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CMV-associated CD4^{+}CD28^{null} 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 ("$CD4^+CD28^{null}$")(4). Although also recognised in patients with autoimmune conditions such as multiple sclerosis, rheumatoid arthritis and vasculitis (5-7), these $CD4^+CD28^{null}$ cells are unresponsive to candidate autoantigens (5, 6), but rather respond to CMV-derived proteins. Furthermore, one study demonstrated emergence of $CD4^+CD28^{null}$ 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 ("$CD4^+CD28^+$"), $CD4^+CD28^{null}$ 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; thrombocytopaenia; 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 $CD4^+CD28^{null}$ cells and their $CD4^+CD28^+$ 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 $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^{pos}$ 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-CD28pos 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 37^0C 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 (eBiosicences) 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 33^0C, then at 37^0C for at least 24h prior to addition of T cells.

PBMCs were isolated from patients and incubated for 16h with either CMV lysate, an α3-domain derived HLA Class I peptide, or culture media. $CD4^{+}CD27^{-}CD28^{null}$ and $CD4^{+}CD27^{-}CD28^{pos}$ cells were then isolated as described above, with 10^5 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

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/1.73m^2, 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).

$CD4^+CD28^{null}$ cell frequencies, expansion and CMV status

Circulating $CD4^+CD28^{null}$ 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. $CD4^+CD28^{null}$ 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 $CD4^{+}CD28^{null}$ cell frequencies. In the entire cohort, although development of CMV infection (detection of DNAemia or "silent" seroconversion) was associated with $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ frequencies in these patients (ratio:9.67 (95%CI:3.30-28.40;p<0.001). Figure 1D demonstrates the evolution of $CD4^{+}CD28^{null}$ cells in these 10 specific patients.

Episodes of inflammation are associated with expansion of $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ frequencies between these timepoints. Peak hsCRP values demonstrated positive skewing (median:21mg/L;range 0.3-140mg/L); delta $CD4^{+}CD28^{null}$ 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

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 $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ 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 characeristics. 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 $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells ($p>0.05$ for all comparisons). Specifically, despite increased expression of NK markers on $CD4^+CD27^-CD28^{null}$ cells, expression of the Vα24Vβ11 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 $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells, in keeping with the effector-memory characteristics of both these cell subsets. Despite the "late differentiation" status of $CD4^+CD27^-CD28^{null}$ cells, expression of the inhibitory costimulation molecules KLRG-1 or PD-1, considered markers of T-cell "exhaustion", was not increased.

$CD4^+CD27^-CD28^{null}$ cells from CMV-seropositive transplant recipients proliferate in response to CMV lysate but not HLA-derived peptide

$CD4^+CD27^-CD28^{null}$ 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).

$CD4^+CD27^-CD28^{null}$ 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 ($p\leq0.01$ for all comparisons).

The lack of proliferation of $CD4^+CD27^-CD28^{null}$ 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 (κ-statistic=1.0; p=0.008).

Glomerular endothelial cell (GEnC) injury following exposure to $CD4^+CD27^-CD28^{null}$ 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 $CD4^+CD27^-CD28^{null}$ or $CD4^+CD27^-CD28^+$ 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 $CD4^+CD27^-CD28^{null}$ cells pre-exposed to CMV lysate. This was not seen with $CD4^+CD27^-CD28^{null}$ cells pre-incubated with HLA-derived peptide, nor with $CD4^+CD27^-CD28^+$ 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 $CD4^+CD27^-CD28^{null}$ cells ($p<0.05$ for all comparisons).

