Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Killer cell immunoglobulin receptor profile on $CD4^+$ $CD28^-$ T cells and their pathogenic role in non-dialysis-dependent and dialysis-dependent chronic kidney disease patients

Behnam Zal,[1] Nihil Chitalia,[1,2] Yin Sing Ng,[1] Verna Trieu,[1] Sana Javed,[1] Rachelle Warrington,[1] Juan Carlos Kaski,[1] Debasish Banerjee[1,2] and Christina Baboonian[1]

[1]*Division of Clinical Sciences, St George's University of London, London, and* [2]*Department of Renal Medicine and Transplantation, St George's Hospital NHS Trust, London, UK*

doi:10.1111/imm.12429
Received 31 May 2013; revised 29 November 2014; accepted 02 December 2014.
Nihil Chitalia and Behnam Zal are joint first authors.
Correspondence: Dr Christina Baboonian, Division of Clinical Sciences, St George's University of London, London SW17 0RE, UK.
Email: cbabooni@sgul.ac.uk
Senior author: Dr Christina Baboonian.

Summary

There is a progressive increase in cardiovascular disease with declining renal function, unexplained by traditional risk factors. A $CD4^+$ T-cell subpopulation ($CD4^+$ $CD28^-$), activated by human heat-shock protein 60 (hHSP 60), expands in patients with acute coronary syndrome and is associated with vascular damage. These cells exhibit cytotoxicity via expression of activating killer cell-immunoglobulin-like receptor KIR2DS2, mainly in the absence of inhibitory KIR2DL3. We investigated expansion of these cells and the pathogenic role of the KIR in non-dialysis-dependent chronic kidney disease (NDD-CKD) and end-stage haemodialysis-dependent renal disease (HD-ESRD) patients. $CD4^+$ $CD28^-$ cells were present in 27% of the NDD-CKD and HD-ESRD patients (8–11% and 10–11% of $CD4^+$ compartment, respectively). $CD4^+$ $CD28^-$ cells were phenotyped for KIR and DAP12 expression. Cytotoxicity was assessed by perforin and pro-inflammatory function by interferon-γ expression on $CD4^+$ $CD28^-$ clones (NDD-CKD $n = 97$, HD-ESRD $n = 262$). Thirty-four per cent of the $CD4^+$ $CD28^-$ cells from NDD-CKD expressed KIR2DS2 compared with 56% in HD-ESRD patients ($P = 0{\cdot}03$). However, 20% of clones expressed KIR2DL3 in NDD-CKD compared with 7% in HD-ESRD patients ($P = 0{\cdot}004$). DAP12 expression in $CD28^-$ $2DS2^+$ clones was more prevalent in HD-ESRD than NDD-CKD (92% versus 60%; $P < 0{\cdot}001$). Only $2DS2^+$ $2DL3^-$ $DAP12^+$ clones were cytotoxic in response to hHSP 60. $CD4^+$ $CD28^-$ cells exhibited increased KIR2DS2, reduced KIR2DL3 and increased DAP12 expression in HD-ESRD compared with NDD-CKD patients. These findings suggest a gradual loss of expression, functionality and protective role of inhibitory KIR2DL3 as well as increased cytotoxic potential of $CD4^+$ $C28^-$ cells with progressive renal impairment. Clonal expansion of these T cells may contribute to heightened cardiovascular events in HD-ESRD.

Keywords: $CD4^+$ $CD28^-$ T cells; chronic kidney disease; killer cell immunoglobulin receptors.

Introduction

A population of $CD4^+$ T lymphocytes that lack the CD28 co-stimulatory marker, $CD4^+$ $CD28^-$ T cells, was first identified in patients with acute coronary syndrome (ACS) by Liuzzo *et al.* in 1999.[1] These cells were reported to be present in unstable coronary artery plaques as well as in the peripheral blood of patients with ACS.[2,3] $CD4^+$ $CD28^-$ T cells are phenotypically and functionally separate from classic $CD4^+$ cells. They express killer cell immunoglobulin-like receptors (KIRs), a characteristic of natural killer cells, and have significant pro-inflammatory and cytotoxic function, releasing interferon-γ (IFN-γ) and perforin upon activation.[4] Recent studies have shown that patients with end-stage renal disease (ESRD) also have increased numbers of circulating $CD4^+$ $CD28^-$ T cells and the presence of these cells is independently associated with atherosclerotic disease in these patients.[5–7]

Studies carried out in our laboratories have shown that the $CD4^+$ $CD28^-$ T cells present in patients with ACS are specifically reactive to the ubiquitously expressed intracellular chaperone protein, human heat-shock protein 60 (hHSP 60).[8] Because of their ability to produce high levels of IFN-γ involved in monocyte and macrophage activation, $CD4^+$ $CD28^-$ cells are thought to play an important pathogenic role in the progression of atherosclerotic disease.[3] Activated macrophages induce expression of matrix metalloproteinases, which are implicated in degradation of the atherosclerotic plaque cap leading to thrombus formation.[3] Perforin release, on the other hand, causes direct lysis of smooth muscle cell and the endothelial cell component of plaques leading to destabilization and rupture.[9] Although $CD4^+$ $CD28^-$ T cells are rare in healthy individuals, in patients with ACS these T cells can comprise more than 50% of the total $CD4^+$ T-cell repertoire and have been shown to increase progressively with subsequent coronary artery events.[2] Our previous studies have also confirmed that $CD4^+$ $CD28^-$ cells from healthy individuals are phenotypically and functionally different to those found in chronic inflammatory conditions and constitute a non-pathogenic T-cell population.[8,10] Recent studies have shown that prevalence of circulating $CD4^+$ $CD28^-$ T cells is associated with early atherosclerotic damage in patients with ESRD.[7]

