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CMV-associated CD4+CD28null cells in NKG2D-dependent glomerular endothelial injury and kidney allograft dysfunction

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Abbreviations: CMV, cytomegalovirus; eGFR, estimated glomerular filtration rate; hs-CRP, high sensitivity C reactive protein, GENC, glomerular endothelial cells; EMRA, effector memory cells re-expressing RA; PBMCs, peripheral blood mononuclear cells

ABSTRACT

Emerging data suggests that expansion of a circulating population of atypical, cytotoxic CD4$^+$ T-cells lacking costimulatory CD28 ("CD4$^+$CD28$^{null}$" cells) is associated with latent CMV infection. The purpose of the current study was to increase the understanding of the relevance of these cells in 100 unselected kidney transplant recipients followed prospectively for a median of 54 months. Multicolour flow cytometry of PBMCs prior to transplantation and serially post-transplantation was undertaken. CD4$^+$CD28$^{null}$ cells were found predominantly in CMV-seropositive patients, and expanded in the post-transplant period. These cells were predominantly effector-memory phenotype, and expressed markers of endothelial homing (CX3CR1) and cytotoxicity (NKG2D and perforin). Isolated CD4$^+$CD27 CD28$^{null}$ cells proliferated in response to PBMCs previously exposed to CMV-derived (but not HLA-derived) antigens, and following such priming incubation with glomerular endothelium resulted in signs of endothelial damage and apoptosis (release of fractalkine and von Willebrand factor; increased caspase 3 expression). This effect was mitigated by NKG2D-blocking antibody. Increased CD4$^+$CD28$^{null}$ cell frequencies were associated with delayed graft function, and lower eGFR at end follow-up. This study suggests an important role for this atypical cytotoxic CD4$^+$CD28$^{null}$ cell subset in kidney transplantation, and points to strategies that may minimize the impact on clinical outcomes.
INTRODUCTION

Cytomegalovirus (CMV) is a ubiquitous β-herpesvirus which may cause significant clinical disease in transplanted patients. It is also now appreciated that CMV can exert a range of “indirect effects”, including allograft dysfunction, vasculopathy and glomerulopathy, although the mechanisms underlying these phenomena are unclear (1-3).

Latent CMV infection in patients with end-stage renal disease (ESRD) is associated with expansion of circulating, late-differentiated, cytotoxic CD4+ T-cells. These are characterised by absence of the costimulatory molecule CD28 from the cell surface (“CD4+CD28null”) (4). Although also recognised in patients with autoimmune conditions such as multiple sclerosis, rheumatoid arthritis and vasculitis (5-7), these CD4+CD28null cells are unresponsive to candidate autoantigens (5, 6), but rather respond to CMV-derived proteins. Furthermore, one study demonstrated emergence of CD4+CD28null cells in 4 kidney transplant recipients following primary CMV infection (8), although evaluation of these cells’ reactivity to alloantigen has not yet been studied.

In contrast to CD28-expressing CD4+ cells (“CD4+CD28+”), CD4+CD28null cells express cytotoxic mediators such as perforin (4, 5, 8), and cells isolated from patients with acute coronary syndromes promote damage to human umbilical vein endothelial cells (HUVEC) in vitro (9). Interestingly, exposure of CD4+ cells to CMV antigens (but not other viral antigens) promotes expansion of CD4+ cells expressing the killer lectin-like receptor NKG2D (10, 11), also uncommonly expressed on CD4+ T-cells (10). Upon ligation, NKG2D initiates an intracellular cascade culminating in perforin exocytosis and consequent cytotoxicity.

The purpose of this study was to gain greater understanding of the epidemiology, biology, and clinical consequences of these cytotoxic CD4+ cells in the relatively understudied field of kidney transplantation. We evaluated kidney transplant patients from the time of transplantation,
undertaking serial examination of circulating CD4^+CD28^{null} cell frequencies, demonstrating a close relationship with CMV-serostatus. These cells proliferated to CMV-derived antigens, but not to HLA-derived antigens, and demonstrated toxicity to glomerular endothelial cells in vitro, highlighting the role of NKG2D. Elevated cell frequencies were associated with relevant clinical outcomes to 5 years post-transplantation.

**CONCISE METHODS**

**Clinical Cohort**

One hundred unselected recipients of solitary kidney transplants were enrolled into this prospective study during 2009. All patients underwent transplantation and follow-up at Queen Elizabeth Hospital Birmingham. Local ethics committee approval was granted for the study.

Prior to transplantation, baseline donor and recipient information was collected: donor and recipient age and sex, inflammatory cause of renal failure (glomerulonephritis; vasculitis; lupus), HLA mismatch (class I and class II), source of transplant (live related; live unrelated; deceased donor following brain death [DBD]; deceased donor following cardiac death [DCD]); dialysis modality prior to transplantation (pre-emptive versus haemodialysis versus peritoneal dialysis). Transplantation proceeded provided the cross match between donor and recipient was negative by flow cytometry and cytotoxicity.

Immunosuppression regimen was identical for all participants, and consisted of Basiliximab induction followed by maintenance tacrolimus (prograf; trough level 5-8ng/ml initially, measured by liquid chromatography-tandem mass spectrometry), mycophenolate mofetil (cellcept; 2g daily initially) and prednisolone (20mg daily, reducing to 5mg maintenance by 3 months post transplantation). Data collected subsequently in the course of standard clinical care was evaluated:
Post operative events of delayed graft function (requirement for dialysis during the first post operative week) and biopsy proven acute rejection (any time; any histological grade) were collected.

Estimated glomerular filtration rate (eGFR) was calculated using the 4 variable MDRD (Modification of Diet in Renal Disease) equation, with IDMS (Isotope Dilution Mass Spectrometry) aligned creatinine measurements; early morning urine albumin:creatinine ratio (UACR) measurement on a “spot” urine sample was used as the measure of proteinuria, and high sensitivity C-reactive protein (hsCRP) as the marker of inflammation. These were collected at each clinic review following transplantation.

**Assessment of CMV serostatus, infection, and disease**

Pre-transplant CMV serostatus of the recipients and their donors was collected. CMV prophylaxis with 100 days of valganciclovir was given to the D+R- group only, with dose adjustment for renal function. Serial whole blood samples were taken for CMV DNA PCR in all patients at day 0 (prior to transplantation), and then weeks 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 34, 40, 46, and 52. The clinical team remained unaware of these results and no changes in clinical management ensued (it is not unit policy to undertake viral load testing and pre-emptive therapy in the context of asymptomatic CMV infection, as is the case in most centers, and so these assessments were undertaken for research purposes only). Additional sampling was undertaken at the time of clinical suspicion of CMV disease, which was then diagnosed according to international guidelines and was based on one or more of the following in association with the finding of CMV viraemia: fever; new onset severe malaise; leucopaenia; thrombocytopenia; hepatitis (alanine transaminase or aspartate transaminase levels greater than twice the upper limit of normal); tissue invasive disease proven by histology. For our laboratory, a copy rate of >500 CMV genome copies/ml of whole blood represents significant CMV viraemia.
Patients who were CMV seronegative at the time of transplantation underwent repeat serological testing at 12 months post transplantation to identify those who have developed asymptomatic infection within the first year, for whom no DNAemia was detected by protocolised testing as above.