Increased circulating $CD4^+CD28^{null}$ cells are independently associated with delayed graft function and inferior medium-term allograft function

$CD4^+CD28^{null}$ 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 $CD4^+CD28^{null}$ cell frequencies (averaged over follow-up), with 7.3ml/min lower eGFR for each $\log_{10}$ increase in $CD4^+CD28^{null}$ 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

(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 $CD4^+CD27^-CD28^{null}$ 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 $CD4^+CD27^-CD28^{null}$ 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 $CD4^+CD27^-CD28^{null}$ 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 $CD4^+CD28^{null}$ 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-seropositve 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 $CD4^{+}CD28^{null}$ cell expansion at baseline and the development of DGF, and also between $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ cells and renal function in a cross sectional study of stable patients with renal vasculitis (6). This link between CMV, inflammation (discussed above) cytotoxic $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ cells is an important biomarker for, and potential mediator of, adverse events following kidney transplantation.

Interventions to avoid and reduce CMV infection (33) and potentially novel strategies to interfere with $CD4^{+}CD28^{null}$ cell toxicity (34) may result in further improvements in allograft outcome.

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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 $CD4^{+}CD28^{null}$ cells is shown. $CD4^{+}CD28^{null}$ 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 3^{rd} and 4^{th} quartiles (ie 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 $CD4^{+}CD28^{null}$ cells in CMV seropositive recipients over the first year post-transplantation, which was not see 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 $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ 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 $CD4^+CD28^{null}$ 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 $CD4^+CD28^{null}$ 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 $CD4^+CD28^{null}$ cell count, whereby this effect of inflammation-associated $CD4^+CD28^{null}$ 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 $CD4^+CD28^{null}$ and $CD4^+CD28^+$ cell populations are shown (as defined by CD27 and CD45RA co-expression; see methods). In CMV-seropositive recipients the majority of $CD4^+$ $CD28^{null}$ 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

readouts for the evaluation of $CD4^+CD28^{null}$ cell phenotype are shown in Figures 2I and 2J. Figure 2I shows data from a CMV-seropositive patient sampled at month 12 ($CD4^+CD28^{null}$ 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; $CD4^+CD28^{null}$ 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 $CD4^+$ $CD28^{null}$ cell frequencies.

Figure 3

Example gating strategy and summary data demonstrating phenotypic comparison between $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells in 49 CMV seropositive recipients alive with graft function at 12 months post-transplantation. Initial gating to define $CD4^+CD27^-CD28^{null}$ 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 $CD4^+CD27^-CD28^{null}$ 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 between $CD4^+CD27^-CD28^{null}$ 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 $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells from CMV positive renal transplant patients (n=10). Proliferation of $CD4^+CD27^-CD28^{null}$ 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-CD28null 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).

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Table 1. Patient demographics

Recipient Age	48±14 years
Recipient Sex	55 male
Recipient ethnicity	
White	74
Indo-Asian	15
African-Caribbean	11
Cause of Renal Failure	
Glomerular	29
Cystic	19
Diabetes	14
Hypertension	19
Other	19
Pre Transplant Modality	
Haemodialysis	38
Peritoneal Dialysis	35
Pre-emptive	27
Repeat transplantation	23
Donor Age	47±15 years
Transplant Source	
Deceased donor	56
(DBD[1])	(44)
(DCD[2])	(12)
Live donor	44
Donor-Recipient HLA Mismatch	
Class I (HLA-A+B)	2.1±0.9 Ag
Class II (HLA-DR)	0.9±0.7 Ag
Donor-Recipient CMV Serostatus	
D-R-	25
D-R+	22
D+R+	32
D+R-	21
Biopsy proven Acute rejection	25
Delayed graft Function[3]	23
Cytomegalovirus	
Asymptomatic infection[4]	18
Disease[5]	7

[1] Donation after Brain Death

[2] Donation after Cardiac death

[3] Requirement for dialysis within the first week post transplantation

[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 $CD4^{+}CD28^{null}$ cell frequencies

