Journal of Nephrology & Therapeutics
ISSN: 2161-0959

Gandolfo et al., J Nephrol Therapeutic 2011, S3
DOI: 10.4172/2161-0959.S3-005

Research Article Open Access

Lower Γδ T Cell Number As a Possible Risk Factor for Cytomegalovirus Activation in Renal Transplanted Patients.

Maria Teresa Gandolfo[1], Loredana Alberti[1], Antonio Leoni[1], Luisa Berardinelli[2], Andrea Artoni[3], Giovanna Lunghi[4], Deborah Mattinzoli[1,5], Maria Pia Rastaldi[1,5] and Piergiorgio Messa[1]*

[1]*Department of Nephrology, Dialysis and Renal Transplant, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 28, Milan, 20122, Italy.*
[2]*Renal Transplant Surgery, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 28, Milan, 20122, Italy.*
[3]*Department of Medicine and Medical Specialities, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 28, Milan, 20122, Italy.*
[4]*Virology Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 28, Milan, 20122, Italy.*
[5]*Nephrology Research Laboratory–Fondazione D'Amico, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 28, Milan, 20122, Italy*

Abstract

The role of gamma-delta (γδ) T cells in renal transplantation (RT) is not well defined. The aim of the present study was to investigate the kinetics of peripheral blood γδ T cells in the first year after RT and the relationships between γδ T cells and the main clinical events.

In 31 kidney transplanted patients, peripheral blood T cell phenotype, as well as clinical and biochemical parameters, included Cytomegalovirus (CMV) DNA assessment, were serially evaluated. Total numbers of γδ T cells increased from day 90 onward, as compared with baseline ($p<0.01$-0.05); CD8α expression on γδ T cells increased transiently as well, with higher percentages on day 60 and 90 ($p<0.05$ versus day 0). T cell numbers inversely correlated with serum creatinine values in renal transplanted patients during the first year after RT ($p<0.001$).

Sixteen of the kidney transplanted patients developed CMV activation. These patients presented lower γδ T cell numbers and percentages in the early period after RT, preceding CMV activation, compared to patients that never experienced it (γδ T cell numbers: day 15 $p<0.01$, day 30 $p<0.05$; percentages: day 0, 15, 30 and 45 $p<0.05$). After CMV activation, γδ T cells expanded, achieving the levels of patients without CMV activation; transient increased CD8α expression was observed in these patients on day 90 compared to baseline ($p<0.05$) and on day 60 and 90 compared to patients that did not developed CMV activation ($p<0.05$). Three out of 31 patients experienced an acute rejection episode. No unique behaviour of γδ T cells was observed in them.

Our results suggest that lower γδ T cell numbers and percentages might represent a risk factor for developing CMV activation in the early period after RT.

Keywords: γδ T cell; Lymphocyte; Renal transplantation; Cytomegalovirus

Introduction

γδ T cells and the immune system

Gamma-delta (γδ) T cells represent a minority of T cells that express T Cell Receptor (TCR) composed of γ and δ chains, instead of the widespread α and β chains (αβ T cells) [1-2]. In the peripheral blood of healthy individuals, γδ T cells account for the 0.5-6% of total circulating T cells, but represent a more substantial fraction of T lymphocytes in specific anatomical localizations, such as the small intestinal mucosae [3-5]. The majority (50-95%) of circulating γδ T cells presents the same TCR V region pair Vγ9-Vδ2 and is generally CD4 and CD8 negative, on the contrary in the small intestine γδ T cells express Vδ1 and CD8αα homodimer [6-8].

Though γδ T and αβ T cells have several homologies, γδ T lymphocytes have peculiar functional characteristics [9-10]. Specific TCR-mediated antigen recognition by γδ T cells does not necessarily require peptide presentation by professional antigen-presenting cells (APCs) [11-13]; γδ T lymphocytes in fact more often recognize unconventional antigens such as lipids and phosphorylated microbial antigens that do not need for presentation to be linked to HLA I and II.

Moreover, γδ T cells directly contribute to the innate immune response, through NKG2D (a member of the natural cytotoxicity receptors) and Toll-like receptors [14-16], by which they can recognize stress- or tumor- induced self-antigens and viral and bacterial antigens [17-19]. They have also been shown to act as professional APCs, by presenting peptides to conventional αβ T cells [20-22]. Finally, recent evidences demonstrated that human peripheral γδ T lymphocytes are a potent type of regulatory T cells, capable of T helper cell suppression [23].

γδ T cells and renal transplantation

The behaviour of γδ T cells after renal transplantation (RT) and their possible role in the main clinical complications of transplanted patients (namely, acute and chronic rejection or infections) are far from having been defined. Initially it was suggested that γδ T cells did not play any role in RT [24-25]. More recently, sparse reports showed similar numbers of peripheral γδ T cells in stable patients after RT compared to controls; however, reduction in $CD8^+$ γδ T cell numbers

***Corresponding author:** Piergiorgio Messa, Department of Nephrology, Dialysis and Renal Transplant - Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via Commenda 15, Milan, 20122, Italy, Tel: 0039 0255034551/2; Fax: 0039 0255034550; E-mail: pmessa@policlinico.mi.it

Received November 21, 2011; **Accepted** December 19, 2011; **Published** December 21, 2011

Citation: Gandolfo MT, Alberti L, Leoni A, Berardinelli L, Artoni A, et al. (2011) Lower Γδ T Cell Number As a Possible Risk Factor for Cytomegalovirus Activation in Renal Transplanted Patients. J Nephrol Therapeutic S3:005. doi:10.4172/2161-0959.S3-005

was observed in patients suffering from acute or chronic rejection compared to the patients with stable evolution and controls, suggesting a possible role for these cells in allograft mantainance [26].

Consistently, oral exposure to donor antigens induced the generation of regulatory T cells, that were represented by IL-10 secreting $CD8^+$ γδ T cells, and determined prolongation of kidney allograft survival in a rat model [27].

γδ T cells and CMV infection

Human cytomegalovirus (CMV) is a widespread β-herpesvirus with primary infection being followed by life-long persistence in the human host. Generally CMV infection is asymptomatic in immunocompetent individuals but can determine life-threatening consequences in immunologically immature or compromised individuals. Previous reports demonstrated that, in both immunocompetent and immunocompromised patients, increased blood levels of $V\delta2^-$ γδ T cells with phenotype of effector/memory cells was a specific signature of CMV infection [28-29].