**Immunophenotyping**

Multicolour flow cytometry was used to undertake detailed phenotyping of patients’ peripheral blood mononuclear cells (PBMCs) prior to transplantation and then 14 days, 3 months, and 12 months post-transplantation. PBMCs were isolated from heparinised blood of the transplant recipient by density gradient centrifugation using Ficoll-Paque (Fisher, UK) and cryopreserved in fetal calf serum (FCS) containing 10% DMSO. This was performed within 6 hours following venepuncture.

Based on surface expression of CD27 and CD45RA, the broad differentiation status of CD4⁺CD27null cells and their CD4⁺CD27⁺ counterparts was defined as follows. Naïve-like: CD27⁺CD45RA⁺; Central memory-like (CM): CD27⁺CD45RA⁻; Effector memory-like (EM): CD27⁻CD45RA⁺; Effector memory cells re-expressing RA (“EMRA”): CD27⁻CD45RA⁺. The panels of antibodies used for flow cytometric analyses are outlined in the supplementary data.

**Isolation of CD4⁺CD27⁻CD28null and CD4⁺CD27⁻CD28⁺ Cells**

At 12-months post transplantation, heparinised blood was taken from patients shown to have a high CD4⁺CD27⁻CD28null cell count (>10% total CD4⁺ T-cells) and PBMCs were isolated by Ficoll-Paque (Fisher, UK) gradient. CD4⁺CD27⁻CD28null and CD4⁺CD27⁻CD28⁺ cells were isolated first by positive selection using a CD4⁺ T Cell Isolation Kit, followed by selection of CD27 negative cells (using anti-CD27 human microbeads) and separation of CD28⁺ and CD28null cells within this population using a CD28 Microbead Kit (Miltenyl Biotec, UK), in accordance to manufacturer’s instructions. These cells were used in 2 experiments to investigate i) their proliferative capacity in response to
CMV-antigen and HLA-derived peptides, and ii] their ability to induce damage on purified glomerular endothelial cells, as described next.

**i] Proliferation Assays (CMV- and HLA-derived peptides)**

CD4^+^CD27^−^CD28^null^ and CD4^+^CD27^−^CD28^pos^ sorted cells were labeled with 0.5µM CFSE (Molecular Probes/Invitrogen) in PBS for 5 min at 37°C followed by 5 min in ice-cold RPMI 1640 containing 5% human AB serum (Sigma, UK). Cells were then seeded at 10^5^ with 10^5^ antigen-pulsed irradiated autologous PBMCs in RPMI 1640 containing 5% human AB serum and incubated at 37^0^C with 5% CO_2_ for 5 days. These autologous PBMCs had been pulsed for 4h prior to irradiation with either i) CMV lysate [from fibroblasts infected with CMV strain AD69], ii) α_3^-domain derived Class-I HLA peptides (also used in ELISPOT assay described below; Thermo, UK,) or iii) control culture media. Cells were surface stained with CD3-APC, CD4-APC-Cy7 (eBioscences) and Yellow Dead Cell Stain Kit ((Molecular Probes/Invitrogen), then analysed using a Cyan Flow Cytometer (Beckman Coulter).

The α_3^-domain derived Class-I HLA peptides used in the second part of this experiment are known targets for cellular alloresponses, and their use allows inter-patient assay standardization (20,21). The responding cells are CD28-expressing effector-memory CD4+ cells which recognise cryptic, autologous HLA-derived peptide epitopes, such that inter-patient standardisation can be undertaken without using distinct HLA peptides tailored to the donor and recipient HLA type (20).

**ii] Analysis of Damage to Glomerular Endothelial Cells**

Conditionally immortalized human glomerular endothelial cells (GEnC) were kindly gifted by Dr S. Satchell, Bristol, UK. These were maintained in supplemented endothelial basal medium-2 (Lonza, UK) as described previously (26). GEnC cells were grown until 90% confluence at 33^0^C, then at 37^0^C for at least 24h prior to addition of T cells.

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PBMCs were isolated from patients and incubated for 16h with either CMV lysate, an α3-domain derived HLA Class I peptide, or culture media. CD4⁺CD27⁺CD28null and CD4⁺CD27⁻CD28pos cells were then isolated as described above, with 10⁵ cells per well incubated with GEnC, and seeded in 24 well plates for 1 hour using transwell inserts to determine the role for chemokines involved in lymphocyte migration (specifically fractalkine in this study). One well per patient was incubated with either 1µg/ml antiNKG2D antibody or isotype control (BD Biosciences) for 2h prior to addition of T cells. Cells were stained for activated caspase 3 (as a marker of apoptosis; BD Biosciences) following T-cell migration, whereby GEnC and T cells were differentiated by staining for cell-specific markers anti-CD31-APC and anti-CD4-PE (eBiosciences) respectively, and analysed using Cyan flow cytometer (Beckman Coulter); In addition, fractalkine production was measured in the supernatants collected from GEnC incubated with T-cells by Recombinant Human CX3CL1/Fractalkine ELISA Kit according to manufacturer’s instructions (R & D Systems, UK); Von Willebrand Factor (vWF) was also measured in supernatants by sandwich ELISA, using unconjugated (capture) and conjugated (detection) vWF antibodies (DAKO, UK).

Enzyme Linked Immunosorbent Spot Assay to detect anti-HLA cellular response

A γ-interferon ELISPOT assay to evaluate the cellular immune response of transplant recipients to non-polymorphic HLA-derived peptides was undertaken as previously described (20, 21), and described briefly above.

Statistical analysis

Data are presented as mean ± standard deviation, unless otherwise stated; the figures demonstrate data distribution as mean, standard error, median, range, interquartile range and percentiles as described in figure legends. Non-normal distributed data underwent transformation as necessary prior to analysis. Of note, CD4+CD27-CD28null cell frequencies displayed heavily positive skewing, such that straightforward attempts (such as logarithmic transformation) to transform into a more

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normally distributed scale were not possible. Instead, these data were evaluated using negative binomial regression analysis. Other continuously distributed end-point data was evaluated using linear regression analysis; categorical endpoints were evaluated by logistic regression; time to event outcomes were analysed using a Cox proportional hazards model. For all analyses, initially the effect of each predictor variable on the outcome was considered separately in a series of univariate analyses. Post-transplant events of acute rejection and CMV infection were analysed as time-dependent variables as required. Variables showing some effect in the univariate analysis (p<0.15) were included in a subsequent multivariable analysis, with a stepwise backwards selection procedure to retain only the statistically significant variables in the final model. For all analyses, a type 1 error rate below 5% (p<0.05) was considered statistically significant.