We have also demonstrated that $CD4^+$ $CD28^-$ cells in ACS patients exhibit selective effector function upon interaction of the activating KIR2DS2 receptor with hHSP 60 and may contribute to progressive atherosclerotic disease.[10] The cytotoxic drive of $CD4^+$ $CD28^-$ cells has been shown to be mediated by the expression of the adaptor molecule DAP12 on these cells.[10] The pro-inflammatory function of $CD4^+$ $CD28^-$ cells, on the other hand, has been demonstrated to be T-cell-receptor-mediated and in the context of MHC class II presentation of the antigen.[4] Although recent data suggest that cytomegalovirus (CMV) infection may be strongly associated with the emergence of the $CD4^+$ $CD28^-$ cells, in patients with ACS these cells are hHSP 60 specific and do not respond to CMV antigens presented by MHC II or MHC I and no cytotoxic response is observed in this cell population when exposed to CMV antigen-expressing autologous cells.[8,11]

Chronic kidney disease (CKD) represents a heightened inflammatory state and is associated with a progressive increase in cardiovascular mortality as the renal function declines.[12,13] The highest mortality rates are observed in patients with ESRD on dialysis. Traditional risk factors only partially explain this disproportionate increase in cardiovascular risk.[14] Heat-shock proteins, because of their ability to stimulate directly a T-cell-mediated immune response, have been implicated in vascular injury and atherosclerosis.[15,16] Renal failure may trigger a heat-shock protein response as a consequence of stress, ischaemia and oxidative injury. Indeed, heat-shock protein expression is up-regulated in patients on haemodialysis and in non-dialysis-dependent CKD.[17–19] The aim of this study was to further characterize the antigen specificity as well as pro-inflammatory and KIR-mediated cytotoxic function of these cells. Non-dialysis-dependent CKD (NDD-CKD) patients were compared with haemodialysis-dependent ESRD (HD-ESRD) patients to explore the possible changes in $CD4^+$ $CD28^-$ T-cell functionality with worsening kidney failure.

Methods

Patient population

Fifteen NDD-CKD patients (mean estimated glomerular filtration rate 30 ± 15 ml/min/1·73 m^2) and 15 maintenance HD-ESRD patients aged between 18 and 75 years were recruited for the study from the general nephrology clinics and haemodialysis unit at St George's Hospital. Patients with active inflammation, history of autoimmune disease, malignancy, heart failure and those with acute coronary syndrome (diagnosed with a Troponin-T rise > 0·05 ng/ml) within the last 3 months were excluded. Ten healthy volunteers were included in the study as controls. The study was approved by the local research ethics committee (REC No: 054/Q0803/185) and conforms to the Declaration of Helsinki.

Peripheral blood mononuclear cell separation and T-cell isolation

Peripheral blood mononuclear cells were separated from peripheral blood by Lymphoprep (Axis-Shield, Oslo, Norway). $CD4^+$ $CD28^-$ cells were then separated magnetically using MACS LS and MS columns and the $CD4^+$ T-cell isolation kit according to the manufacturer's instruction and as described previously (Miltenyi Biotec, Surrey, UK).[10]

$CD4^+$ $CD28^-$ cell cloning

Cloning was performed at a dilution of 0·5 cell/well of 96-well plates using Iscove's modified Dulbecco's medium (Life Technologies, Paisley, UK) and irradiated allogeneic feeder cells as described previously.[10,20] Cultures were incubated at 37° for 4 days then supplemented with fresh medium and single clones were identified on day 12.

Phenotyping of $CD4^+$ $CD28^-$ cell clones

$CD4^+$ $CD28^-$ cell clones were phenotyped for the expression of activating and inhibitory receptors KIR2DS2, KIR2DL2, KIR2DL3 and the adaptor molecule DAP12. Using the Trizol reagent, RNA was extracted from each

clone and cDNA synthesis was carried out using Superscript II reverse transcriptase and random hexamers (Life Technologies) according to our previously published methodology.[8]

Amplification of the receptor transcripts was performed using the primers and conditions as previously described.[10]

$CD4^+$ $CD28^-$ T-cell responses to antigen stimulation

Autologous monocyte-derived target cells were prepared from peripheral blood mononuclear cells using a previously described methodology.[10,20] Target cells were pulsed with 5 μg/ml hHSP 60 at 37° overnight and reseeded into 96-well plates at a density of 3×10^4 cells/well. T-cell clones expressing different receptors were added to target cells at an effector to target cell ratio of 30 : 1 in 100 μl final volumes.[10] After 5 hr of incubation at 37°, perforin and IFN-γ mRNA transcriptions were assessed for each well using RT-PCR. Each well containing the same number of target and effector cells was used for RNA extraction and cDNA synthesis, which were carried out as described above. The amplification of the activation markers, IFN-γ and perforin was performed using 100 ng of cDNA and AmpliTaq Gold polymerase.[8] The positive and negative controls included phytohaemagglutinin-stimulated (10 μg/ml) and unstimulated (no hHSP 60) target cells.

Perforin responses of $2DS2^+$ $DAP12^-$ and $2DS2^+$ $DAP12^-$ T-cell clones

CytoTox96 Cytotoxicity Assay kit (Promega, Southampton, UK) was used to assess the cytotoxic function of $CD4^+$ $CD28^-$ clones. The kit measures the amount of lactate dehydrogenase released in the culture medium upon lysis of the target cells. Hence, assessment of lactate dehydrogenase release reflects target cell lysis levels as a measure of T-cell cytotoxicity. Replicas of $2DS2^+$ $DAP12^+$ and $2DS2^+$ $DAP12^-$ clones that were used for the measurement of perforin mRNA were analysed for their cytotoxic responses against monocytes pulsed with 5 μg/ml hHSP 60. The procedures employed for the preparation of effector and target cells, as well as the antigen pulsing, were as described in the previous section.[10] The CytoTox96 assay protocol was then followed according to the manufacturer's instruction (Promega) and as previously described.[10] After exposure of target to effector T cells for 5 hr, the absorbance values were recorded at 492 nm and cytotoxicity was calculated using the formula provided in the manufacturer's manual (Promega).