In the specific field of renal transplantation, a dramatic expansion of γδ T cells was observed in the peripheral blood of kidney transplanted patients who experienced CMV infection [30-32]. Expansion of γδ T cells involved only the Vδ1 and Vδ3 subsets, while the size of Vγ9-Vδ2 population was not altered [28-29, 33]. Moreover, expansion was associated with γδ T cell activation and in particular higher expression of CD8α antigen, one of the hallmarks of intraepithelial T cell activated state [29].

Objective

The aim of the current observational prospective study was, initially, to define the kinetics of peripheral blood γδ T cells in kidney transplant recipients during the first year after RT.

We found that γδ T lymphocytes increased in the peripheral blood of kidney allograft recipients starting from day 90 after RT; the expression of CD8α on γδ T cells increased transiently as well, with higher percentages on day 60 and 90 compared to baseline.

Subsequently we explored the possibility of a relationship between γδ T cell changes and the main clinical events in kidney allograft recipients. We demonstrated that γδ T cells were reduced in the early period after RT in patients that were going to experience CMV activation and that they expanded after the activation itself, showing transient increased CD8α expression.

Patients and Methods

Patients

From the 1st of April 2008 to 31st of December 2008, 33 patients were submitted to cadaveric donor and 4 patients to living donor RT in our Transplant Centre. Of these 37 renal transplanted patients, 31 allograft recipients were included in the present study, after giving their informed consent. Six cadaveric donor recipients were not included in the study due to organizational problems or to the lack of consent by the patient.

The 31 patients included in the study had the following characteristics: 12 females; mean age 45.4±12.4 years (range: 17-62 years); 27 received a cadaveric donor kidney, 2 a living related and 2 a living unrelated donor kidney. The median value for HLA-match was 2 and for HLA-mismatch 4. The causes of the pre-transplant renal disease were: autosomic dominant polycystic kidney disease (9 patients), IgA nephropathy (3 patients), chronic glomerulonephritis (1 patient), chronic pyelonephritis (5 patients), extracapillary glomerulonephritis (1 patient), membranous glomerulonephritis (1 patient) pre-eclampsia (1 patient), IgM nephropathy (1 patient), diabetic nephropathy (1 patient), hypertensive nephroangiosclerosis (1 patient), nephropathy of unknown origin (3 patients), CAKUT (1 patient) and renal stone disease (1 patient). Before transplantation, the patients spent 52.3±30.4 months on dialytic treatment (range 3-132 months).

Twenty-six of these patients were recipients of a first graft, the remaining 5 patients of a second graft. All kidney transplanted patients were submitted to the immune-suppressive regimen in use in our centre (basiliximab, tacrolimus, mofetil mycophenolate, steroids). No patients received ATG or monoclonal antibodies, other than basiliximab, as induction therapy or to treat acute rejection episodes.

At the time of RT, all the studied patients had undetectable CMV-DNA levels (<500 copies/ml). Six recipients were CMV-IgG negative at time of RT and had received a graft from a CMV-IgG positive donor. They all were submitted to prophylactic treatment with valgancyclovir over 45 days after RT. The remaining 25 recipients were CMV-IgG positive at the time of RT.

Nineteen healthy subjects were included as normal controls (NC, 7 females, age 35.4±10.4 years, range 23-55 years). Nine chronic dialysis patients (DC; 3 females, age 49.0±2.8 years, range 39-67 years) present on our waiting list for renal transplantation were also considered as controls.

Study design

We conducted a prospective observational study. All the enrolled patients were submitted to routine biochemical evaluation according to the protocol in use in our Unit. In addition, fasting peripheral blood samples were collected in EDTA-containing vials for T cell subset assessment, at the time of surgery (time 0) and thereafter on day 15, 30, 45, 60, 90, 120, 180 and 360 after transplantation. At the same observational times, CMV-DNA titer and clinical related parameters were simultaneously and serially evaluated. Any major clinical event was also recorded.

Antibodies and flow cytometry analysis

The following murine anti-human fluorochrome-labeled monoclonal antibodies (mAbs) were obtained from BD Biosciences (San José, CA, USA): anti-CD3 PerCP (SK7), anti-CD4 FITC (RPA-T4), anti-CD8 APC (α chain; RPA-T8), anti-αβ TCR PE (T10B9,1A-31), anti-γδ TCR PE (B1).

Peripheral blood samples from patients were collected in EDTA and lymphocyte count was determined using an automatic hemocytometer (ABX Diagnostics, ROCHE, Milan, Italy). Fifty µl of each sample were incubated for 10 minutes with lysing solution (BD Biosciences), and then centrifugated at 1500 x g for 5 minutes. Supernatant was discarded and the cells were resuspended in 50 µl of Phosphate Buffered Saline. The different conjugated mAbs were added to the cell suspension at the appropriate concentration in accordance with the manufacturer's instructions and incubated for 30 minutes at 4°C. Background fluorescence levels were established using correspondent conjugated isotype antibodies (BD Biosciences). Four-color immunofluorescence

staining was analyzed using FACSCalibur instrument and CellQuest software (BD Biosciences). Lymphocytes were gated using forward and side scatter and 5,000 events were acquired in each assay. T cell subsets are expressed either as percentages of $CD3^+$ T lymphocytes or as absolute numbers/µl of blood, that were calculated by multiplying the absolute lymphocyte count by the percentages obtained by flow cytometry analysis (Figure 1).

CMV DNA assessment

CMV DNA was extracted from whole blood with an Automated Nucleic Acid Extraction Kit (EasyMag Biomerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. CMV DNA quantitative real time PCR was carried out with CMV Alert (Nanogen, Torino, Italy) according to the manufacturer's instructions.

Clinical definitions

Diagnosis of acute rejection was proven by renal biopsy and scored according to Banff Classification [34], in the presence of an acute reduction of renal function.

CMV-DNA activation was defined as the presence of any confirmed positive CMV-DNA titer that was at least two-fold higher compared to the threshold of the method (method threshold = 500 copies/ml). CMV infection was defined as the appearance of clinical symptoms associated with CMV activation, that required specific therapy (gancyclovir or valgancyclovir). Evaluated Glomerular Filtration Rate (eGFR) was calculated according to the Cockcroft-Gault formula.

Statistical analyses

Results are presented as mean ± standard deviation. Data were analysed by t-test for paired data and ANOVA for repeated measures, with post-hoc analysis. To study the relationship between two normally distributed variables, linear correlation analysis was used. The log transformation of some variables was performed when they were not normally distributed. A p value ≤ 0.05 was considered statistically significant.