Another feature of the data was the fact that there were several measurements for each subject. To allow for the non-independence of the data, multilevel statistical methods were used for data analysis in the linear regression and negative binomial models. Two-level models were used with individual measurements nested within patients. This was implemented using the xtnbreg procedure with the Stata statistical software package. For time to event analyses, predictor variable were analysed as time-dependent covariates; for analyses of single endpoints (either categorical or continuously distributed), averaged values (over the course of the study) for predictor variables were entered.

For other analyses, continuously distributed parametric data was compared using Pearson’s correlation coefficient and Student’s t-test; multiple-group, independent, non-parametric data was analysed by Kruskall-Wallis testing, with post hoc analysis for individual group comparisons; multiple-group, non-independent (multiple comparisons over time), non-parametric data was analysed by Friedman test. Categorical data was compared using Chi-square testing. Inter-test concordance was evaluated using the kappa (κ) statistic.
RESULTS

Clinical outcomes

The clinical cohort is described in Table 1. Over the study duration, three patients died (from sepsis), and four lost their grafts (primary non-function with nephrectomy 2 months post-transplantation, early acute rejection, recurrent disease, late [14 months] acute rejection); all patients underwent serial sampling (as described in “methods”) until death or graft failure (95 patients were alive with graft function at 12 months post-transplantation). For the entire cohort, median follow-up was 54 months (range 2-60 months). For the 93 patients alive with graft function at the end of the study, mean eGFR at the end of follow-up was 52±17ml/min/1.73m², and median UACR was 2.6 mg/mmol (IQR:1.0-6.6; range 0.3-280). Twenty-three patients experienced DGF, and twenty-five acute rejection (≥Banff grade 1).

In the D-R- group, 1 patient developed detectable CMV DNAemia (with clinical disease) and 1 seroconverted without prior detectable DNAemia. In the D+R- group (prophylaxed for 100 days), 5 patients developed detectable DNAemia (2 clinical disease) and 3 seroconverted without detectable DNAemia. In the D-R+ group, 6 patients developed detectable DNAemia (1 clinical disease). In the D+R+ group, 9 patients developed detectable DNAemia (3 clinical disease).

CD4⁺CD28null cell frequencies, expansion and CMV status

Circulating CD4⁺CD28null cell frequencies (expressed as a percentage of total CD4⁺ cells), and the flow cytometry gating strategy these results are based on, are shown in Figure 1. CD4⁺CD28null frequencies were higher in seropositive recipients than seronegative recipients at all timepoints (Kruskall-Wallis p<0.05 for all timepoints; Figure 1B). This was particularly evident at the 12-month timepoint, as there was expansion of CD4⁺CD28null frequencies in CMV-seropositive recipients over time (irrespective of donor CMV status; p<0.001 by Friedman test) that was not seen in the CMV-seronegative patients (p=NS). The cell frequencies of these CMV-seronegative patients are also shown in Figure 1C, the y-axis of which is set to a smaller scale reflecting the low frequencies of cells.
in these patients. Multivariable analysis (Table 2) showed recipient serostatus and time post
transplantation as the significant independent predictors of CD4⁺CD28null cell frequencies. In the
entire cohort, although development of CMV infection (detection of DNAemia or “silent”
seroconversion) was associated with CD4⁺CD28null expansion on univariate analysis, this did not hold
in the multivariate analysis.

Although as mentioned above, there was no expansion of CD4⁺CD28null cells in CMV-seronegative
recipients, the individuals who did experience primary CMV infection within the first year (either
with or without clinical disease as described above; n=10), displayed increases in CD4⁺CD28null
frequencies from pre-transplant (median 0.1 [range 0.0-0.6]) to 12 months post-transplantation
(median 2.1 [range 0.1-6.0]; p=0.009). Multiple regression analysis (adjusted for variables in Table 2)
demonstrated prior CMV infection as the only predictor of CD4⁺CD28null frequencies in these patients
(ratio:9.67 (95%CI:3.30-28.40;p<0.001). Figure 1D demonstrates the evolution of CD4⁺CD28null cells in
these 10 specific patients.

**Episodes of inflammation are associated with expansion of CD4⁺CD28null cells in CMV-seropositive
recipients**

*In vitro* work and clinical data from patients with critical illness suggests importance of inflammation
in driving CMV reactivation (12-15). Because information was lacking in regard to inflammatory
events or markers prior to transplantation, we undertook a separate “delta analysis” identifying
factors associated with changes (“deltas”) in cell frequencies between sampling timepoints (i.e.
baseline to 14 days; 14 days to 3 months; 3 months to 12 months). Analysis focused on the
relationship between the highest recorded (peak) hsCRP measurement during these timepoints and
the delta CD4⁺CD28null frequencies between these timepoints. Peak hsCRP values demonstrated
positive skewing (median:21mg/L;range 0.3-140mg/L); delta CD4⁺CD28null frequencies were normally
distributed (mean:1.0±3.8;range -11.1 to 16.7). In the entire cohort, some association between
hsCRP and delta CD4⁺CD28⁻null cells was seen (coefficient:0.5;95% CI:0.1-1.1;p=0.07). A significant statistical interaction between peak hsCRP and recipient CMV-serostatus was seen (p<0.05), with a significant relationship between hsCRP and delta count only in CMV-seropositive recipients (coefficient:1.0; 95% CI:0.2-1.8;p=0.02). Figure 1E (left panel) shows the line of best fit demonstrating the relationship between hsCRP and expansion of CD4⁺CD28⁻null cells (based on the output from the regression analysis) in both serostatus groups. Indeed hsCRP was the only independent predictor of delta count in seropositive recipients when adjusted for variables in Table 2, including the development of CMV viremia between timepoints. Furthermore, in subgroup analysis of these CMV-seropositive patients in whom CD4⁺CD28⁻null cell expansion was seen, a further interaction between hsCRP and timepoint was evident (p=0.007), and suggested episodes of inflammation had a greater effect upon CD4⁺CD28⁻null cell expansion at times beyond 14 days post-transplantation. This relationship is demonstrated in Figure 1E (right panel). In summary, this suggests episodes of inflammation may drive expansion of CD4⁺CD28⁻null cells in CMV-seropositive recipients, particularly beyond 14 days post-transplantation.

In CMV-seropositive recipients CD4⁺CD28⁻null cells display predominant effector memory-like or EMRA-like phenotype

In CMV-seropositive individuals the majority of CD4⁺CD28⁻null cells displayed either an EM or EMRA phenotype compared to CD4⁺CD28⁺ cells (Figure 2; Panels A/B versus Panels C/D;p<0.001). This confirms CD4⁺CD28⁻null cells in CMV-seropositive individuals as a “late-differentiated”, antigen-experienced population; this data is compatible with the concept that CD4⁺ cells lose surface CD27 and then CD28 during differentiation (4-8).