IFN-γ responses of $2DS2^+$ and $2DS2^-$ T-cell clones

The RayBio® Human IFN-γ ELISA kit (RayBiotech, Norcross, GA) was used to detect the amount of IFN-γ released in culture supernatant of $2DS2^+$ and $2DS2^-$ T-cell clones upon antigen stimulation. This kit provides a quantitative measurement of human IFN-γ using a specific antibody immobilized on a 96-well plate. Replicas of $2DS2^+$ and $2DS2^-$ clones that were used for the measurement of IFN-γ mRNA were tested for their IFN-γ release against monocytes pulsed with 5 μg/ml hHSP 60. The protocols for the preparation of effector and target cells, as well as the antigen pulsing, were as described in the previous section.[10]

T-cell clones were stimulated with hHSP 60 and supernatants were separately transferred into the wells of the assay plate. The ELISA was then completed according to the manufacturer's instructions (RayBio) after addition of biotinylated anti-human IFN-γ and horseradish peroxidase-conjugated streptavidin followed by the substrate solution. The absorbance values were recorded at 450 nm and the amount of IFN-γ bound was calculated from the calibration curve. The detection limit for IFN-γ was 15 pg/ml.

Statistical analyses

All continuous normally distributed variables are expressed as mean ± 1SD. Non-parametric variables are expressed as median ± interquartile range. Categorical variables are expressed as *n* (%). Independent sample *t*-test was used to test statistical significance for parametric continuous variables, whereas the Mann–Whitney *U*-test was used to test statistical significance for non-parametric continuous variables. A χ^2-test was used to test statistical significance for categorical variables. A two-sided *P*-value of $< 0{\cdot}05$ was considered for statistical significance.

Results

The demographic and clinical characteristics of patients are summarized in Table 1. The NDD-CKD ($n = 15$) and HD-ESRD patients ($n = 15$) were similar in age (55 ± 12 versus 57 ± 13 years, $P = 0{\cdot}70$), gender (60% males in both groups) and ethnicity. No significant differences were observed for presence of hypertension defined as blood pressure ≥ 130/80 mmHg or on antihypertensive medication. All other parameters evaluated, including antihypertensive treatments, statin therapy, anti-platelet therapy, diabetes status, hypercholesterolaemia, previous vascular disease, current smokers, and high sensitivity C-reactive protein levels (hsCRP) were comparable in the two groups.

Table 2 shows the demographic and clinical characteristics of patients and controls. The patients and controls were age, gender and ethnicity matched. NDD-CKD and HD-ESRD patients had a higher proportion of hypertension ($P < 0{\cdot}001$) compared with healthy volunteers.

Table 1. Characteristics of patients with non-dialysis-dependent chronic kidney disease and haemodialysis patients

Variables	Non-dialysis-dependent CKD (n = 15)	Haemodialysis (n = 15)	P-value
Age	55 ± 12	57 ± 13	0·70
Gender (males) (%)	9 (60)	9 (60)	1·00
Ethnicity (%)			0·67
Caucasian	5 (33)	5 (33)	
Afro Caribbean	4 (27)	6 (40)	
South Asian	6 (40)	4 (27)	
Hypertension (%)	14 (93)	12 (80)	0·59
Diabetes mellitus (%)	0	3 (20)	0·22
Hypercholesterolaemia (%)	8 (53)	5 (33)	0·26
Current smokers (%)	2 (13)	1 (6)	1·00
History of vascular disease (%)	1 (7)	4 (27)	0·33
hsCRP (mg/l)	4 ± 3·15	6·9 ± 8·7	0·38
Systolic BP (mmHg)	133 ± 12	129 ± 22	0·54
Diastolic BP (mmHg)	81 ± 7	76 ± 13	0·24
ACE inhibitor or ARB therapy (%)	12 (80)	7 (47)	0·055
Statin therapy (%)	9 (60)	6 (40)	0·27
Anti-platelet therapy (%)	3 (20)	2 (13)	1·00
Calcium channel blockers (%)	10 (67)	7 (47)	0·26
β-blockers (%)	3 (20)	4 (27)	1·00

Abbreviations: ACE inhibitor, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BP, blood pressure; CKD, chronic kidney disease; hsCRP, high-sensitivity C-reactive protein.
Hypertension defined as BP ≥ 130/80 mmHg or on anti-hypertensives. Hypercholesterolaemia defined as total cholesterol > 5 mmol/l or on statin therapy. History of vascular disease defined as presence of ischaemic heart disease, peripheral vascular disease or previous cerebrovascular event.

Table 2. Comparison of demographic and clinical characteristics between patients and controls

Variables	Patients NDD-CKD, n = 15 and HD-ESRD, n = 15	Controls Healthy volunteers (n = 10)	P-value
Age (years)	56 ± 13	51 ± 12	0·31
Gender (males) (%)	18 (60)	5 (50)	0·58
Ethnicity (%)			0·59
Caucasian	10 (33)	5 (50)	
Afro Caribbean	10 (33)	2 (20)	
Asian	10 (33)	3 (30)	
Diabetes (%)	3 (10)	0 (0)	0·29
Hypertension (%)	26 (87)	0 (0)	< 0·001*

Abbreviations: HD-ESRD, end-stage haemodialysis-dependent renal disease; NDD-CKD, non-dialysis-dependent chronic kidney disease.
Hypertension defined as blood pressure ≥ 130/80 mmHg or on antihypertensive medications.
*Denotes a significant difference in proportion of hypertension between healthy subjects and chronic kidney disease patients.