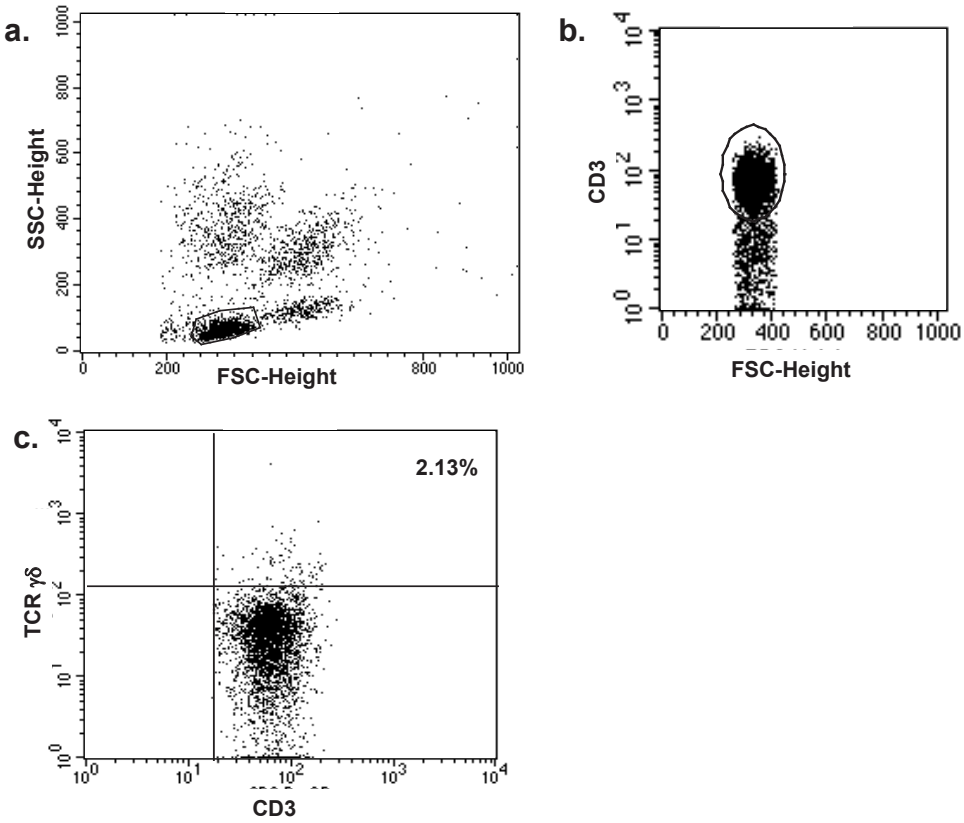
Results

Renal function in renal transplanted patients during the first year after RT

The 31 kidney transplanted patients admitted in the study were monitored for 360 days (see *Materials and Methods*). Kidney function data (creatinine, eGFR, proteinuria) were recorded at each time of observation and are presented in (Table 1). Recorded haemoglobin levels are shown in (Table 1) as well.

Total numbers of leucocytes and lymphocytes in renal transplanted patients during the first year after RT

Total numbers of leucocytes significantly increased in kidney



a. Lymphocytes were initially gated according to forward- and side-scatter height (and here identified by the circle in the dot plot). **b.** $CD3^+$ T cell subset was identified in the lymphocyte area (and here identified by the circle in the dot plot). **c.** Subsequently γδ expression was examined in this population; the percentage of **γδ** T cells was obtained in the upper right quadrant in the dot plot.

Figure 1: Gating strategy for γδ T cells.

transplanted patients on day 15 after RT, as compared with time 0 and with controls (NC and DC). No difference was observed in leucocytes numbers on day 30 and on the following time-points, in comparison with day 0 significant difference persisted until day 30 between transplanted patients and all controls (NC and DC) and until day 45 between transplanted patients and NC only (Table 2).

Total numbers of lymphocytes was significantly lower at time 0, as compared with any other observation time and with NC; no significant difference was observed in comparison with DC (Table 2).

T lymphocytes in renal transplanted patients: γδ T cell numbers increase after 90 days from RT

We subsequently focused our attention on $CD3^+$ T cells and the different T cell subsets. Similarly to total lymphocytes, T cell total numbers and $TCR\alpha\beta^+$ (αβ) T cell numbers were significantly lower at time 0, as compared with any other observation time and with NC. However, no difference in the percentage of αβ among total T cells was observed during the whole observational study and in comparison with controls (Table 2).

Total number of $TCR\gamma\delta^+$ (γδ) T cells, on the contrary, significantly increased from day 90 to the last time-point, as compared with basal levels (Table 2), with an increase of the percentage values at the later observation times compared with the early ones (p<0.05 day 120 *versus* day 30; p =0.02, day 180 and day 360 *versus* day 30). The expression of CD8α, marker of intraepithelial T cell activation, transiently increased on γδ T cells, with higher percentages on day 60 and 90 compared to baseline (day 0: 25.5±11.0%, day 60: 29.2±12.7% day 90: 30.2±14.5%, p<0.05).

γδ T cell numbers inversely correlate with serum creatinine values in renal transplanted patients during the first year after RT

At any observation time, γδ T cell numbers, pooled from all patients, were significantly and inversely related to serum creatinine values, by a logarithmic relationship (log γδ T = 4.55 -0.48 x Creat; r= -0.230; p<0.001). The correlation persisted also when the patients suffering from a rejection episode were excluded from the analysis (p = 0.02). However, when the relationship between γδ T cells and eGFR was considered, a significant correlation was no longer observed (r = 0.072; p = 0.309). To better explore this apparent contradiction, we checked for the possible influence of body weight, age, and gender (variables which are entered in the eGFR calculation) on γδ T cells. We found that all these three variables were significantly related to γδ T cell numbers, with older, male and higher body weight patients having the lowest γδ T cell numbers (age: r = -0.277, p < 0.001; body weight : r = -0.272, p<0.001; gender: F 6.28, p = 0.01). At multivariate analysis level, however, only age (p < 0.001) and body weight (p<0.01) significantly affected γδ T cells numbers.