CMV-seronegative recipients’ CD4⁺CD28⁻null cells more commonly expressed CD27 than those from seropositive individuals, thereby displaying a ‘naïve-like’ or ‘CM-like’ phenotype. This pattern of loss of CD28 without loss of CD27 may represent recent CD4⁺-cell activation and temporary

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downregulation of CD28 in isolation, as previously suggested (16). This is shown in Figure 2 (panels E and F) which show data from CMV-seronegative patients excluding patients who experienced CMV infection in the first 12 months post transplantation (as described above); these data therefore demonstrate the ‘natural history’ of the (lack of) evolution of CD4⁺CD28null cells across the first year post-transplantation in recipients who were CMV-seronegative at the time of transplantation. It should be specifically highlighted that the low CD4⁺CD28null cell frequencies in CMV-seronegative patients means that the surface phenotyping results in these patients require cautious interpretation (representative flow cytometry readouts for seropositive and seronegative patients are shown in Figures 2I and 2J respectively).

We next analysed those CMV-seronegative patients who developed primary CMV infection during the first year post-transplantation (described above and Figure 1D). In these patients, phenotype characteristics resembled those of CMV-seropositive patients, with an EM or EMRA phenotype in 44±8% and 31±6% of these patients at 12 months respectively. These proportions were greater than the proportion of EM or EMRA seen at baseline in these patients, and also greater than the proportions in CMV-seronegative patients who did not experience CMV infection during the first year (p<0.05 for all comparisons).

CD4⁺CD28⁺ cells from CMV-seronegative and CMV-seropositive individuals displayed a similar phenotype as shown in Figure 2 panels C/D and G/H respectively (the latter again excluding patients experiencing CMV infection in the first 12 months post-transplantation). These described phenotype characteristics were stable during the first year post-transplantation.
CD4\(^+\) CD27 CD28\(^{null}\) cells display markers of endothelial homing and cytotoxic potential

Further detailed immune phenotyping of CD4\(^+\) CD28\(^{null}\) and their comparator CD4\(^+\) CD28\(^+\) cells was then undertaken. As CD4\(^+\) CD28\(^{null}\) cells were essentially limited to CMV-seropositive patients, phenotyping was undertaken 12 months post-transplantation in this group (49 patient samples available from the 52 CMV-seropositive patients alive with graft function at 12 months). As also described above, the majority of CD4\(^+\) CD28\(^{null}\) cells in CMV-seropositive individuals display an effector-memory phenotype, whereas many CD4\(^+\) CD28\(^+\) cells display naïve- or CM-like characteristics. Therefore, we compared specifically the flow cytometry characteristics of the effector-memory subsets of both cell types, i.e. CD4\(^+\) CD27 CD28\(^{null}\) versus CD4\(^+\) CD27 CD28\(^+\) cells, with CD4\(^+\) CD27 CD28\(^{null}\) cells representing a later differentiated population of effector-memory cells. We did not attempt to differentiate EM and EMRA in these analyses.

Of particular interest, CD4\(^+\) CD27 CD28\(^{null}\) cells displayed increased expression of the natural killer (NK) cell marker NKG2D and also of perforin. Figure 3A shows the gating strategy for these phenotype characteristics. Figure 3B shows the summary data for these and other surface phenotype characteristics for which differences between CD4\(^+\) CD27 CD28\(^{null}\) and CD4\(^+\) CD27 CD28\(^+\) cells were seen. CD4\(^+\) CD27 CD28\(^{null}\) cells also displayed increased expression of the late-differentiation marker CD57. Another NK receptor, CD56, was also expressed at higher levels although the difference was not as marked as for NKG2D. Furthermore, expression of the fractalkine receptor CX3CR1 was almost exclusive to CD4\(^+\) CD27 CD28\(^{null}\) cells, suggesting capability of homing to fractalkine-releasing inflamed endothelium. Of relevance to endothelial homing and tissue invasion potential was increased expression of CD11a (component of LFA-1) and CD49d (component of VLA-4) in the CD4\(^+\) CD27 CD28\(^{null}\) population. It is worth noting that CD11a and CD49d may also act as costimulation molecules as well as purely adhesion molecules (17, 18). CD4\(^+\) CD27 CD28\(^{null}\) cells also expressed higher levels of other costimulation receptors CD134 (OX-40) and CD137 (4-1BB), albeit at low absolute levels in the resting state.
In comparison, Figure 3C shows surface characteristics which did not differ between CD4⁺CD27⁻CD28null and CD4⁺CD27⁻CD28⁺ cells (p>0.05 for all comparisons). Specifically, despite increased expression of NK markers on CD4⁺CD27⁻CD28null cells, expression of the Va24β11 TCR, which is characteristically expressed on iNKT cells, was low and comparable to that in CD4⁺CD27⁻CD28⁺ cells.

No difference in expression of the lymph node homing molecule CD62L was seen between CD4⁺CD27⁻CD28null and CD4⁺CD27⁻CD28⁺ cells, in keeping with the effector-memory characteristics of both these cell subsets. Despite the “late differentiation” status of CD4⁺CD27⁻CD28null cells, expression of the inhibitory costimulation molecules KLRG-1 or PD-1, considered markers of T-cell “exhaustion”, was not increased.

CD4⁺CD27⁻CD28null cells from CMV-seropositive transplant recipients proliferate in response to CMV lysate but not HLA-derived peptide

CD4⁺CD27⁻CD28null and CD4⁺CD27⁻CD28⁺ cells were isolated from CMV-seropositive recipients 12 months post-transplantation (n=10), and examined for their proliferative responses to irradiated autologous PBMCs alone (“control”), or PBMCs previously exposed to either CMV-derived or HLA-derived peptides. Figure 4 demonstrates representative flow cytometry analyses (left panel) and summary data (right hand panels).

CD4⁺CD27⁻CD28null proliferation in response to CMV antigen was evident in all patients, whereas no response to HLA peptide was observed in any (p<0.01). Differences in proliferation across groups was seen (p<0.005), with proliferation of CD4⁺CD27⁻CD28null cells pulsed with CMV lysate significantly increased compared to either control (no antigen), HLA pulsed CD4⁺CD27⁻CD28null cells, or CD4⁺CD27⁻CD28⁺ cells pulsed with CMV lysate (ps0.01 for all comparisons).