Assessment of the $CD4^+$ $CD28^-$ cell expansion

Four NDD-CKD patients had increased numbers of $CD4^+$ $CD28^-$ cells that constituted 8–11% of the $CD4^+$ compartment. There was no difference in age and hsCRP levels in patients with $CD4^+$ $CD28^-$ cells compared with those without. Expanded levels of pathogenic $CD4^+$ $CD28^-$ cells were also detected in four ESRD patients on haemodialysis, which represented 10–11% of the $CD4^+$ population. Compared with the rest of the dialysis patients they had similar hsCRP and total $CD4^+$ cell count, but were older (70 ± 3·5 versus 59·9 ± 8·7 years, P = 0·045). Low level presence of non-pathogenic $CD4^+$ $CD28^-$ cells was detected in six of the 10 healthy controls constituting 1% of the $CD4^+$ compartment.

Assessment of KIRs and DAP12 distribution

KIR2DS2 is the predominant receptor mediating $CD4^+$ $CD28^-$ cell cytotoxicity.[10] We therefore determined

the expression of KIR2DS2 and its corresponding inhibitory receptors, KIR2DL2 and KIR2DL3, on individual T-cell clones from patients and control groups. Analysis of 97 T-cell clones established from three NDD-CKD patients revealed the presence of KIR2DS2 in 36 (35%), 41 (40%) and 29 (28%) clones. In comparison, investigation of the 262 clones from four HD-ESRD patients showed expression of KIR2DS2 in 107 (41%), 238 (91%), 134 (51%) and 113 (43%) clones ($P = 0{\cdot}03$). The co-expression of the inhibitory KIR2DL3 was present in 20%, 25% and 17% compared with only 10%, 3%, 6% and 9% of the $2DS2^+$ $CD4^+$ $CD28^-$ cell clones in NDD-CKD and HD-ESRD patients, respectively ($P = 0{\cdot}004$). DAP12 adaptor molecule is an essential component of 2DS2-mediated effector function. Screening for the presence of DAP12 showed lower expression of the corresponding mRNA in $CD28^-$ $2DS2^+$ cells from NDD-CKD patients compared with HD-ESRD patients (62·5%, 55% and 62·5% versus 87%, 95%, 94% and 93%, $P < 0{\cdot}001$). The inhibitory receptor KIR2DL2 was not detected on the clones from either group. The prevalence of $2DS2^+$ clones in healthy individuals was 24%, but its expression largely coincided with the presence of the inhibitory receptors (92%) and complete absence of DAP12 expression.

Assessment of IFN-γ and perforin responses of $CD4^+$ $CD28^-$ cells by RT-PCR

The analysis of the total $CD4^+$ $CD28^-$ T-cell fractions isolated from peripheral blood mononuclear cells showed the antigen specificity of these T cells for hHSP 60 by up-regulation of IFN-γ and perforin transcription. We then investigated the pro-inflammatory and cytotoxic responses of the T-cell clones expressing KIR2DS2 by RT-PCR. Assessment of T-cell clones established from both groups of patients revealed that the cytotoxic response, manifested by perforin transcription upon hHSP 60 exposure, was dependent on expression of both KIR2DS2 and the DAP12 molecule (Fig. 1). Clones lacking KIR2DS2 were also investigated and found to be non-reactive. The pro-inflammatory IFN-γ response was not, however, dependent on the expression of either of these molecules (Fig. 2). Perforin response always coincided with IFN-γ up-regulation, although in some clones the latter response was present in the absence of the former. These data demonstrate that IFN-γ production by $CD4^+$ $CD28^-$ cells is independent of KIR expression and MHC class I pathway confirming our previous findings that cytokine activation occurs in the context of MHC class II antigen presentation.[10]

Analysis of $CD4^+$ $CD28^-$ $2DS2^+$ clones from healthy controls showed that these cells were non-reactive to hHSP 60 by perforin or IFN-γ responses. Our previous work on $CD4^+$ $CD28^-$ cell clones isolated from healthy volunteers has also confirmed the non-responsiveness of

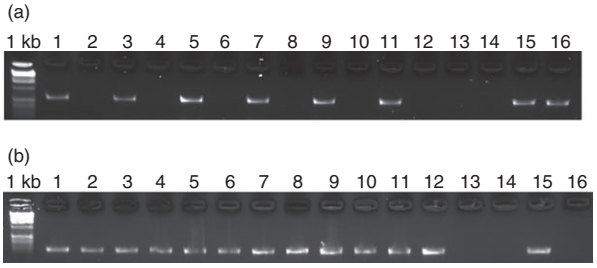

Figure 1. Perforin reactivity of $CD4^+$ $CD28^-$ $2DS2^+$ T-cell clones against human heat-shock protein 60 (hHSP 60)-pulsed autologous monocytic target cells. (a) Expression of perforin transcripts by T-cell clones in the presence (wells 1, 3, 5, 7, 9 and 11) and absence (wells 2, 4, 6, 8, 10 and 12) of antigen exposure. Each test was performed using replica clones. Wells 15 and 16 represent $2DS2^+$ $DAP12^+$ and $2DS2^+$ $DAP12^-$ clones stimulated with phytohaemagglutinin, respectively. (b) Expression of DAP12 in relation to hHSP 60 reactivity of $CD4^+$ $CD28^-$ $2DS2^+$ clones in (a). $2DS2^+$ $DAP12^-$ clones did not produce perforin mRNA in response to hHSP 60-pulsed (well 13) and un-pulsed target cells (well 14) in both panels. Transcript sizes for perforin and DAP12 are 589 bp and 236 bp, respectively.

the T-cell clones to hHSP 60 stimulation.[10] This agrees with the published literature in confirming that these cells constitute a non-pathogenic population of $CD4^+$ cells and their expansion is a result of age-related replicative senescence.[21]