Kidney transplanted patients: time after renal transplantation (Days)									
	0	**15**	**30**	**45**	**60**	**90**	**120**	**180**	**360**
Serum creatinine (mg/dl)	7.3 ±2.9	1.0 ±0.5#	1.4±0.4#	1.4 ±0.4#	1.4±0.5#	1.4 ±0.4#	1.4 ±0.4#	1.4 ±0.5#	1.4 ± 0.4#
u-prot (g/day)	1.2±1.5	0.5 ±1.1#	0.2 ±0.1#	0.3 ±0.3#	0.3 ±0.4#	0.3 ±0.2#	0.2±0.1#	0.3 ±0.3#	0.4± 0.5#
eGFR (ml/min/1.73 m²)	13.3 ±6.8	60.5 ±15.8#	60.6 ±16.9#	60.5 ±17.5#	59.7 ±16.5#	61.4 ±17.7#	60.0 ±22.8#	60.4 ±19.8#	61.8 ± 18.4#
Hb (g/dl)	10.1 ±1.5	10.5 ±1.8	11.4 ±1.4	11.6 ±1.5	12.0 ±1.7#	12.6 ±1.6#	12.4 ±1.6#	12.2 ±1.6#	12.7 ± 1.9#

Means±SD. # p<0.001 versus Day 0

Table 1: Renal graft function and haemoglobin levels in renal transplanted patients in the first year after transplantation.

Kidney transplanted patients: time after renal transplantation (Days)										DC	NC
	0	**15**	**30**	**45**	**60**	**90**	**120**	**180**	**360**		
Total leukocytes (cells/μL) [a]	7474 ±3107	9735 ±2786	8159 ±2922	7803 ±2465	7168 ±1839	7008 ±2146	6263 ±2165	6748 ±1553	6659±1580	6122 ±1180	6300 ±1434
		°°° *** ^^	° *	*							
Total lymphocytes (cells/μL) [a]	947 ±423	1803 ±888	1724 ±789	1733 ±920	1686 ±704	1711 ±732	1804 ±821	1890 ±613	1855± 679	1422 ±446	1784 ±425
	***	^^^	^^^	^^^	^^^	^^^	^^^	^^^	^^^		^^^
T cells (cells/μL) [a]	677 ±356	1415 ±767	1375 ±667	1360 ±739	1327 ±600	1383 ±658	1429 ±743	1532 ±555	1506± 635	1052 ±351	1345 ±371
	***	^^^	^^^	^^^	^^^	^^^	^^^	^^^ °	^^^ °		^^^
αβ T cells (cells/μL) [a]	627 ±329	1311 ±715	1294 ±626	1259 ±685	1241 ±570	1268 ±606	1293 ±656	1405 ±526	1396± 612	942 ±308	1220 ±361
	*	^^	^^^	^^^	^^^	^^^	^^^	^^^ °	^^^ °		^^
αβ T cells (%) [b]	92.5 ±5.0	92.3 ±4.9	94.0 ±4.3	92.7 ±5.0	93.3 ±4.8	91.7 ±6.2	90.8 ±7.1	91.3 ±6.7	92.4± 6.9	90.2 ±7.1	91.4 ±4.7
γδ T cells (cells/μL) [a]	34.7 ±43.9	59.9 ±57.9	53.9 ±47.1	61.2 ±60.0	61.6 ±43.9	80.6 ±98.2	96.3 ±116.1	96.4 ±85.9	74.8 ± 60.2	81.8 ±83.3	75.3 ±45.9
						^	^^	^^	^^		
γδ T cells (%) [b]	4.8 ±4.9	4.6 ±4.0	4.1 ±3.5	4.5 ±3.6	4.8 ±3.2	5.7 ±5.8	6.5 ±5.7	6.6 ±5.7	5.6± 4.4	7.3 ±6.7	5.6 ±3.1

Means± SD. [a] absolute cell count/μL of blood; [b] results are expressed as % of $CD3^+$ T cells; * p<0.05 ** p<0.01 *** p<0.001 vs NC ; ° p<0.05 °° p<0.01 °°° p<0.001 vs DC ^ p<0.05 ^^ p<0.01 ^^^ p<0.001 vs Time 0.

Table 2: Peripheral blood total leucocytes, total lymphocytes and T lymphocyte subsets in kidney transplanted patients in the first year after transplantation, in normal controls (NC) and chronic dialysis patients (DC).

T lymphocyte subsets and CMV activation in renal transplanted patients: lower γδ T cell percentages are present in CMV-activated patients in the early period post-RT

In 16 of the 31 studied patients, some degree of CMV activation (DNA copies > 1,000/ml) was observed (45,386 ± 101,014 copies/ml) (CMV-act); three of them were CMV-IgG negative at the time of RT, received a kidney from a CMV-IgG positive donor and were submitted to prophylaxis with valgancyclovir. The mean first CMV activation time occurred at about 45 days after RT (45.9±22.9 days; range: day 15-90). Ten out of the 16 CMV-act patients reached CMV-DNA levels >20,000 copies/ml and were therefore treated with valgancyclovir until CMV-DNA was undetectable, according to the policy in course at the time of the beginning of the study protocol in our centre. None of the CMV-act patients developed biochemical and/or general clinical symptoms related to CMV infection.

We analyzed the changes in the total numbers and percentages of T cell subsets in both CMV-act and CMV-neg patients. We did not observe any difference in αβ T cells, expressed as total numbers or percentages of T cells, between the two groups during the whole study period (Table 3).

We then studied the changes in γδ T cells in both CMV-act and CMV-neg patients. Significant difference in γδ T cell total numbers between CMV-act and CMV-neg patients was found on day 15 and day 30 (before any activation was evident), when γδ T cells were significantly lower in CMV-act patients compared with CMV-neg (day 15: CMV-neg 79.6±60.4 cells/μl, CMV-act 29.7±16.9 cells/μl, $p< 0.01$; day 30: CMV-neg 71.2±56.6 cells/μl, CMV-act 34.0±21.7 cells/μl, $p< 0.05$); γδ T cell numbers were similar between the groups at the other observation times. When we focused our attention on the percentages of this specific T subsets, we observed that CMV-act patients presented significant lower γδ T cell percentages compared to CMV-neg patients during the first 45 days after RT (Figure 2). The percentage of γδ T cells in the CMV-act group increased over time, with values significantly higher at the later observation times compared with earlier ones ($p<0.05$ day 90 versus day 15; $p=0.02$ day 120 and 180 versus day 15; $p<0.05$ day 180 versus day 30). On the contrary, the percentages of γδ T cells in CMV-neg patients remained stable throughout the entire observation period.