The lack of proliferation of CD4⁺CD27⁻CD28null cells to HLA peptide was not a reflection of assay sensitivity, as CD4⁺CD27⁻CD28⁺ cells from 50% (5/10) of patients demonstrated proliferative
responses to HLA-derived peptides (p=0.01). Interestingly, there was complete inter-patient concordance between this assay of CD4+CD27-CD28+ cell proliferation to HLA-derived peptides and the responses in the IFN-γ ELISPOT assay (“raw” data not shown), with all 5 patients responding in one assay also responding in the other, and all 5 patients unresponsive in one assay being similarly unresponsive in the other (K-statistic=1.0; p=0.008).

**Glomerular endothelial cell (GEnC) injury following exposure to CD4+CD27-CD28null cells**

PBMCs from CMV-seropositive transplant recipients (n=4) were incubated for 16h in media alone (control) or with either CMV lysate or HLA peptide. Thereafter, 10^5 CD4+CD27-CD28null or CD4+CD27-CD28null-CD28+ cells were isolated and incubated with GEnC for 6 hours. Figure 5 demonstrates release of vWF and fractalkine from GEnC, and increased intracellular active caspase 3 expression within GEnC following incubation with CD4+CD27-CD28null cells pre-exposed to CMV lysate. This was not seen with CD4+CD27+CD28null cells pre-incubated with HLA-derived peptide, nor with CD4+CD27-CD28null cells pre-incubated with either CMV-derived HLA-derived peptide (p<0.05 for all comparisons). Of particular note, all effects were attenuated by addition of NKG2D-blocking antibody to GEnC for 2 hours prior to, and then during their incubation with CD4+CD27-CD28null cells (p<0.05 for all comparisons).

**Increased circulating CD4+CD28null cells are independently associated with delayed graft function and inferior medium-term allograft function**

CD4+CD28null frequencies immediately prior to transplantation were significantly associated with post-operative DGF. This relationship held when adjusted for baseline demographics, including donor/recipient CMV serostatus (multivariate model shown in Table 3).

Graft failure was uncommon, and so not investigated as an outcome. However, eGFR at the end of follow up was associated with increased CD4+CD28null cell frequencies (averaged over follow-up), with 7.3ml/min lower eGFR for each log_{10} increase in CD4+CD28null cells in the multivariate analysis.
which included adjustment for DGF and also for peak hsCRP averaged over time (p=0.03; Table 3).

No statistical interaction between CD4^+^CD28\^null cells and either DGF or acute rejection was seen for the eGFR endpoint. Results were similar when CD4^+^CD28\^null cell frequencies specifically at 12 months were analysed as the predictor variable (Beta = -7.8 (-14.9, -0.7); p=0.02).

No association was found between CD4^+^CD28\^null cells (averaged over follow-up) and log-transformed UACR at end of follow-up, or between baseline CD4^+^CD28\^null cells and time to acute rejection (final multivariate models shown in Table 3).

DISCUSSION

This study represents a detailed, prospective, longitudinal examination of CD4^+^CD28\^null cells in unselected kidney transplant recipients. Our work demonstrates CD4^+^CD28\^null T-cell expansion is driven by inflammation on a background of latent CMV infection. CD4^+^CD28\^null cells isolated from kidney transplant recipients responded to CMV-derived antigens in vitro, but not to HLA-derived antigens. Detailed phenotyping of CD4^+^CD28\^null cells confirmed their late differentiation status and potential for endothelial adhesion, tissue invasiveness, and cytotoxicity. In addition, exposure of CD4^+^CD28\^null cells to glomerular endothelial cells in vitro resulted in NKG2D-dependent endothelial cell activation and apoptosis. This mechanism might plausibly explain the association between increased CD4^+^CD28\^null cells and important clinical endpoints such as DGF and eGFR 5 years post-transplantation. Taken together these data point to the importance of this cell subset in kidney transplantation, particularly in regard to “indirect” effect of CMV on kidney allograft function and outcome (1-3).

Expansion of circulating CD4^+^CD28\^null cells is recognized for CMV-seropositive patients with ESRD (4). The current study extends the available evidence in kidney transplantation, again showing this circulating subset as “pathognomonic” of latent CMV at the time of transplantation. A novel finding
is that episodes of significant inflammation are associated with subsequent expansion of
CD4^+CD28^{null} cells. This resonates with increased CD4^+CD28^{null} cell frequencies in CMV-seropositive
patients with ESRD (a state of chronic inflammation) (4), and in other autoinflammatory conditions
(5-7). In CMV-seropositive recipients, no independent association between CD4^+CD28^{null} cell
expansion and the development of prior CMV DNAemia was seen, suggesting the latter was not a
“pre-requisite” for expansion of these cells. One plausible explanation for this might be that
episodes of inflammation drive “abortive” viral replication, whereby inflammation-induced dendritic
cell expression of early viral antigens is not followed by overt viraemia (13), but might nevertheless
incite an immune response (in this case CMV-specific CD4^+CD28^{null} cells). Regarding this, knowledge
of specific viral proteins to which CMV-specific CD4^+ cells respond would be informative, but is not
currently available and was beyond the current study’s scope. But of relevance many healthy CMV-
seropositive individuals control CMV effectively with a CD8^{+} T-cell repertoire predominantly directed
against early-expressed viral proteins, rather than later structural components(19).

The relationship between CD4^+CD28^{null} cells and CMV-serostatus is likely more than mere association,
as purified CD4^+CD28^{null} cells (specifically CD4^+CD27^{−}CD28^{null} cells) isolated from patients proliferated
to CMV antigens in vitro. Importantly, CD4^+CD27^{−}CD28^{null} cells demonstrated no evidence of HLA-
alloreactivity, this being confined to the CD28-expressing CD4^+CD27^{−}CD28^{+} subset. The robustness of
this important and novel finding is supported by the perfect concordance between this proliferation
assay and IFN-γ release in a “confirmatory” ELISPOT assay, which is specifically relevant because
previous studies showed the responding cells in this ELISPOT assay also demonstrate an EM-like
CD4^+CD28^{−} phenotype (20, 21).

Further detailed immune phenotyping was undertaken on samples available 12 moths post
transplantation. And so although the temporal dynamics of the findings cannot be assessed (and was
not a primary aim of the study), we can confirm CD4^+CD27^{−}CD28^{null} cells as late-differentiated

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(expressing CD57), effector memory cells with increased cytotoxic potential (expressing NKG2D and perforin). They express very low levels of the lymph node homing receptor CD62L, but high levels of the receptor for fractalkine (a chemokine of endothelial origin) along with increased expression of adhesion molecules, collectively suggesting capabilities of endothelial homing and tissue-invasiveness.