Pathological assessment of KIR2DS2 and its inhibitory receptor KIR2DL3

KIR2DS2 is expressed on $CD4^+$ $CD28^-$ cells largely in the absence of the corresponding inhibitory receptors KIR2DL2 and KIR2DL3. However, in patients with ACS it has been shown that the presence of these inhibitory receptors had no functional role in opposing the activation signals generated by KIR2DS2 upon antigen encounter.[10]

The inhibitory receptor present in the KIR2DS2-expressing $CD4^+$ $CD28^-$ cells in NDD-CKD and HD-ESRD patients is KIR2DL3. Analysis of 10 $CD4^+$ $CD28^-$ clones co-expressing KIR2DS2 and KIR2DL3 in NDD-CKD patients showed that KIR2DL3 expression in eight clones inhibited the perforin response of the KIR2DS2. Upon exposure to hHSP 60-pulsed autologous cells no up-regulation of perforin transcription was noted by these cells. Two KIR2DS2 and KIR2DL3 positive clones, however, responded to autologous hHSP60-expressing cells by perforin response. A representative set of results is shown in Fig. 3. Analysis of the clones expressing KIR2DS2 in the absence of the KIR2DL3 showed that all the clones ($n = 10$) reacted to antigen-pulsed target cells by up-regulation of perforin transcription. A representation of the results is shown in Fig. 4. This suggests that unlike in

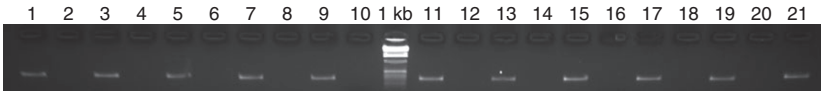

Figure 2. Pro-inflammatory response of $2DS2^+$ $2DL3^-$ $DAP12^+$ and $2DS2^-$ $2DL3^-$ $DAP12^+$ clones from non-dialysis-dependent chronic kidney disease (NDD-CKD) patients. Panels 1–10 and 11–20 represent interferon-γ (IFN-γ) response from $2DS2^+$ $2DL3^-$ $DAP12^+$ and $2DS2^-$ $2DL3^-$ $DAP12^+$ cells, respectively, against target cells pulsed with human heat-shock protein 60 (hHSP 60). The negative wells represent replica clones exposed to target cells in the absence of the antigen. The data shows that the pro-inflammatory response of $CD4^+$ $CD28^-$ cells is present irrespective of KIR2DS2 expression. Well 21 represents a $2DS2^+$ $2DL3^-$ $DAP12^+$ clone stimulated with phytohaemagglutinin as a positive control. Transcript size for IFN-γ is 356 bp.

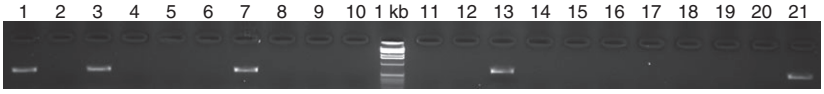

Figure 3. The effect of KIR2DL3 expression on the cytotoxic response of $CD4^+$ $CD28^-$ $2DS2^+$ $DAP12^+$ clones from non-dialysis-dependent chronic kidney disease (NDD-CKD) and end-stage haemodialysis-dependent renal disease (HD-ESRD) patients. Wells 1, 3, 5, 7 and 9 represent the perforin response by the $2DL3^+$ clones from HD-ESRD patients against target cells pulsed with human heat-shock protein 60 (hHSP 60). Wells 11, 13, 15, 17 and 19 represent this response by $2DL3^+$ clones from NDD-CKD patients. Replica clones exposed to target cells in the absence of the antigen are included adjacent to each test sample and represent negative controls. Well 21 represents PHA-stimulated T cells as positive control. The inhibitory function of 2DL3 was more pronounced in the NDD-CKD patients. Transcript size for perforin is 589 bp.

ACS, the inhibitory KIR2DL3 on the majority of $CD4^+$ $CD28^-$ cells from NDD-CKD patients are functional and capable of opposing the KIR2DS2-mediated reactivity to autologous antigens and the non-discriminate killing potential of these T cells.

We then addressed the protective role of KIR2DL3 in patients on haemodialysis. Antigen stimulation of the 10 $2DS2^+$ $2DL3^+$ clones revealed a reduction of the inhibitory function by KIR2DL3 with only four $2DS2^+$ $2DL3^+$ clones being non-reactive, whereas the rest (60%) exhibited an increased perforin transcription response. A representative set of results is shown in Fig. 3. All the $2DS2^+$ $2DL3^-$ clones ($n = 10$) reacted to hHSP 60-pulsed cells by up-regulation of perforin mRNA. A representation of the results is shown in Fig. 4. The significance of these findings is further addressed in the discussion.

Selective lysis of hHSP 60-pulsed monocytes by $2DS2^+$ $DAP12^+$ clones

Following the assessment of the perforin response of $2DS2^+$ $DAP12^+$ clones at the mRNA level, we conducted further experiments to assess the cytolytic capability of the same clones using the CytoTox96 assay (Promega). This kit quantitatively measures lactate dehydrogenase released upon cell lysis.[10] Replicas of $2DS2^+$ $DAP12^+$ and $2DS2^+$ $DAP12^-$ clones were investigated using hHSP 60-pulsed monocytes as target cells (Fig. 5). A total of eight T-cell clones, consisting of four $2DS2^+$ $DAP12^+$ and four $2DS2^+$ $DAP12^-$ clones from NDD-CKD and HD-ESRD patients were investigated. These represented the replicas of the same clones that had already been used for the measurement of perforin transcript levels following antigen stimulation. The mean percentage cytotoxicity was 90·5% for $2DS2^+$ $DAP12^+$ clones, calculated according to the manufacturer's instructions (Promega). $2DS2^+$ $DAP12^-$ clones and control wells containing effector clones in the absence of antigen prompted no cytotoxicity. Duplicate clones were tested to validate our findings. These results support our mRNA data on the cytotoxic capability of $CD4^+$ $CD28^-$ T cells co-expressing 2DS2 and DAP12 receptors against hHSP 60 and demonstrate that these T cells can induce an effector response by releasing perforin granules. The results further confirm that the cytotoxicity of $CD4^+$ $CD28^-$ $2DS2^+$ cells is dependent on the expression of DAP12 by these T cells.