Furthermore, taking in consideration only the 16 CMV-act patients, we grouped the assessed γδ T cell percentages in five main periods:

		Kidney transplanted patients: time after renal transplantation (Days)								
		0	15	30	45	60	90	120	180	360
αβ T cells (cells/μL) [a]	CMV act	619±249^	1238±812	1131±628	1309±740	1285±601	1363±677	1332±724	1425±565	1509±712
	CMV neg	693±561^	1390±613	1445±580	1219±597	1246±530	1184±487	1263±568	1389±481	1290±504
αβ T cells (%) [b]	CMV act	94.4±2.4	93.8±2.8	95.8±3.0	94.4±3.0	94.0±3.0	92.7±3.8	92.5±5.1	92.6±4.6	92.0±8.4
	CMV neg	92.2±6.4	90.8±6.2	93.2±5.1	91.5±6.1	93.4±6.1	91.9±7.6	90.8±8.1	91.2±8.0	92.8±5.4

Means± SD. [a] absolute cell count/μL of blood; [b] results are expressed as % of $CD3^+$ T cells;

^ = $p<0.05$ versus all the other observation times

Table 3: Total numbers and percentages of peripheral αβ T cells in kidney transplanted patients who experienced CMV activation (CMV-act) and patients who did not (CMV-neg) in the first year after renal transplantation.

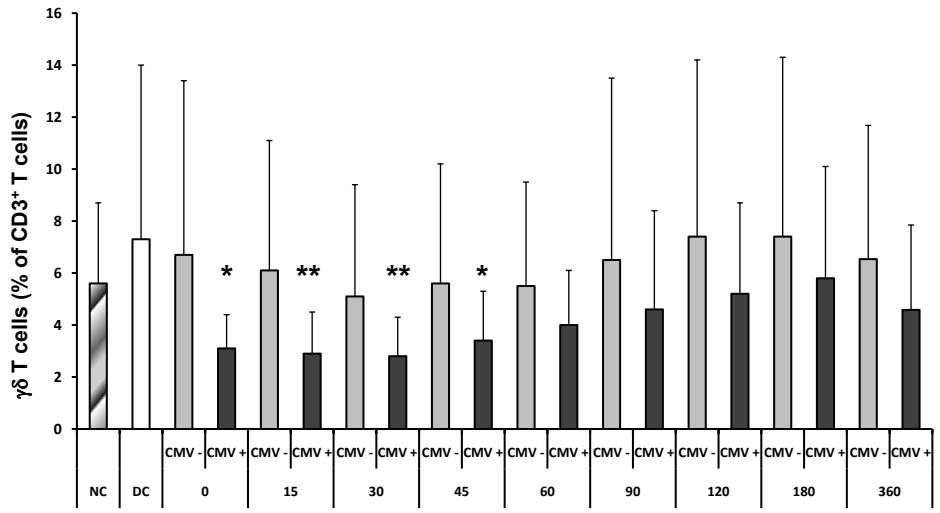


Figure 2: Percentages of γδ T cells in CMV-act (CMV +) and in CMV-neg (CMV -) patients in the year after renal transplantion (RT), in normal controls (NC) and chronic dialyzed patients (DC).

Significant difference in γδ T cell percentages between CMV-act and CMV-neg patients was observed in the first 45 days after RT. Mean ± SD; * $p<0.05$, ** $p<0.01$ *versus* CMV-neg

The percentage of γδ T cells in the CMV-act group increased over time, with values significantly higher at the later observation times compared with earlier ones ($p<0.05$ day 90 *versus* day 15; $p=0.02$ day 120 and 180 *versus* day 15; $p<0.05$ day 180 *versus* day 30).

the first corresponding to times not immediately preceding CMV activation (basal); the second one to the time immediately preceding CMV activation (pre-activation); the third one to the period when CMV activation was present (activation); the fourth one to the time immediately after the negativization of CMV (early negativization) and the fifth one corresponding to observation times occurring later in the negativization process (late negativization) (Figure 3). A significant increase in γδ T cell percentages was observed in the early negativization period.

We next evaluated the expression of CD8α on γδ T cells in the two different groups (Figure 3). In CMV-neg patients, the level of CD8α expression remained stable over time; on the contrary, in CMV-act patients, we observed a transient increase in CD8α expression on day 90 compared to baseline ($p<0.05$); moreover the percentage of $CD8^+$ γδ T cells was increased also on day 60 and 90 compared to the observation times within the first month after RT (day 60 versus day 15: $p<0.01$, day 90 versus day 15: $p=0.01$, day 90 versus day 30: $p<0.001$). Consequently the percentage of $CD8\alpha^+$ γδ T cells was significantly higher in CMV-act

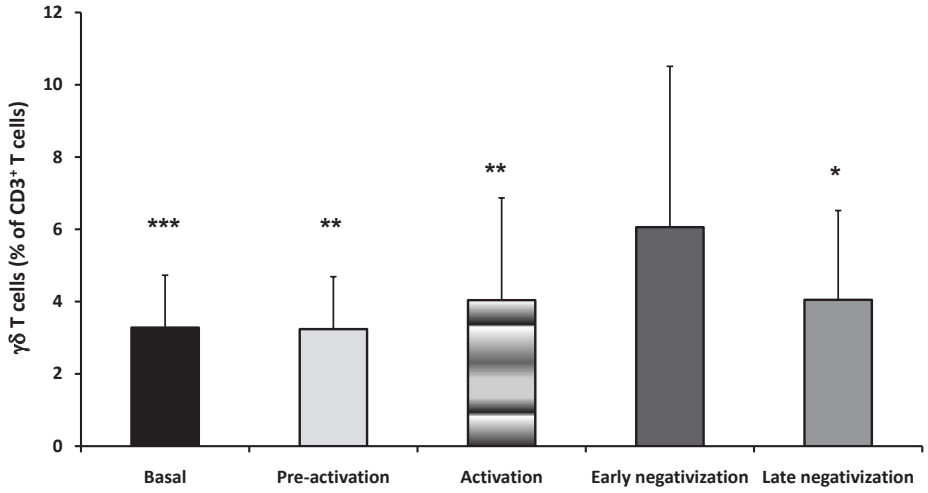


Figure 3: Percentage of γδ T cells in the 16 CMV-act patients at different periods of CMV activation.

The assessed γδ T cell percentages in the 16 CMV-act patients were grouped in five main periods: the first corresponding to times not immediately preceding CMV activation (basal); the second one to the time immediately preceding CMV activation (pre-activation); the third one to the period when CMV activation was present (activation); the fourth one to the time immediately after the negativization of CMV (early negativization) and the fifth one corresponding to observation times occurring later in the negativization process (late negativization.

A significant increase in γδ T cell percentages was observed in the early negativization period.

Mean ± SD; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, all vs early negativization period.