Increased CD4⁺CD27⁻CD28null expression of fractalkine receptor (CX3CR1) makes biological sense regarding CMV cellular immunity, as endothelium is a key site of CMV latency and reactivation (22). However, the current study suggests that an unwanted consequence of CMV immunosurveillance may be development of endothelial injury, which was mediated by CD4⁺CD27⁻CD28null cells in vitro. Preincubation of PBMCs with CMV antigen was required to induce this injury, which was mitigated by addition of NKG2D-blocking antibody. This NK receptor represents an integral component of CMV immunosurveillance and immunoevasion (23), was upregulated on CD4⁺CD27⁻CD28null cells isolated from patients in the current study, and we propose as an important component of the cytotoxic effects (either protective or pathogenic) of these cells. Indeed HUVEC injury by CD4⁺CD28null cells in the context of coronary disease is independent of conventional T-cell receptor ligation (9), and so the current study extends these observations specifically to glomerular endothelial injury, highlighting the importance of CMV and NKG2D in the process. Although not specifically addressed in the current study, it is recognized that human endothelial cells express NKG2D ligands (24), and it is likely (albeit unproven) that the same holds for GEnC. These results also align with other broader observations (from non-transplant cohorts) including induction of NKG2D on CD4⁺ cells exposed to CMV antigen (10, 11), degranulation of NKG2D-expressing CD4⁺ cells in the presence of endothelial cells (24), endothelial fractalkine release following exposure to CD4⁺ cells from CMV-seropositive individuals (albeit not further characterised phenotypically) (25), and CMV-induced endothelial damage in a rodent model which is independent of endothelial infection (26).
The use of GEnC is relevant as these glomerular cells display unique structural and functional properties which are not shared by endothelial cells of different vessels, anatomical locations, or species (27). The conditionally immortalized cells (“ciGEnC”) used in this study retain similar morphological and physiological characteristics as primary culture human GEnC (27), and therefore give informative insights into events occurring in vivo. Indeed a major component in the pathogenesis of both DGF and chronic allograft dysfunction is endothelial injury (28, 29), and therefore the mechanism of endothelial injury described above may explain the independent associations between CD4⁺CD28null cell expansion at baseline and the development of DGF, and also between CD4⁺CD28null cell numbers during the first year post-transplantation and renal function at 5 years post-transplantation. Further insight into this may be gained from detailed histological evaluation, which might demonstrate precise anatomic localization of CD4⁺CD28null cells, and also further characterize their phenotype, specifically in regard to whether they represent a subpopulation of CD4 tissue-resident memory cells. This will be the focus for future work.

Although sampling was not continued beyond the first year, the intensive protocol and detailed phenotyping conducted during this critical period adds robustness and relevance to these findings, which are novel to transplantation but resonate with the inverse association between circulating CD4⁺CD28null cells and renal function in a cross sectional study of stable patients with renal vasculitis (6). This link between CMV, inflammation (discussed above) cytotoxic CD4⁺CD28null cell expansion, endothelial injury and allograft dysfunction may contribute to explaining the well-recognised but incompletely understood association between inflammation and graft outcome (30, 31), and may even go towards explaining the so-called “transplant paradox” whereby improvements in acute rejection rates have not been mirrored by long-term improvement in graft survival (32).

In summary, this study reveals that expansion of CMV-related, cytotoxic CD4⁺CD28null cells is an important biomarker for, and potential mediator of, adverse events following kidney transplantation.

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Interventions to avoid and reduce CMV infection (33) and potentially novel strategies to interfere with CD4⁺CD28null cell toxicity (34) may result in further improvements in allograft outcome.

ACKNOWLEDGMENTS

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DISCLAIMER

The Research was carried out at the National Institute for Health Research (NIHR)/Wellcome Trust Birmingham Clinical Research Facility. The views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR or the Department of Health.

DISCLOSURE

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

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Supplemental Materials and Methods
FIGURE LEGENDS

Figure 1

The gating strategy (Figure 1A) and results (Figure 1B) for the flow cytometry identification of CD4⁺CD28null cells is shown. CD4⁺CD28null cell frequencies (expressed as a proportion of total CD4 cells) over time are shown in Figures 1B and 1C as median (line) and interquartile range (boxes). Outliers were defined as having values greater than 1.5 interquartile ranges above the value differentiating the upper 3rd and 4th quartiles (i.e., the top of the interquartile range/box). The whiskers of the plots demonstrate the range of values not including these outlying values. Figure 1B (and accompanying table) demonstrates significant differences in cell frequencies across CMV-serostatus subgroups at all timepoints (Kruskall-Wallis p<0.05 for all) and evidence of expansion of CD4⁺CD28null cells in CMV seropositive recipients over the first year post-transplantation, which was not seen in their CMV-seronegative counterparts (Friedman test p<0.001 for D-/?R+ and D+/R+ groups).

Figure 1C demonstrates the same data as Figure 1B for CMV-seronegative recipients, but with an approximately 10-fold difference in y-axis scale reflecting the far lower cell frequencies in these patients.

Figure 1D demonstrates expansion of CD4⁺CD28null cells in 10 patients who were seronegative at the time of transplant and who developed CMV infection during the first 12 months post-transplantation (2 received kidneys from CMV-seronegative donors, and 8 from CMV-seropositive donors).

Figure 1E shows the association between delta CD4⁺CD28null cell count and peak hsCRP across sampling time points. Sampling time points were baseline to 14 days; 14 days to 3 months; and 3 months to 12 months. The “delta count” (as described in “methods”) represents the absolute...
change in CD4⁺CD28null frequency (expressed as the percentage of total CD4⁺ cells) between time points. Curves shown represent lines of best fit from the regression analyses undertaken. Figure 2A demonstrates the relationship between delta count (all time points considered) and hsCRP in all studied patients. This shows the influence of inflammation on CD4⁺CD28null cell expansion was only evident in CMV-seropositive recipients. Further analysis of this CMV-seropositive group (only) is represented in Figure 2B. This shows the influence of time post-transplantation on the relationship between hsCRP and delta CD4⁺CD28null cell count, whereby this effect of inflammation-associated CD4⁺CD28null cell expansion is only seen beyond 14 days post-transplantation in this CMV-seropositive group.

Samples available and analysed for 100 patients pre-transplant, 94 patients at 14 days, 92 patients at 3 months, and 90 patients at 12 months.

Figure 2

Frequencies of naïve, central memory (CM), effector memory (EM) and effector memory re-expressing RA (EMRA) cells within the CD4⁺CD28null and CD4⁺CD28⁺ cell populations are shown (as defined by CD27 and CD45RA co-expression; see methods). In CMV-seropositive recipients the majority of CD4⁺CD28null cells display an EM-like or EMRA-like phenotype (A and B), contrasting with their phenotype in CMV-seronegative recipients (E and F), and also with the phenotype of CD4⁺CD28⁺ cells from either seropositive or seronegative recipients (C, and D and G and H). Samples available and analysed for 100 patients pre-transplant, 94 patients at 14 days, 92 patients at 3 months, and 90 patients at 12 months. Data normally distributed and displayed as mean and standard error. To demonstrate the “natural history” of the evolution of cell frequencies in CMV-seronegative patients, the data shown in Panels E through H exclude patients who were CMV-seronegative at the time of transplant and who developed evidence of CMV infection during the first year post transplantation (total n=10 as described further in text). “Representative” flow cytometry

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readouts for the evaluation of CD4⁺CD28null cell phenotype are shown in Figures 2I and 2J. Figure 2I shows data from a CMV-seropositive patient sampled at month 12 (CD4⁺CD28null cells comprised 4% of total CD4⁺ population); Figure 2J shows data from a CMV-seronegative patient also sampled at month 12 (donor CMV-seronegative; no CMV disease, infection or seroconversion by 12 months; CD4⁺CD28null cells comprised 0.6% of total CD4⁺ population). Although the results from CMV-seronegative patients are interpretable, results are not as robust as for the case of seropositive patients displaying higher CD4⁺CD28null cell frequencies.