IFN-γ secretion of $2DS2^+$ and $2DS2^-$ T cells

To assess the capability of $CD4^+$ $CD28^-$ $2DS2^+$ and $CD4^+$ $CD28^-$ $2DS2^-$ T cells to secrete IFN-γ and to confirm our earlier mRNA data, cytokine secretion was determined from the supernatant of $2DS2^+$ and $2DS2^-$ clones following hHSP 60 stimulation. The RayBio® Human IFN-γ ELISA was used for this purpose. Six clones, three expressing the 2DS2 receptor and three lacking the expression of this receptor from NDD-CKD patients were analysed. The results (Fig. 6) confirm our mRNA data and further demonstrate that the pro-inflammatory function of $CD4^+$ $CD28^-$ T cells is present regardless of the expression of KIR2DS2. No significant difference was observed between $2DS2^+$ and $2DS2^-$ responses in terms of IFN-γ secretion (Fig. 6). $2DS2^+$ clones exposed to target cells in the absence of antigen prompted no IFN-γ response. These data are in line with our previous findings that differential pathways govern the pro-inflammatory and cytotoxic function of these T cells.[10]

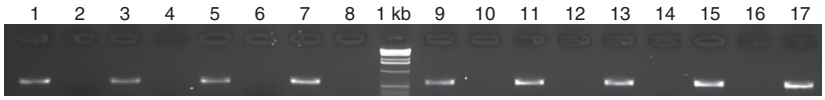

Figure 4. The cytotoxicity of $CD4^+$ $CD28^-$ $2DS2^+$ $DAP12^+$ clones lacking KIR2DL3 from end-stage haemodialysis-dependent renal disease (HD-ESRD) and non-dialysis-dependent chronic kidney disease (NDD-CKD) patients. Wells 1–8 and 9–16 represent clones from HD-ESRD and NDD-CKD patients, respectively. The data show all KIR2DL3$^-$ T cells exhibiting a perforin response against target cells pulsed with human heat-shock protein 60 (hHSP 60). The negative wells represent replica clones exposed to target cells not pulsed with hHSP 60. Well 17 represents cells stimulated by phytohaemagglutinin as positive control. Transcript size for perforin is 589 bp.

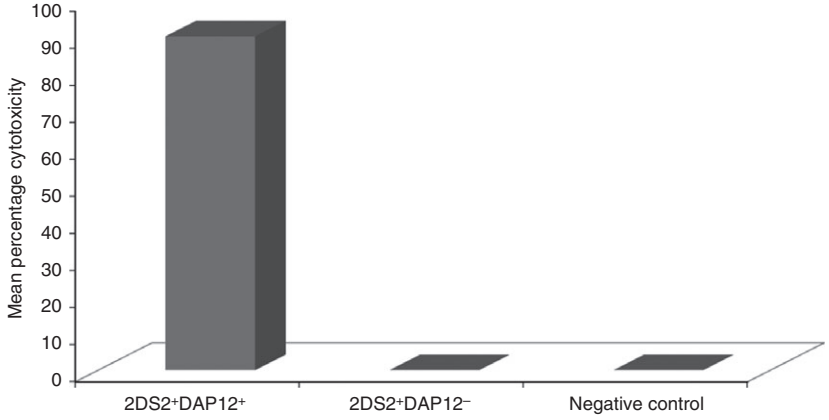

Figure 5. Selective lysis of human heat-shock protein 60 (hHSP 60)-pulsed target cells by $2DS2^+$ $DAP12^+$ clones. $2DS2^+$ $DAP12^+$ ($n = 4$) and $2DS2^+$ $DAP12^-$ clones ($n = 4$) from non-dialysis-dependent chronic kidney disease (NDD-CKD) and end-stage haemodialysis-dependent renal disease (HD-ESRD) patients were investigated for their cytotoxic function using hHSP 60-pulsed monocytes as target cells. Using the CytoTox96 assay kit (Promega), the mean percentage cytotoxicity, was calculated to be 90·5% for $2DS2^+$ $DAP12^+$ clones. This represented a percentage cytotoxicity of 94%, 91·5%, 89·5% and 87% for individual clones tested. $2DS2^+$ $DAP12^-$ clones and control wells containing T-cell clones in the absence of antigen prompted no cytotoxicity.

Discussion

Patients with chronic inflammatory conditions often experience an expansion of $CD4^+$ $CD28^-$ cells. The emergence of these cells is considered to be a consequence of chronic antigen stimulation that induces the down-regulation of CD28 in an attempt to re-establish tolerance.[22] These T cells have been identified in patients with ACS, rheumatoid arthritis and CKD patients on haemodialysis.[3,5,6,23–26] Our findings are consistent with the observations in ACS patients and suggest the presence of these killer cells in CKD patients and their hHSP 60 reactivity may have a pathogenic role in the development of cardiovascular disease in these patients.