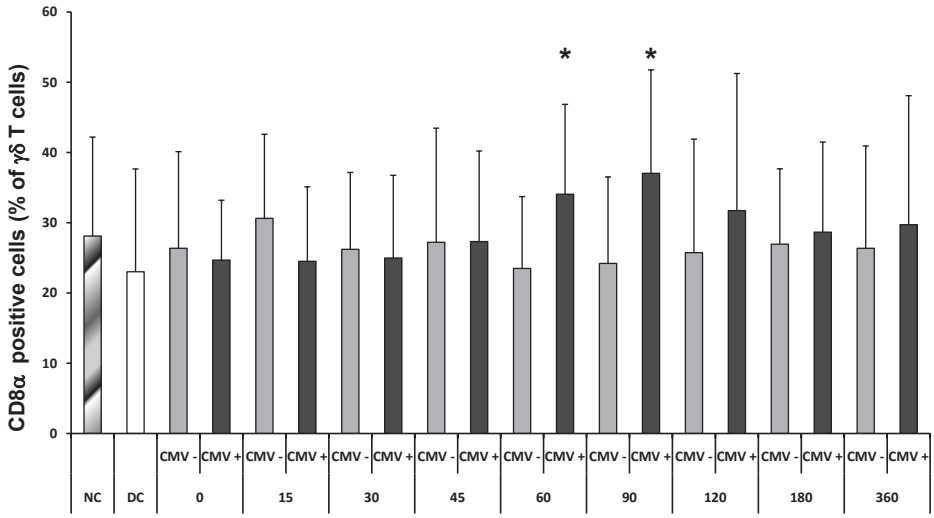


Figure 4: CD8α expression on γδ T cells in in CMV-act (CMV +) and in CMV-neg (CMV -) patients in the year after renal transplantion (RT), in normal controls (NC) and chronic dialyzed patients (DC).

The percentage of $CD8\alpha^+$ γδ T cells was significantly higher in CMV-act compared to CMV-neg patients on day 60 and 90 after RT. In CMV-neg patients, the level of CD8α expression remained stable over time; on the contrary, in CMV-act patients, we observed a transient increase in CD8α expression on day 90 compared to baseline ($p<0.05$). Moreover the percentage of $CD8^+$ γδ T cells was increased also on day 60 and 90 compared to the observation times within the first month after RT (day 60 *versus* day 15: $p<0.01$, day 90 *versus* day 15: $p=0.01$, day 90 *versus* day 30: $p<0.001$).

Mean ± SD; * $p<0.05$ *versus* CMV-neg.

		Kidney transplanted patients: time after renal transplantation (Days)								
		0	**15**	**30**	**45**	**60**	**90**	**120**	**180**	**360**
αβ T cells (cells/μL) [a]	**Inf. patients**	634±496^	1188±593	1317±652	1210±571	1162±469	1200±429	1250±553	1356±439	1333±495
	Not-Inf. patients	690±384^^	1401±796	1300±596	1313±749	1482±574	1345±728	1337±714	1451±582	1448±705
αβ T cells (%) [b]	**Inf. patients**	94.2±4.9	92.9±4.6	95.4±2.8	93.8±6.0	93.9±5.5	92.3±6.0	92.7±7.3	92.8±6.9	93.2±4.9
	Not-Inf. patients	91.9±5.0	91.9±5.2	93.5±5.2	92.2±3.9	93.5±4.0	92.1±6.5	90.2±6.6	91.0±6.5	91.8±8.3
γδ T cells (cells/μL) [a]	**Inf. patients**	20.6±15.1	54.3±54.9	39.2±32.3	53.0±68.1	46.0±38.9	67.9±82.3	79.8±119.2	77.1±100.4	73.53±70.0
	Not-Inf. patients	47.4±57.4	60.1±61.2	62.2±53.9	65.4±49.9	71.2±44.5	84.8±109.7	99.2±106.8	102.0±68.0	116.75 ± 171
γδ T cells (%) [b]	**Inf. patients**	3.5±3.1	4.3±3.6	2.80±1.57	3.95±4.1	4.26±3.4	5.26±5.82	5.37±6.1	5.18±5.8	5.43±4.3
	Not-Inf. patients	5.68±6.0	4.76±4.5	4.87±4.3	4.77±3.1	4.73±3.1	5.43±5.4	6.74±5.1	7.13±5.5	7.55±8.3

Means± SD. [a] absolute cell count/μL of blood; [b] results are expressed as % of $CD3^+$ T cells; ^ p<0.05, ^^ p<0.01 versus all the other observation times

Table 4: Peripheral αβ and γδ T cells in kidney transplanted patients who experienced at least one non-CMV infectious episode (Inf.) or no infections (not-Inf.) in the first year after transplantation.

compared to CMV-neg patients on day 60 and 90 after RT (Figure 4).

T subset numbers and percentages are not related to not-CMV infectious episodes in renal transplanted patients

Over the study period, 13 out of the 31 patients had at least one not-CMV infectious episode: 6 patients were affected by isolated urinary tract infections (UTI); 2 patients by pulmonary infections; 2 patients by cutaneous infections; 2 patients had both UTI and pulmonary infections and 1 patient cutaneous infection plus UTI.

We studied the behaviour of αβ and γδ T cells in the group of the 13 infected (Inf.) patients and in the remaining 18 patients who did not develop any not-CMV infection during the observation period (not-Inf.). No significant difference in the total numbers and in the percentages of αβ and γδ T cells was observed between the 2 groups at any time during the study (Table 4).

γδ T cell percentages and rejection episodes in renal transplanted patients

During the observational period, 3 episodes of biopsy-proven renal allograft acute cellular rejection were observed in 3 patients (2 patients on the 15th day; one patient on the 60th day after RT). They were all treated with pulse methylprednisolone successfully. Due to the extremely low number of patients affected by rejection, no significant correlations were observed between the percentages of γδ T lymphocytes before and during the rejection episodes and the episode itself.

The percentage value of γδ T cells before the rejection episodes in fact was low (0.67%), normal (3.77 %), and high (11.36%) as compared with the mean values of the entire group in each of these three patients respectively. After the rejection episodes, the percentage of γδ T cells levels increased in one patient (day 0: 3.77% day 15: 18.03%) and remained stable in the other two.

Discussion

These data demonstrated that γδ T cells increased in the peripheral blood of kidney transplanted patients starting from day 90 after RT and showed a transient increase in CD8α expression on day 60 and 90. We showed that γδ T cells were reduced in the early period after RT in patients that were going to experience CMV, expanding after CMV activation with transient increased CD8α expression.