Variable	Category	Univariate Ratio (95%CI)	p-value	Multivariate Ratio (95%CI)	p-value
Time	Baseline	1		1	
	14 days	0.64 (0.33, 1.29)		0.58 (0.31, 1.09)	
	3 months	0.90 (0.47, 1.75)		0.75 (0.41, 1.37)	
	12 months	2.31 (1.36, 3.92)	<0.001	1.92 (1.17, 3.14)	<0.001
CMV Status	D-R-	1		1	
	D+R-	0.53 (0.21, 1.36)		0.65 (0.25, 1.68)	
	D-R+	3.16 (1.39, 7.19)		4.45 (1.90, 10.40)	
	D+R+	4.46 (1.86, 10.70)	<0.001	4.28 (1.78, 10.30)	<0.001
CMV Infection*	Yes	4.40 (1.77, 10.9)	0.001		
Recipient Age	Per 10 years	0.9 (0.76, 1.28)	0.91		
Recipient Sex	Male vs Female	1.76 (0.88, 3.55)	0.11		
Inflammatory Diagnosis**	Yes	1.53 (0.69, 3.38)	0.30		
Dialysis Modality	Haemodialysis	1			
	Peritoneal Dialysis	0.54 (0.23, 1.25)			
	Pre-emptive	0.46 (0.19, 1.10)	0.16		
Repeat Transplantation	Yes	1.75 (0.67, 4.58)	0.25		
Class I HLA Mismatch	Per antigen	0.93 (0.65, 1.33)	0.67		
Class II HLA Mismatch	Per antigen	0.92 (0.54, 1.57)	0.76		
Delayed Graft Function***	Yes	1.30 (0.60, 2.83)	0.51		
Rejection****	Yes	1,37 (0.62, 3.05)	0.44		

Table 2 shows the predictors of $CD4^{+}CD28^{null}$ 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 $CD4^{+}CD28^{null}$ 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

Variable	Category	Effect Size	p-value
eGFR at end of follow up[1]			
$CD4^{+}CD28^{null}$ count	Expressed as proportion of total CD4 cells; log10 scale	-7.3 (-15.2, -0.6)	0.03
Biopsy-proven acute rejection	Yes (any grade)	-9.9 (-18.8, -0.9)	0.03
Donor Age	Per 10 years	-6.9 (-11.4, -2.4)	0.01
Delayed Graft function[2]			
$CD4^{+}CD28^{null}$ count	Expressed as proportion of total CD4 cells; log_{10} scale	1.81 (1.11, 2.95)	0.02
Recipient Age	Per 10 years	1.53 (1.04, 2.24)	0.03
Donor Age	Per 10 years	1.39 (1.05, 1.84)	0.03
UACR at end of follow up[1]			
Biopsy-proven acute rejection	Yes (any grade)	3.02 (1.58, 5.81)	0.001
Delayed Graft Function	Yes	2.15 (1.13, 4.12)	0.02
Time to Biopsy Proven Acute Rejection[3]			
Class II HLA Mismatch	Per Antigen	1.93 (1.04, 3.59)	0.04
CMV infection*	Yes	9.91 (2.94, 33.4)	<0.001
dnDSA**	Yes	3.42 (1.69, 6.92)	0.01