In this study we investigated the expression of KIR2DS2, KIR2DL3 and DAP12 molecules and evaluated the functionality of $2DS2^+$ $DAP12^+$ clones in the presence or absence of the inhibitory KIR2DL3 using hHSP 60-pulsed autologous monocyte-derived cells.[27–29] Our data from the pre-dialysis CKD patients showed up-regulation of perforin mRNA upon antigen exposure in all of $2DS2^+$ $2DL3^-$ $DAP12^+$ clones investigated. However, analysis of $2DS2^+$ $2DL3^+$ $DAP12^+$ clones highlighted the role of KIR2DL3 in opposing the activation pathway generated by KIR2DS2 in most clones in which they were expressed. This is an important finding in relation to our previous data on the functionality of $CD4^+$ $CD28^-$ $2DS2^+$ cells in ACS patients. Analysis of the inhibitory KIR2DS2 and 2DL3 in ACS patients revealed that these receptors were unable to inhibit the activation signals generated by KIR2DS2. These results suggest that the inhibitory KIR receptors may exhibit a compromised function in ACS patients, which is not observed in CKD.

When we investigated the functional role of KIR2DL3 on $2DS2^+$ $DAP12^+$ clones in HD-ESRD patients, analysis of $2DS2^+$ $2DL3^+$ $DAP12^+$ clones revealed a different picture. Presence of KIR2DL3 did not inhibit the perforin response in 60% of the clones. Taken together, this is an interesting finding that may suggest a gradual loss of expression level as well as functionality and protective role of the inhibitory KIR2DL3 on $CD4^+$ $CD28^-$ cells with progressive renal disease; however, this may warrant a larger study. This supports the notion that these receptors may gradually acquire a compromised role in ongoing inflammatory conditions.

Other co-factors that have been highlighted in previous investigations are the KIR2DS2 receptors and their interactions with HLA-C molecules as ligands. Any changes in

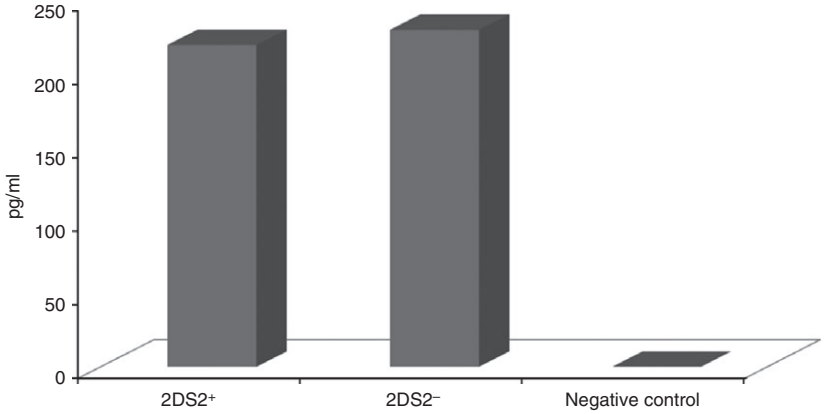

Figure 6. Interferon-γ (IFN-γ) secretion of 2DS2$^+$ and 2DS2$^-$ T-cell clones. Six clones, three expressing the 2DS2 receptor and three lacking the expression of this receptor were established from non-dialysis-dependent chronic kidney disease (NDD-CKD) patients and assessed for their IFN-γ secretion upon exposure to human heat-shock protein 60 (hHSP60) -pulsed monocytes. Using The RayBio® Human IFN-γ ELISA kit, cytokine secretion was determined from the culture supernatant of 2DS2$^+$ and 2DS2$^-$ clones following antigen exposure. The results show that the pro-inflammatory function of CD4$^+$ CD28$^-$ T cells is independent of KIR2DS2 expression. The IFN-γ secreted in the culture medium of 2DS2$^+$ and 2DS2$^-$ clones after antigen stimulation were 220 and 230 pg/ml, respectively.

HLA-C subtypes may prevent activatory or inhibitory function of the CD4$^+$ CD28$^-$ T cells.[30] Studies in our laboratories however, have shown no significant enrichment of HLA-C variants in patients with ACS. Commonly KIR2D-recognized variants of HLA-C (*01, *03 and *07), as well as, *04 and *05 have been identified in these patients.[10] These data suggest that the HLA-C variation is not likely to be a contributory factor in the functional differences observed in the CD4$^+$ CD28$^-$ T cells.

Investigation of the pro-inflammatory and cytotoxic function of CD4$^+$ CD28$^-$ cells from both groups showed a similar pattern of IFN-γ response which was unaffected by KIR expression and confirms our previous findings of differential responses mediated by KIR and the T-cell receptor.[10] The co-expression of 2DS2/DAP12 molecules on CD4$^+$ CD28$^-$ cells was necessary for the cytotoxic function of these T cells. The IFN-γ ELISA data further complemented the mRNA results in that the cytokine response was independent of KIR2DS2 expression and confirmed our previous findings that the cytotoxic and pro-inflammatory activation pathways of CD4$^+$ CD28$^-$ cells are independent of each other.[10]

To our knowledge this is the first study to provide an insight into the potential pathogenic role of hHSP 60-reactive CD4$^+$ CD28$^-$ 2DS2$^+$ cells in NDD-CKD and HD-ESRD patients on maintenance haemodialysis. These cells in haemodialysis patients exhibited reduced expression of inhibitory KIR2DL3 and increased presence of KIR2DS2 and the DAP12 molecule compared with the NDD-CKD group. In addition, the expression of KIR2DL3 in NDD-CKD patients was associated with significant inhibition of the cytotoxic response compared with HD-ESRD patients. Together, this may be responsible for the heightened cardiovascular event rate in HD-ESRD patients compared to NDD-CKD patients. This study is significant in highlighting a possible role of these cells in the inflammation–atherosclerosis cascade that characterizes patients with kidney disease. CKD patients suffer from a low-grade systemic inflammation, the cause of which is not clear. There is a low level rise of CRP which worsens with advancing CKD and is associated with endothelial dysfunction and atherosclerotic changes.[31] Our functional data suggest that CD4$^+$ CD28$^-$ cells may target endothelial cells expressing hHSP 60, causing direct endothelial injury. Furthermore, oxidative stress, a risk factor in patients on haemodialysis, may result in an increased expression of hHSP 60 and activation of CD4$^+$ CD28$^-$ cells.[32]

We are proposing an inflammatory mechanism outside the traditional risk factors that may account for increased cardiovascular mortality in CKD patients. Furthermore, our data are important in explaining the specific antigen–receptor interactions and pathways involved in the generation of pro-inflammatory and effector responses that may lead to the development of cardiovascular complications in CKD. Our findings may have implications for novel molecular and cellular therapeutic approaches to the management of CKD. Further investigations on the frequency of CD4$^+$ CD28$^-$ cells and their receptor expression may also help to provide new strategies for monitoring CKD progression.

