886 and A111950) from the Ministry for Health, Welfare, and Family Affairs in South Korea.

DECLARATION OF CONFLICTING INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

- Grinyo JM, Bestard O, Torras J, Cruzado JM. Optimal immunosuppression to prevent chronic allograft dysfunction. *Kidney Int* 2010;**78**:S66–70
- de Fijter JW. Rejection and function and chronic allograft dysfunction. *Kidney Int* 2010;**78**:S38–41
- Nickel P, Bestard O, Volk HD, Reinke P. Diagnostic value of T-cell monitoring assays in kidney transplantation. *Curr Opin Organ Transplant* 2009;**14**:426–31
- Brehm MA, Mangada J, Markees TG, Pearson T, Daniels KA, Thornley TB, Welsh RN, Rossini AA, Greiner DL. Rapid quantification of naive alloreactive T cells by TNF-alpha production and correlation with allograft rejection in mice. *Blood*. 2007;**109**:819–26
- Brouard S, Mansfield E, Braud C, Li L, Giral M, Hsieh SC, Baeten D, Zhang M, Ashton-Chess J, Braudeau C, Hsieh F, Dupont A, Pallier A, Moreau A, Louis S, Ruiz C, Salvatierra O, Souillou JP, Sarval M. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A* 2007;**104**:15448–53
- Wallgren AC, Andersson B, Backer A, Karlsson-Parra A. Direct allorecognition promotes activation of bystander dendritic cells and licenses them for Th1 priming: a functional link between direct and indirect allosensitization. *Scand J Immunol* 2005;**62**:234–42
- van Besouw NM, Zijderwijk JM, Vaessen LM, Balk AH, Maat AP, van der Meide PH, Weimar W. The direct and indirect allogeneic presentation pathway during acute rejection after human cardiac transplantation. *Clin Exp Immunol* 2005;**141**:534–40
- Okumi M, Fishbein JM, Griesemer AD, Gianello PR, Hirakata A, Nobori S, et al. Role of persistence of antigen and indirect recognition in the maintenance of tolerance to renal allografts. *Transplantation* 2008;**85**:270–80
- Ford ML, Kirk AD, Larsen CP. Donor-reactive T-cell stimulation history and precursor frequency: barriers to tolerance induction. *Transplantation* 2009;**87**:S69–74
- Lechler RI, Garden OA, Turka LA. The complementary roles of deletion and regulation in transplantation tolerance. *Nat Rev Immunol* 2003;**3**:147–58
- Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, Lechler RI. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* 2001;**167**:7199–206
- Schlienger K, Craighead N, Lee KP, Levine BL, June CH. Efficient priming of protein antigen-specific human CD4(+) T cells by monocyte-derived dendritic cells. *Blood* 2000;**96**:3490–8
- Waanders MM, Heidt S, Koekkoek KM, Zoet YM, Doxiadis II, Amir A, Heemskerk MH, Mulder A, Brand A, Roelen DL, Claas FH. Monitoring of indirect allorecognition: wishful thinking or solid data? *Tissue Antigens* 2008;**71**:1–15
- Tambur AR. Monitoring indirect presentation of alloantigens by utilizing the autologous processing machinery of dendritic cells in-vitro. *J Immunol Methods* 2003;**283**:215–23
- Lee PC, Zhu L, Terasaki PI, Everly MJ. HLA-specific antibodies developed in the first year posttransplant are predictive of chronic rejection and renal graft loss. *Transplantation* 2009;**88**:568–74
- Hornick PI, Mason PD, Baker RJ, Hernandez-Fuentes M, Frasca L, Lombardi G, Taylor K, Weng L, Tose ML, Yacoub MH, Batchelor R, Lechler RI. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation* 2000;**101**:2405–10
- Bestard O, Nickel P, Cruzado JM, Schoenemann C, Boenisch O, Seifrin A, Grinyo JM, Volk HD, Reinke P. Circulating alloreactive T cells correlate with graft function in longstanding renal transplant recipients. *J Am Soc Nephrol* 2008;**19**:1419–29
- Jeras M, Bergant M, Repnik U. In vitro preparation and functional assessment of human monocyte-derived dendritic cells-potential antigen-specific modulators of in vivo immune responses. *Transplant Immunol* 2005;**14**:231–44
- Bender A, Sapp M, Schuler G, Steinman RM, Bhardwaj N. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J Immunol Methods* 1996;**196**:121–35
- Pei R, Lee JH, Shih NJ, Chen M, Terasaki PI. Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities. *Transplantation* 2003;**75**:43–9
- Thakker P, Leach MW, Kuang W, Benoit SE, Leonard JP, Marusic S. IL-23 is critical in the induction but not in the effector phase of experimental autoimmune encephalomyelitis. *J Immunol* 2007;**178**:2589–98
- Famulski KS, Einecke G, Reeve J, Ramassar V, Allanach K, Mueller T, Hidalgo LG, Zhu LF, Halloran PF. Changes in the transcriptome in allograft rejection: IFN-gamma-induced transcripts in mouse kidney allografts. *Am J Transplant* 2006;**6**:1342–54
- Aharinejad S, Krenn K, Zuckermann A, Schafer R, Gmeiner M, Thomas A, Aliabadi A, Schneider B, Grimm M. Serum matrix metalloproteinase-1 and vascular endothelial growth factor—a predict cardiac allograft rejection. *Am J Transplant* 2009;**9**:149–59
- Goriely S, Goldman M. The interleukin-12 family: new players in transplantation immunity? *Am J Transplant* 2007;**7**:278–84
- Giavedoni LD. Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using luminex technology. *J Immunol Methods* 2005;**301**:89–101
- Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004;**22**:745–63
- Zhou L, Tougouz M, Donckier V, Andrien M, Troisi R, de Hemptinne B, Le Moine A, Dupont E, Goldman M, Stordeur P. A rapid test to monitor alloreactive responses in whole blood using real-time polymerase chain reaction. *Transplantation* 2005;**80**:410–3
- Guitard J, Ribes D, Kamar N, Cointault O, Lavayssiere L, Esposito L, Rostaing L, Muscari F, Suc B, Peron JM. Predictive factors for chronic renal failure one year after orthotopic liver transplantation. *Ren Fail* 2006;**28**:419–25
- Peng W, Chen J, Jiang Y, Shou Z, Chen Y, Wang H. Non-invasive detection of acute renal allograft rejection by measurement of vascular endothelial growth factor in urine. *J Int Med Res* 2007;**35**:442–9
- Slavcev A, Honsova E, Lodererova A, Pavlova Y, Sajdlova H, Vitko S, Skibova J, Striz I, Viklicky O. Soluble CD30 in patients with antibody-mediated rejection of the kidney allograft. *Transpl Immunol* 2007;**18**:22–7
- Sawitzki B, Bushell A, Steger U, Jones N, Risch K, Siepert A, Lehmann M, Schmitt-Knosalla I, Vogt K, Gebuhr I, Wood K, Volk HD. Identification of gene markers for the prediction of allograft rejection or permanent acceptance. *Am J Transplant* 2007;**7**:1091–102
- Lachmann N, Terasaki PI, Budde K, Liefeldt L, Kahl A, Reinke P, Pratschke J, Rudolph B, Schmidt D, Salama A, Schronemann C. Anti-human leukocyte antigen and donor-specific antibodies detected by luminex posttransplant serve as biomarkers for chronic rejection of renal allografts. *Transplantation* 2009;**87**:1505–13
- Riethmuller S, Ferrari-Lacraz S, Muller MK, Raptis DA, Hadaya K, Rusi B, Laube G, Schneider G, Fehr T, Villard J. Donor-specific antibody levels and three generations of crossmatches to predict antibody-mediated rejection in kidney transplantation. *Transplantation* 2010;**90**:160–7
- Jordan SC, Vo AA. Donor-specific antibodies in allograft recipients: etiology, impact and therapeutic approaches. *Curr Opin Organ Transplant* 2014;**19**:591–7
- Hidalgo LG, Campbell PM, Sis B, Einecke G, Mengel M, Chang J, Sellares J, Reve J, Halloran PF. De novo donor-specific antibody at the time of kidney transplant biopsy associates with microvascular pathology and late graft failure. *Am J Transplant* 2009;**9**:2532–41
- Filippone EJ, Farber JL. Humoral immune response and allograft function in kidney transplantation. *Am J Kidney Dis*. Epub ahead of print 2015
- Ashokkumar C, Shapiro R, Tan H, Ningappa M, Elinoff B, Fedorek S, Sun Q, Higgs BW, Randhawa P, Humar A, Sindhi R. Allospecific CD154+ T-cytotoxic memory cells identify recipients experiencing acute cellular rejection after renal transplantation. *Transplantation* 2011;**92**:433–8