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

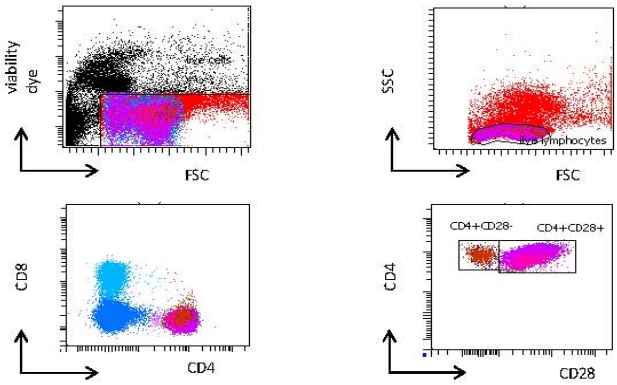
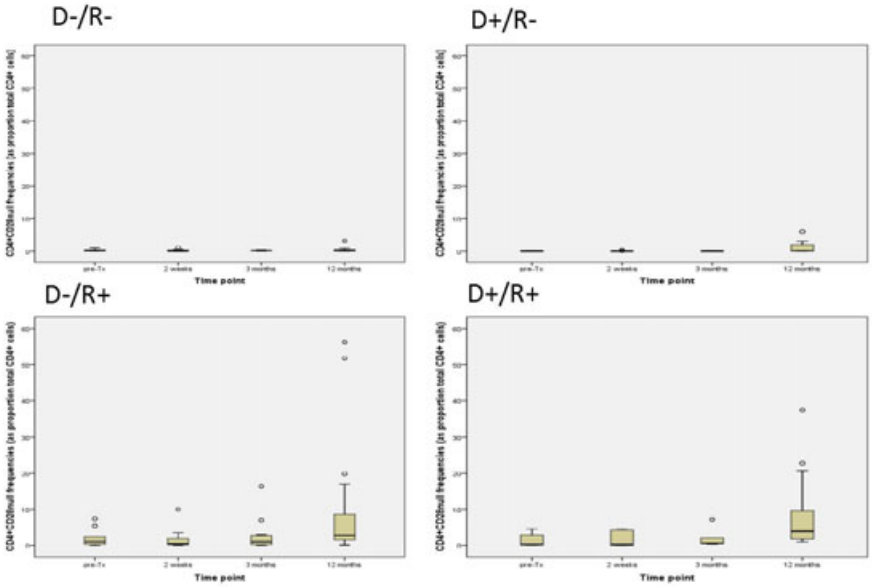


Figure 1A

Figure 1B



	Pre-Transplant	14 days	3 months	12 months
-/-	0.20 (0.0-1.1)	0.3 (0.0-1.1)	0.3 (0.1-0.5)	0.3 (0.0-3.1)
+/-	0.1 (0.0-0.2)	0.1 (0.0-0.5)	0.1 (0.0-0.3)	0.2 (0.0-6.0)
-/+	1.1 (0.0-7.3)	0.3 (0.0-9.9)	1.0 (0.0-16.4)	2.7 (0.1-56.2)
+/+	0.7 (0.0-4.5)	0.2 (0.0-4.5)	0.5 (0.3-7.1)	3.8 (0.9-37.4)