The small subset represented by γδ T cells play a multi-faceted role in the immune defence, combining properties of both adaptive and innate immunities [9, 11, 35]. γδ T cells have been considered as cells of immunosurveillance playing a significant role in the immunity against pathogens and tumors. These cells can also act as professional APCs and behave as regulatory T cells, in part through the enhancement of dendritic cell maturation and the induction of T helper polarization [20, 36].

The role of γδ T cells in RT has not been extensively studied; few studies in fact have analysed the kinetics of these cells after RT and their possible role in the modulation of the main clinical events which occur after RT.

Expansion of γδ T cells in the peripheral blood of transplanted patients was observed by Volk et al. [24], but without any clear association with specific clinical outcomes. The presence of γδ T lymphocytes was not increased in the fine-needle aspiration biopsies from transplanted patients in the early period after RT [25]. Moreover, a significant decrease in the percentage of γδ T cells was observed in peripheral blood in the first year after transplantation.

In our study, we evaluated the kinetics of T cell subsets in the peripheral blood in the first year after RT. We observed a significant increase in γδ T cell numbers starting from day 90 and continuing at the later end-points. On the other hand, αβ T cells were lower on day 0 and then immediately increased, maintaining stable values until the end of the study period. Whether these results were related to the direct effect of immunological mounting response after RT or to the effects of immunosuppressive therapy cannot be defined on the basis of the results of this and previous studies.

We next observed that, not only γδ T cell numbers increased at the end of the first trimester, but also a transient increased activation status was present on day 60 and 90, as suggested by the increased expression of CD8α at those end-points.

In murine models of chronic nephropathy the proportion of γδ T cells was positively related to histological damage and serum creatinine; depletion of γδ T cells resulted in worsened kidney damage and function, suggesting a role of these lymphocytes in the regulation of inflammation [37].

In the current study, in the first year after RT, we demonstrated that γδ T cell numbers in the peripheral blood were inversely related to serum creatinine levels. A relationship between γδ T cell numbers

and renal function was however not substantiated since the correlation was no longer significant if eGFR was used instead of serum creatinine. We therefore hypothesized that the relationship with creatinine was an indirect one. In fact, in the calculation of eGFR by the CockCroft-Gault formula, in addition to creatinine, also age, body weight and gender are taken into account. Both age and gender have been reported to affect γδ T cells [38]; in our patients, γδ T cell numbers were related to age and gender, with older male patients having the lower numbers. In addition, we also found that patients with higher body weight had unexpectedly lower γδ T numbers; by multivariate analysis, age and body weight were the only variables significantly related with γδ T cell numbers. Subsequently we suggested that the association of lower γδ T cell numbers with higher creatinine concentration was mainly based on the dependence of the latter on body mass more than on renal function.

After demonstrating expansion of γδ T cells with transient increase of activation status during the first year after RT, we investigated the possible link between γδ T cell kinetics and the main clinical events which follow RT.

A marked increase in circulating γδ T cells (from <5 up to 50% of total T cells) has been described in kidney transplanted patients with active CMV infection [30]. CMV infection has also been reported to be characterized by long-term expansion of effector/memory γδ T cells in patients irrespective of the immunological competence [28-29]. These studies clearly state for a crucial role for γδ T cells in the adaptive immune response against CMV.

In our study, we consistently observed a significant increase in γδ T cell percentages in patients that had developed CMV activation; in contrast, patients that did not experience increase of CMV titer showed stable γδ T cell percentages. No difference, on the other hand, was observed in αβ T cell numbers and percentages at each observation time between the groups. Moreover, in CMV-act patients, a transient increased activation status of γδ T cells, as defined by CD8α expression, was observed in the late first trimester, when CMV activation had already taken place in the majority of the recipients.

Intriguingly, we also proved that the patients that were going to develop CMV activation were characterized by lower γδ T cell numbers and percentages in the very early period after RT, preceding the virus activation itself, compared to patients that never developed it. After CMV activation, γδ T cells expanded, but simply achieving the percentages present in CMV-neg patients. These data point out for the first time to the possibility that lower γδ T cell numbers and percentages may represent a risk factor for CMV activation in the early period after RT.

Déchanet et al. previously demonstrated that $V\delta2^-$ γδ T cell subset was the one involved in the immune response against CMV and was characterized by increased expression of CD8α, a specific marker of intraepithelial lymphocyte activation [29]. A limit of our study was not to phenotype the different γδ T cell subsets. Our data about CD8α expression indirectly pointed out the prevalent role of $V\delta2^-$ T .

Thirteen of our patients developed a non-CMV infection during the first year after RT. No significant difference was evident between patients with and without infection at any observation time as far as either αβ or γδ T cells numbers and percentages were analyzed.

The importance of γδ T cells in cellular rejection has been debated in previous studies. Malan Borel et al. [26] suggested a protective role for these cells, [26], since they observed a significant decrease of a sub-group of γδ T cells (CD8 γδ) in the peripheral blood of patients with acute or chronic rejection, in contrast with patients with stable allograft function who maintained unchanged CD8 γδ cell percentages. On the other hand, Raasveld et al. [25] did not find a role for γδ T cells in the acute rejection episodes occurring in kidney transplant recipients.

Only 3 out of 31 patients enrolled in our study experienced an acute rejection episode. No unique behaviour of γδ T cells could be observed in these 3 patients, and, due to the small sample, no conclusion can be drawn on the possible association between γδ T cell variations and acute renal rejection.

In summary, we conducted a small prospective study to carefully investigate the kinetics of peripheral blood γδ T cells during the first year after RT and to record the possible relationship between γδ T cell numbers and percentages and the main clinical events occurring in this period.

Despite the limitations due to small numbers, our results suggest that lower γδ T cell numbers and percentages in kidney transplanted patients can predispose to the development of CMV activation. Further studies on larger cohorts of patients and the analysis of the different γδ T cell subsets might confirm our preliminary results and give some further insights.

Role of Funding Source

This study was supported by the grant 'Project Glomerulonephritis' in memory of Pippo Neglia.

Disclosure Statement

The authors have no financial conflict of interest.