38. Jones ND. Memory T cells: how might they disrupt the induction of tolerance? *Transplantation* 2009;**87**:S74–7
39. Yang J, Brook MO, Carvalho-Gaspar M, Zhang J, Ramon HE, Sayegh MH, Wood KH, Turka LA, Jones ND. Allograft rejection mediated by memory T cells is resistant to regulation. *Proc Natl Acad Sci U S A*. Epub ahead of print 2007
40. Seo W, Ziltener HJ. CD43 processing and nuclear translocation of CD43 cytoplasmic tail are required for cell homeostasis. *Blood* 2009;**114**:3567–77
41. Ohara T, Koyama K, Kusunoki Y, Hayashi T, Tsuyama N, Kubo Y, Kyoizumi S. Memory functions and death proneness in three CD4+CD45RO+ human T cell subsets. *J Immunol* 2002;**169**:39–48.
42. Hikono H, Kohlmeier JE, Takamura S, Wittmer ST, Roberts AD, Woodland DL. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J Exp Med* 2007;**204**:1625–36
43. Tsuda K, Yamanaka K, Kitagawa H, Akeda T, Naka M, Niwa K, Nakanishi T, Kakeda M, Gabazza EC, Mizutani H. Calcineurin inhibitors suppress cytokine production from memory T cells and differentiation of naive T cells into cytokine-producing mature T cells. *PloS One* 2012;**7**:e31465
44. Koenen HJ, Michielsen EC, Verstappen J, Fasse E, Joosten I. Superior T-cell suppression by rapamycin and FK506 over rapamycin and cyclosporine A because of abrogated cytotoxic T-lymphocyte induction, impaired memory responses, and persistent apoptosis. *Transplantation* 2003;**75**:1581–90

(Received May 3, 2015, Accepted July 20, 2015)