Figure 1C

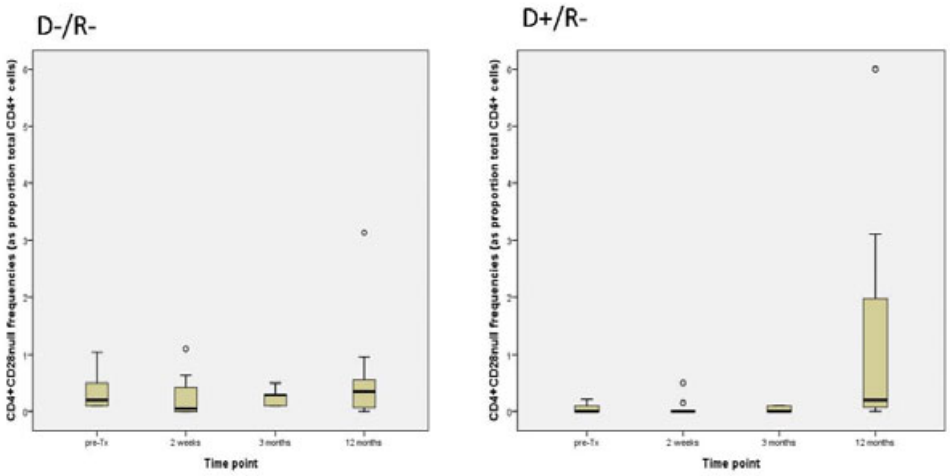


Figure 1D

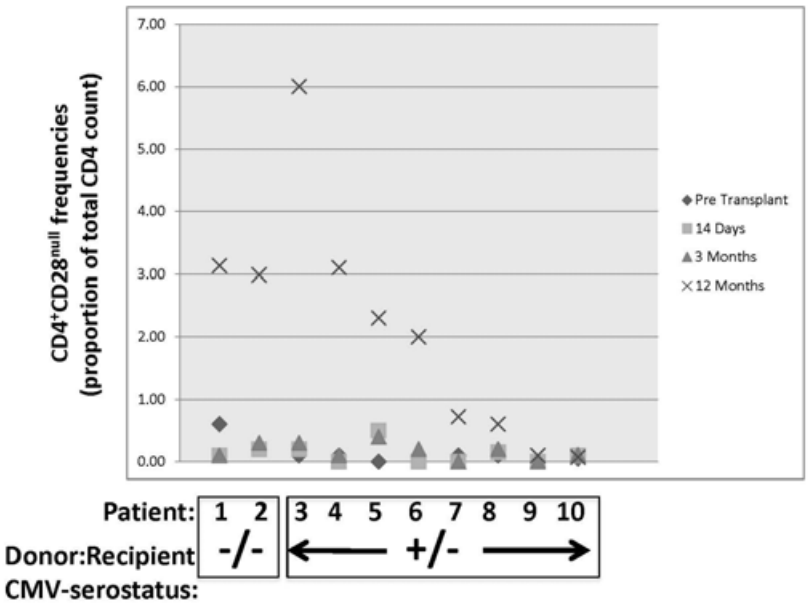


Figure 1E

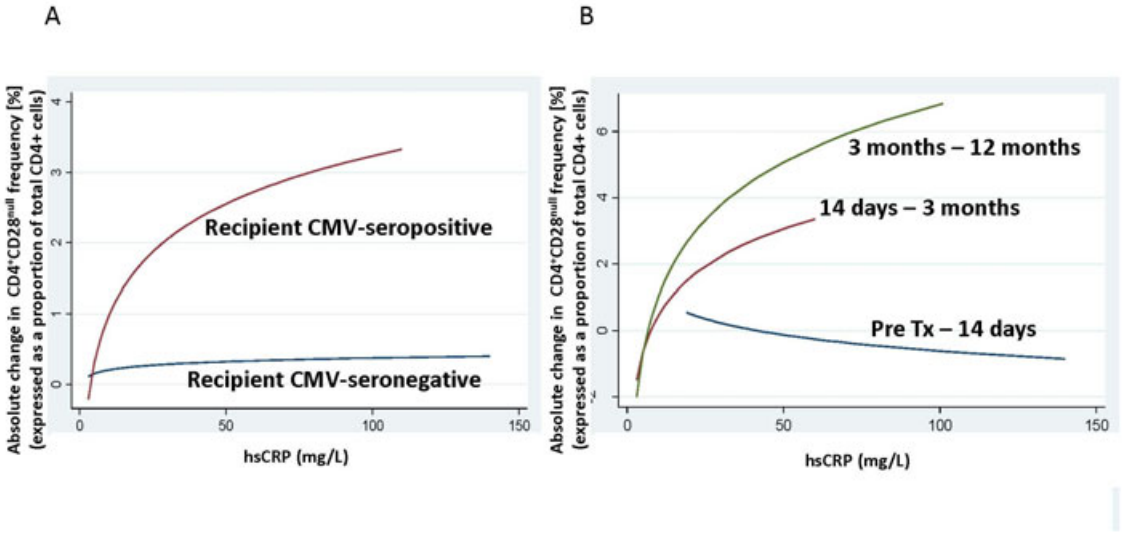