**Figure 3**

Example gating strategy and summary data demonstrating phenotypic comparison between CD4⁺CD27⁻CD28null and CD4⁺CD27⁺CD28⁺ cells in 49 CMV seropositive recipients alive with graft function at 12 months post-transplantation. Initial gating to define CD4⁺CD27⁻CD28null and CD4⁺CD27⁺CD28⁺ cells was conducted as shown in Figure 1A. Figure 3A then demonstrates surface staining for NKG2D and intracellular staining for perforin in CD4⁺CD27⁻CD28null cells (lower panel), which was absent in the CD4⁺CD27⁺CD28⁺ compartment (upper panel).

Figure 3B demonstrates summary data for those phenotype characteristics which differed between CD4⁺CD27⁻CD28null and CD4⁺CD27⁺CD28⁺ cells (p<0.05 for all); Figure 3C demonstrates phenotyping characteristics where no difference was observed between these cell subsets. Data normally distributed and displayed as mean and standard error.

**Figure 4**

Flow cytometric analysis demonstrating proliferation of CD4⁺CD27⁻CD28null and CD4⁺CD27⁺CD28⁺ cells from CMV positive renal transplant patients (n=10). Proliferation of CD4⁺CD27⁻CD28null and
CD4^+^CD27^−^CD28^+^ cells was evaluated by CFSE dilution. Cells were pulsed with irradiated autologous PBMCs previously exposed to either control culture media, CMV lysate, or HLA peptide. Example plots of flow cytometric analysis are displayed in left hand panel with summary data from the 10 subjects shown in right hand panel. Differences in proliferation across groups was seen (p<0.005 by Kruskall-Wallis), with proliferation of CD4^+^CD27^−^CD28^null^ cells pulsed with CMV lysate significantly increased compared to either control (no antigen), HLA pulsed CD4^+^CD27^−^CD28^null^ cells, or CD4^+^CD27^+^CD28^−^ cells pulsed with CMV lysate (p<0.01 for all comparisons; Kruskall-Wallis with post hoc testing).

Data displayed as median (bars), interquartile range (boxes) and range (whiskers).

**Figure 5**

Glomerular Capillary Endothelial cells (GEnC) were incubated with 10^5^ CD4^+^CD27^−^CD28^null^ or CD4^+^CD27^−^CD28^+^ cells isolated from CMV positive renal transplant patients (n=4), incubated for 16h in media alone (Control) or stimulated with CMV lysate or HLA peptide prior to sorting. At 6 hours, release of vWF, release of fractalkine, and frequency of GEnCs expressing active caspase 3 was significantly different across experiments (Kruskall-Wallis p=0.001), and specifically increased following prior incubation of CD4^+^CD27^−^CD28^null^ cells with CMV lysate compared to either control media, HLA peptide (p<0.05 for both comparisons; Kruskall-Wallis with post hoc testing), and also compared with CD4^+^CD27^−^CD28^+^ cells pretreated with either CMV lysate or HLA peptide (p<0.05 for both post hoc comparisons in all experiments). This effect was inhibited by incubation with an NKG2D blocking antibody during and two hours prior to the addition of CD4^+^CD27^−^CD28^null^ cells (post hoc p<0.05 for all experiments). Data displayed as median (bars), interquartile range (boxes) and range (whiskers).
REFERENCES


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32. Meier-Kriesche HU, Schold JD, Kaplan B. Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? Am J Transplant 2004;4(8):1289-1295.


**Table 1. Patient demographics**

<table>
<thead>
<tr>
<th>Recipient Age</th>
<th>48±14 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient Sex</td>
<td>55 male</td>
</tr>
<tr>
<td>Recipient ethnicity</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>74</td>
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<tr>
<td>Indo-Asian</td>
<td>15</td>
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<td>African-Caribbean</td>
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<tr>
<td>Cause of Renal Failure</td>
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<td>Glomerular</td>
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<tr>
<td>Cystic</td>
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</tr>
<tr>
<td>Diabetes</td>
<td>14</td>
</tr>
<tr>
<td>Hypertension</td>
<td>19</td>
</tr>
<tr>
<td>Other</td>
<td>19</td>
</tr>
<tr>
<td>Pre Transplant Modality</td>
<td></td>
</tr>
<tr>
<td>Haemodialysis</td>
<td>38</td>
</tr>
<tr>
<td>Peritoneal Dialysis</td>
<td>35</td>
</tr>
<tr>
<td>Pre-emptive</td>
<td>27</td>
</tr>
<tr>
<td>Repeat transplantation</td>
<td>23</td>
</tr>
<tr>
<td>Donor Age</td>
<td>47±15 years</td>
</tr>
<tr>
<td>Transplant Source</td>
<td></td>
</tr>
<tr>
<td>Deceased donor (DBD&lt;sup&gt;1&lt;/sup&gt;)</td>
<td>56 (44)</td>
</tr>
<tr>
<td>(DCD&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>(12)</td>
</tr>
<tr>
<td>Live donor</td>
<td>44</td>
</tr>
<tr>
<td>Donor-Recipient HLA Mismatch</td>
<td></td>
</tr>
<tr>
<td>Class I (HLA-A+B)</td>
<td>2.1±0.9 Ag</td>
</tr>
<tr>
<td>Class II (HLA-DR)</td>
<td>0.9±0.7 Ag</td>
</tr>
<tr>
<td>Donor-Recipient CMV Serostatus</td>
<td></td>
</tr>
<tr>
<td>D-R-</td>
<td>25</td>
</tr>
<tr>
<td>D-R+</td>
<td>22</td>
</tr>
<tr>
<td>D+R+</td>
<td>32</td>
</tr>
<tr>
<td>D+R-</td>
<td>21</td>
</tr>
<tr>
<td>Biopsy proven Acute rejection</td>
<td>25</td>
</tr>
<tr>
<td>Delayed graft Function&lt;sup&gt;3&lt;/sup&gt;</td>
<td>23</td>
</tr>
<tr>
<td>Cytomegalovirus Asymptomatic infection&lt;sup&gt;4&lt;/sup&gt;</td>
<td>18</td>
</tr>
<tr>
<td>Disease&lt;sup&gt;5&lt;/sup&gt;</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>1</sup> Donation after Brain Death
<sup>2</sup> Donation after Cardiac death
<sup>3</sup> Requirement for dialysis within the first week post transplantation