References

1. Brenner MB, Strominger JL, Krangel MS (1988) The gamma delta T cell receptor. Adv Immunol 43: 133-192.
2. Shin S, El-Diwany R, Schaffert S, Adams EJ, Garcia KC, et al. (2005) Antigen recognition determinants of gammadelta T cell receptors. Science 308: 252-255.
3. Carding SR, Egan PJ (2002) Gammadelta T cells: functional plasticity and heterogeneity. Nat Rev Immunol 2: 336-345.
4. Hayday A, Tigelaar R (2003) Immunoregulation in the tissues by gammadelta T cells. Nat Rev Immunol 3: 233-242.
5. Girardi M (2006) Immunosurveillance and immunoregulation by gammadelta T cells. J Invest Dermatol 126: 25-31.
6. Deusch K, Luling F, Reich K, Classen M, Wagner H, et al. (1991) A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment. Eur J Immunol 21: 1053-1059.
7. Spits H, Paliard X, Vandekerckhove Y, van Vlasselaer P, de Vries JE (1991) Functional and phenotypic differences between CD4+ and CD4- T cell receptor-gamma delta clones from peripheral blood. J Immunol 147: 1180-1188.
8. Morita CT, Verma S, Aparicio P, Martinez C, Spits H, et al. (1991) Functionally distinct subsets of human gamma/delta T cells. Eur J Immunol 21: 2999-3007.
9. Modlin RL, Sieling PA (2005) Immunology. Now presenting: gammadelta T cells. Science 309: 252-253.
10. Brandes M, Willimann K, Lang AB, Nam KH, Jin C, et al. (2003) Flexible migration program regulates gamma delta T-cell involvement in humoral immunity. Blood 102: 3693-3701.
11. Morita CT, Mariuzza RA, Brenner MB (2000) Antigen recognition by human gamma delta T cells: pattern recognition by the adaptive immune system. Springer Semin Immunopathol 22: 191-217.
12. Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, et al. (2003) Microbial isoprenoid biosynthesis and human gammadelta T cell activation. FEBS Lett 544: 4-10.

13. Morita CT, Jin C, Sarikonda G, Wang H (2007) Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev 215: 59-76.

14. Konigshofer Y, Chien YH (2006) Gammadelta T cells - innate immune lymphocytes? Curr Opin Immunol 18: 527-533.

15. Wrobel P, Shojaei H, Schittek B, Gieseler F, Wollenberg B, et al. (2007) Lysis of a broad range of epithelial tumour cells by human gamma delta T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. Scand J Immunol 66: 320-328.

16. Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, et al. (2006) Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. J Immunol 176: 1348-1354.

17. Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, et al. (2000) Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. J Exp Med 191: 937-948.

18. Tanaka Y, Morita CT, Nieves E, Brenner MB, Bloom BR (1995) Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. Nature 375: 155-158.

19. Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, et al. (2003) Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 197: 163-168.

20. Brandes M, Willimann K, Moser B (2005) Professional antigen-presentation function by human gammadelta T Cells. Science 309: 264-268.

21. Moser B, Brandes M (2006) Gammadelta T cells: an alternative type of professional APC. Trends Immunol 27: 112-118.

22. Moser B, Eberl M (2007) Gammadelta T cells: novel initiators of adaptive immunity. Immunol Rev 215: 89-102.

23. Kuhl AA, Pawlowski NN, Grollich K, Blessenohl M, Westermann J, et al. (2009) Human peripheral gammadelta T cells possess regulatory potential. Immunology 128: 580-588.

24. Volk HD, Reinke P, Neuhaus K, Fiebig H, von Baehr R, et al. (1989) Expansion of a CD 3+4-8- TCR alpha/beta- T lymphocyte population in renal allograft recipients. Transplantation 47: 556-558.

25. Raasveld MH, Bloemena E, Surachno S, ten Berge RJ (1992) T gamma/delta lymphocytes in renal transplant recipients. Nephrol Dial Transplant 7: 530-533.

26. Malan Borel I, Racca A, Garcia MI, Bailat A, Quiroga F, et al. (2003) Gammadelta T cells and interleukin-6 levels could provide information regarding the progression of human renal allograft. Scand J Immunol 58: 99-105.

27. Zhou J, Appleton SE, Stadnyk A, Lee TD, Nashan BA (2008) CD8+ gammadelta T regulatory cells mediate kidney allograft prolongation after oral exposure to alloantigen. Transpl Int 21: 679-687.

28. Pitard V, Roumanes D, Lafarge X, Couzi L, Garrigue I, et al. (2008) Long-term expansion of effector/memory Vdelta2-gammadelta T cells is a specific blood signature of CMV infection. Blood 112: 1317-1324.

29. Dechanet J, Merville P, Lim A, Retiere C, Pitard V, et al. (1999) Implication of gammadelta T cells in the human immune response to cytomegalovirus. J Clin Invest 103: 1437-1449.

30. Dechanet J, Merville P, Berge F, Bone-Mane G, Taupin JL, et al. (1999) Major expansion of gammadelta T lymphocytes following cytomegalovirus infection in kidney allograft recipients. J Infect Dis 179: 1-8.

31. Merville P, Dechanet J, Berge F, Bone-Mane G, Taupin J, et al. (2000) Cytomegalovirus infection in kidney allograft recipients is followed by a prolonged expansion of gammadelta T lymphocytes. Transplant Proc 32: 357-359.

32. Lafarge X, Merville P, Cazin MC, Berge F, Potaux L, et al. (2001) Cytomegalovirus infection in transplant recipients resolves when circulating gammadelta T lymphocytes expand, suggesting a protective antiviral role. J Infect Dis 184: 533-541.

33. Halary F, Pitard V, Dlubek D, Krzysiek R, de la Salle H, et al. (2005) Shared reactivity of V{delta}2(neg) {gamma}{delta} T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. J Exp Med 201: 1567-1578.

34. Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, et al. (2007) Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant 7: 518-526.

35. Beetz S, Wesch D, Marischen L, Welte S, Oberg HH, et al. (2008) Innate immune functions of human gammadelta T cells. Immunobiology 213:173-82.

36. Casetti R, Martino A (2008) The plasticity of gamma delta T cells: innate immunity, antigen presentation and new immunotherapy. Cell Mol Immunol 5: 161-170.

37. Wu H, Wang YM, Wang Y, Hu M, Zhang GY, et al. (2007) Depletion of gammadelta T cells exacerbates murine adriamycin nephropathy. J Am Soc Nephrol 18: 1180-1189.

38. Caccamo N, Dieli F, Wesch D, Jomaa H, Eberl M (2006) Sex-specific phenotypical and functional differences in peripheral human Vgamma9/Vdelta2 T cells. J Leukoc Biol 79: 663-666.

This article was originally published in a special issue, **Clinical Nephrology** handled by Editor(s). Dr. Yoshiyuki Morishita, Jichi Medical University, Japan