Figure 2

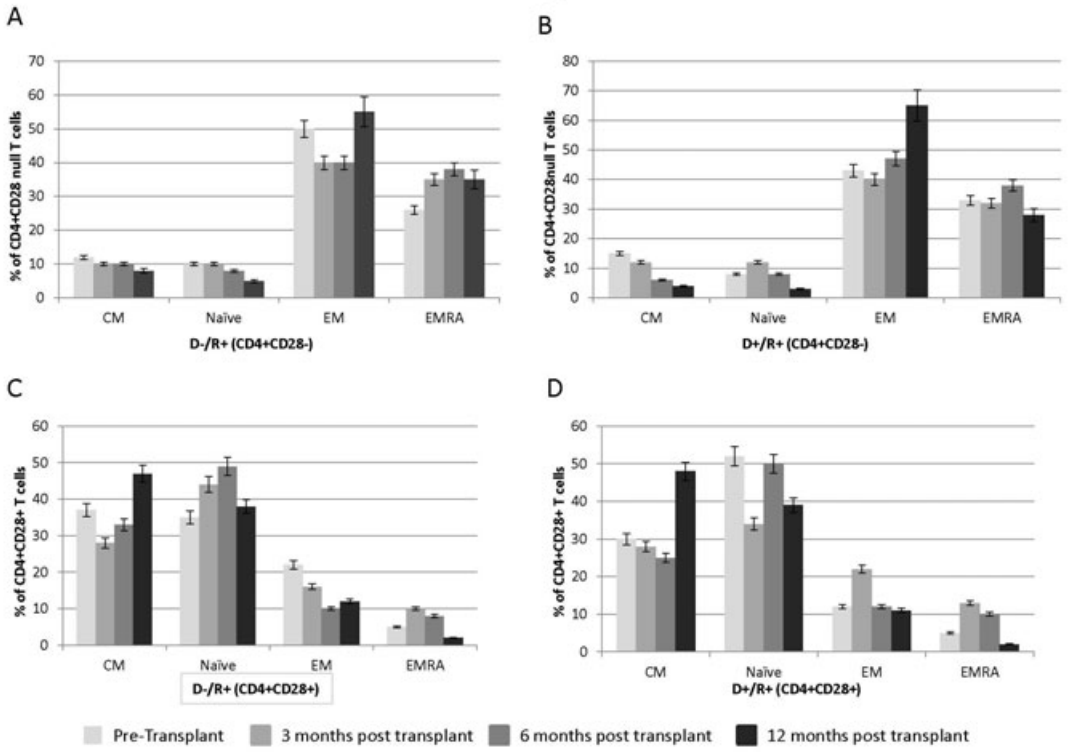


Figure 2

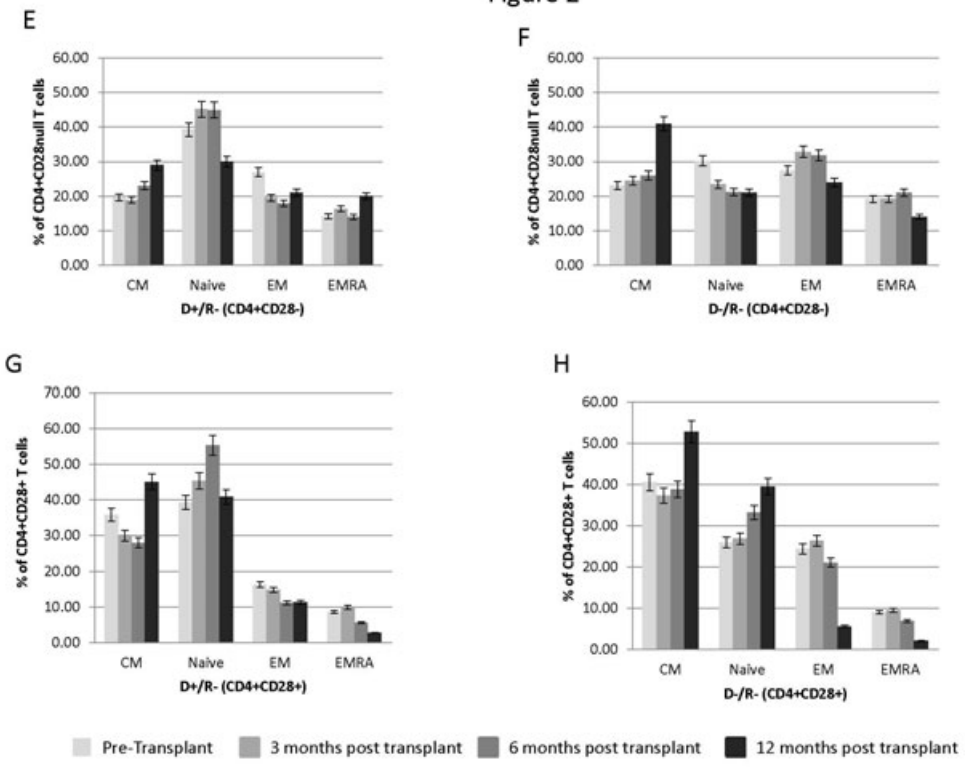


Figure 2