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4 Denotes the development of asymptomatic CMV viremia on protocol sampling, or the de novo development of anti-CMV antibody in those patients who were seronegative at the time of transplantation – none of these patients displayed evidence of clinical disease (see text for details)

5 Denotes the development of symptomatic CMV disease requiring clinical intervention

Table 2. Predictors of CD4⁺CD28null cell frequencies

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Univariate Ratio (95%CI)</th>
<th>p-value</th>
<th>Multivariate Ratio (95%CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>Baseline</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>0.64 (0.33, 1.29)</td>
<td>&lt;0.001</td>
<td>0.58 (0.31, 1.09)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td>0.90 (0.47, 1.75)</td>
<td></td>
<td>0.75 (0.41, 1.37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 months</td>
<td>2.31 (1.36, 3.92)</td>
<td></td>
<td>1.92 (1.17, 3.14)</td>
<td>&lt;0.001</td>
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<tr>
<td>CMV Status</td>
<td>D-R-</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D+R-</td>
<td>0.53 (0.21, 1.36)</td>
<td>&lt;0.001</td>
<td>0.65 (0.25, 1.68)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D+R+</td>
<td>3.16 (1.39, 7.19)</td>
<td></td>
<td>4.45 (1.90, 10.40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D+R+</td>
<td>4.46 (1.86, 10.70)</td>
<td></td>
<td>4.28 (1.78, 10.30)</td>
<td>&lt;0.001</td>
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<tr>
<td>CMV Infection*</td>
<td>Yes</td>
<td>4.40 (1.77, 10.9)</td>
<td>0.001</td>
<td></td>
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<tr>
<td>Recipient Age</td>
<td>Per 10 years</td>
<td>0.9 (0.76, 1.26)</td>
<td>0.91</td>
<td></td>
<td></td>
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<td>Recipient Sex</td>
<td>Male vs Female</td>
<td>1.76 (0.98, 3.55)</td>
<td>0.11</td>
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<tr>
<td>Inflammatory Diagnosis**</td>
<td>Yes</td>
<td>1.53 (0.69, 3.38)</td>
<td>0.30</td>
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<td>Dialysis Modality</td>
<td>Haemodialysis</td>
<td>1</td>
<td></td>
<td>1</td>
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<td></td>
<td>Peritoneal Dialysis</td>
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<td></td>
<td>0.46 (0.19, 1.10)</td>
<td>0.16</td>
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<td></td>
<td>Pre-emptive</td>
<td>0.54 (0.23, 1.25)</td>
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<tr>
<td>Repeat Transplantation</td>
<td>Yes</td>
<td>1.75 (0.67, 4.58)</td>
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<td>Class I HLA Mismatch</td>
<td>Per antigen</td>
<td>0.93 (0.65, 1.33)</td>
<td>0.67</td>
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<tr>
<td>Class II HLA Mismatch</td>
<td>Per antigen</td>
<td>0.92 (0.54, 1.57)</td>
<td>0.76</td>
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<tr>
<td>Delayed Graft Function***</td>
<td>Yes</td>
<td>1.30 (0.60, 2.83)</td>
<td>0.51</td>
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<td>Rejection****</td>
<td>Yes</td>
<td>1.37 (0.62, 3.05)</td>
<td>0.44</td>
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</table>

Table 2 shows the predictors of CD4⁺CD28null cell frequencies (expressed as a proportion of total CD4⁺ cells) in the study population. Results from univariate and multiple regression models displayed. The effect sizes are summarised in the form of ratios. For the categorical variables these represent the ratio of CD4⁺CD28null cell frequencies in each category relative to a baseline category. For the continuous variables, these represent the change in ratio for a one-unit increase in that variable (unless otherwise indicated).

*denotes either i] detection of CMV DNAemia (either clinically manifest or silent) prior to sampling time or ii] recipient seroconversion prior to sampling time

** denotes primary renal disease due to inflammatory nephritis (e.g vasculitis, lupus, glomerulonephritis)

*** defined as dialysis requirement in first week post transplantation

**** Biopsy proven rejection (any grade) prior to sampling time
Table 3. Predictors of clinical endpoints

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Effect Size</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>eGFR at end of follow up</strong></td>
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<td></td>
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<tr>
<td>CD4^+CD28null count</td>
<td>Expressed as proportion of total CD4 cells; log10 scale</td>
<td>-7.3 (-15.2, -0.6)</td>
<td>0.03</td>
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<td>Biopsy-proven acute rejection</td>
<td>Yes (any grade)</td>
<td>-9.9 (-18.8, -0.9)</td>
<td>0.03</td>
</tr>
<tr>
<td>Donor Age</td>
<td>Per 10 years</td>
<td>-6.9 (-11.4, -2.4)</td>
<td>0.01</td>
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<td><strong>Delayed Graft function</strong></td>
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<td></td>
<td></td>
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<tr>
<td>CD4^+CD28null count</td>
<td>Expressed as proportion of total CD4 cells; log10 scale</td>
<td>1.81 (1.11, 2.95)</td>
<td>0.02</td>
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<td>Recipient Age</td>
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<td>1.53 (1.04, 2.24)</td>
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<td>Donor Age</td>
<td>Per 10 years</td>
<td>1.39 (1.05, 1.84)</td>
<td>0.03</td>
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<td><strong>UACR at end of follow up</strong></td>
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<tr>
<td>Biopsy-proven acute rejection</td>
<td>Yes (any grade)</td>
<td>3.02 (1.58, 5.81)</td>
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<td>Yes</td>
<td>2.15 (1.13, 4.12)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Time to Biopsy Proven Acute Rejection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II HLA Mismatch</td>
<td>Per Antigen</td>
<td>1.93 (1.04, 3.59)</td>
<td>0.04</td>
</tr>
<tr>
<td>CMV infection*</td>
<td>Yes</td>
<td>9.91 (2.94, 33.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>dnDSA**</td>
<td>Yes</td>
<td>3.42 (1.69, 6.92)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 3 shows final multivariate model describing relationships between predictor variables and clinical outcomes.

Effect size reported as \(^1\) beta coefficient; \(^2\) odds ratio; \(^3\) hazard ratio

*denotes detectable CMV DNAemia

**de novo anti HLA donor-specific antibody
Figure 1E

A

B

Figure 2

A

B

C

D

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Figure 4

CD28<sup>+</sup> : control
CD28<sup>+</sup> : CMV
CD28<sup>+</sup> : HLA

% of cells seeded proliferating

Figure 5

vWF
Fractalkine
Active Caspase 3

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