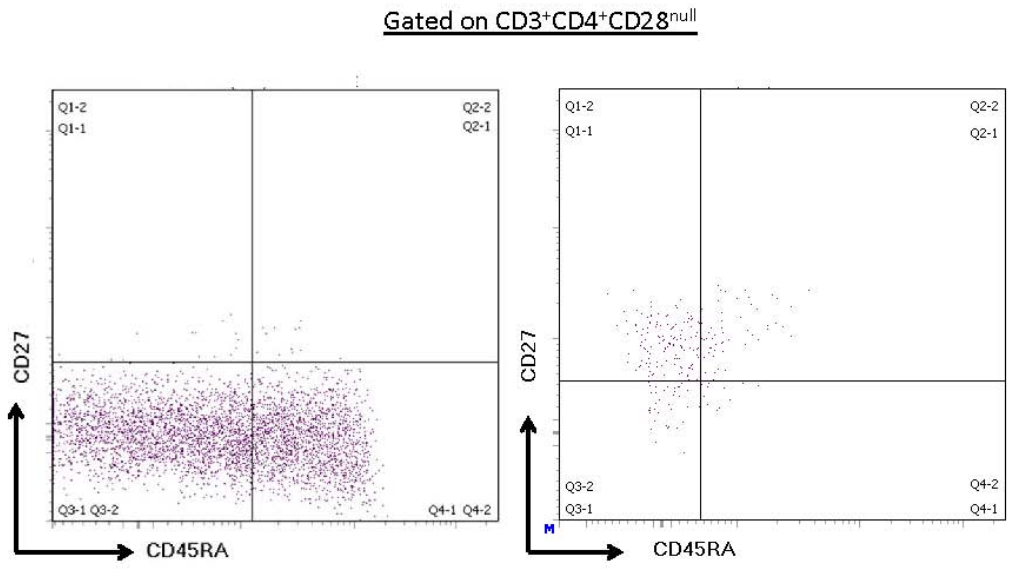


Figure 2I

Figure 2J

Figure 3A

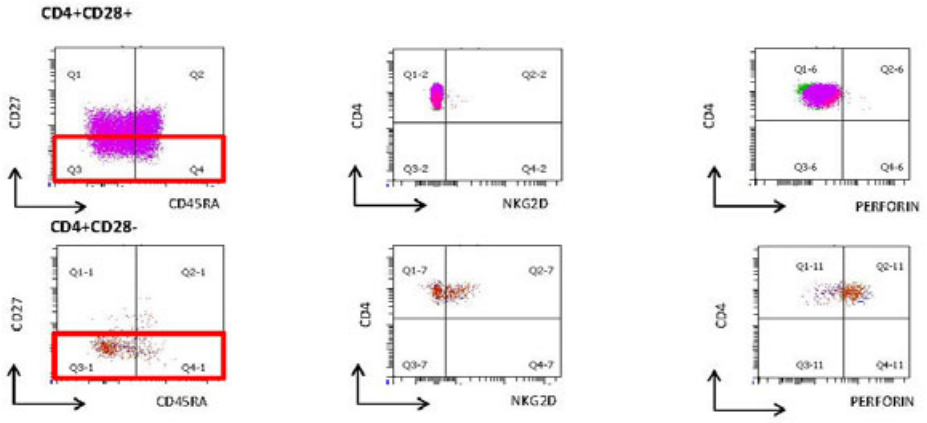


Figure 3B

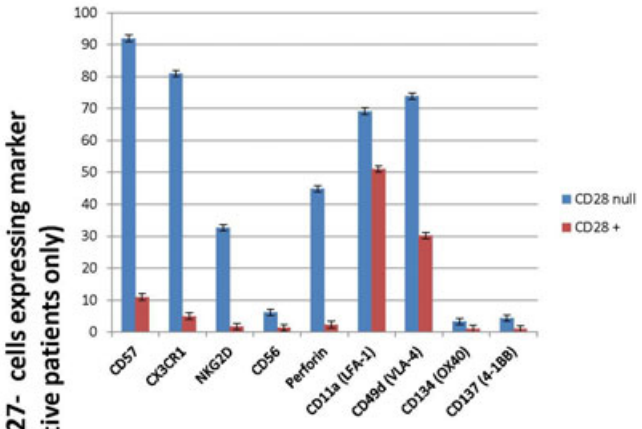


Figure 3C

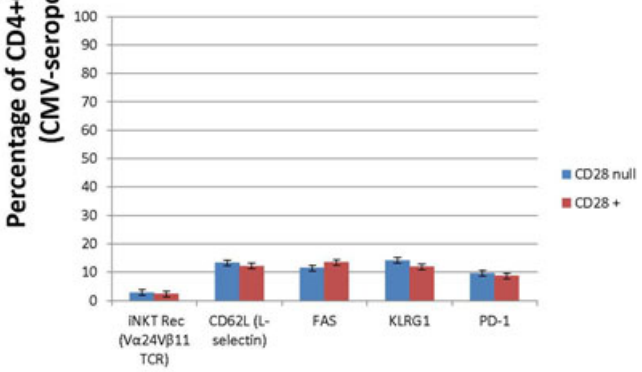


Figure 4

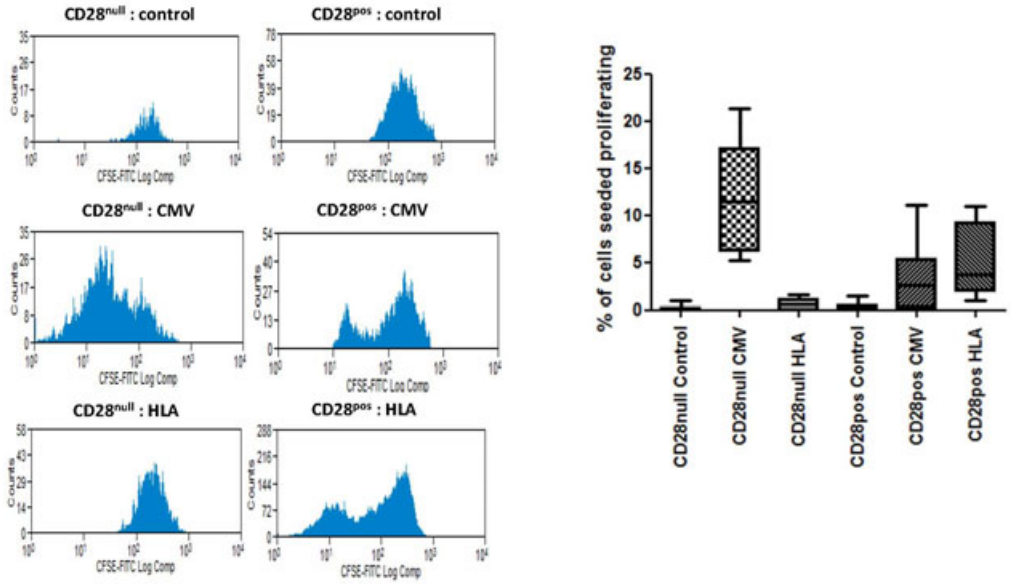


Figure 5