Disclosures

The results presented in this paper have not been published previously in whole or part, except in abstract form. The research was funded by St George's Hospital Charitable Trust.

References

1 Liuzzo G, Kopecky SL, Frye RL, O'Fallon WM, Maseri A, Goronzy JJ, Weyand CM. Perturbation of the T-cell repertoire in patients with unstable angina. *Circulation* 1999; **100**:2135–9.

2 Liuzzo G, Biasucci LM, Trotta G *et al.* Unusual CD4$^+$ CD28^{null} T lymphocytes and recurrence of acute coronary events. *J Am Coll Cardiol* 2007; **50**:1450–8.

3 Liuzzo G, Goronzy JJ, Yang H, Kopecky SL, Holmes DR, Frye RL, Weyand CM. Monoclonal T-cell proliferation and plaque instability in acute coronary syndromes. *Circulation* 2000; **101**:2883–8.

4 Dumitriu IE, Araguas ET, Baboonian C, Kaski JC. CD4$^+$ CD28^{null} T cells in coronary artery disease: when helpers become killers. *Cardiovasc Res* 2009; **81**:11–9.

5 Yadav AK, Jha V. CD4$^+$ CD28^{null} cells are expanded and exhibit a cytolytic profile in end-stage renal disease patients on peritoneal dialysis. *Nephrol Dial Transplant* 2011; **26**:1689–94.

6 Betjes MGH, de Wit EEA, Weimar W, Litjens NHR. Circulating pro-inflammatory CD4^{pos}CD28^{null} T cells are independently associated with cardiovascular disease in ESRD patients. *Nephrol Dial Transplant* 2010; **25**:3640–6.

7 Sun Z, Ye H, Tang B, Shen X, Wu X, Zhong H, Song W. Prevalence of circulating CD4$^+$ CD28^{null} T cells is associated with early atherosclerotic damage in patients with end-stage renal disease undergoing hemodialysis. *Hum Immunol* 2013; **74**:6–13.

8 Zal B, Kaski JC, Arno G *et al.* Heat-shock protein 60-reactive CD4$^+$ CD28^{null} T cells in patients with acute coronary syndromes. *Circulation* 2004; **109**:1230–5.

9 Nakajima T, Goek O, Zhang X, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. *De novo* expression of killer immunoglobulin-like receptors and signaling proteins regulates the cytotoxic function of CD4 T cells in acute coronary syndromes. *Circ Res* 2003; **93**:106–13.

10 Zal B, Kaski JC, Akiyu JP *et al.* Differential pathways govern CD4$^+$ CD28$^-$ T cell proinflammatory and effector responses in patients with coronary artery disease. *J Immunol* 2008; **181**:5233–41.

11 van Leeuwen EM, Remmerswaal EB, Vossen MT, Rowshani AT, Wertheim-van Dillen PM, van Lier RA, ten Berge IJ. Emergence of CD4$^+$ CD28$^-$ granzyme B$^+$, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection. *J Immunol* 2004; **173**:1834–41.

12 Sarnak MJ, Levey AS, Schoolwerth AC *et al.* Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation* 2003; **108**:2154–69.

13 Go AS, Chertow GM, Fan D, McCulloch CE, Hsu C. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004; **351**:1296–305.

14 Schiffrin EL, Lipman ML, Mann JF. Chronic kidney disease: effects on the cardiovascular system. *Circulation* 2007; **116**:85–97.

15 Perschinka H, Mayr M, Millonig G *et al.* Cross-reactive B-cell epitopes of microbial and human heat shock protein 60/65 in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2003; **23**:1060–5.

16 Xiao Q, Mandal K, Schett G *et al.* Association of serum-soluble heat shock protein 60 with carotid atherosclerosis: clinical significance determined in a follow-up study. *Stroke* 2005; **36**:2571–6.

17 Dinda AK, Mathur M, Guleria S, Saxena S, Tiwari SC, Dash SC. Heat shock protein (HSP) expression and proliferation of tubular cells in end stage renal disease with and without haemodialysis. *Nephrol Dial Transplant* 1998; **13**:99–105.

18 Esposito P, Libetta C, Rampino T, Gregorini M, Gabanti E, Portalupi V, Dal Canton A. Autoimmune response to heat shock protein 60 in haemodialysis patients. *J Intern Med* 2010; **267**:440.

19 Musial K, Szprynger K, Szczepanska M, Zwolinska D. The heat shock protein profile in children with chronic kidney disease. *Perit Dial Int* 2010; **30**:227–32.

20 Markovic-Plese S, Cortese I, Wandinger KP, McFarland HF, Martin R. CD4$^+$ CD28$^-$ costimulation-independent T cells in multiple sclerosis. *J Clin Invest* 2001; **108**:1185–94.

21 Vallejo AN, Brandes JC, Weyand CM, Goronzy JJ. Modulation of CD28 expression: distinct regulatory pathways during activation and replicative senescence. *J Immunol* 1999; **162**:6572–9.

22 Vallejo AN, Schirmer M, Weyand CM, Goronzy JJ. Clonality and longevity of CD4$^+$ CD28^{null} T cells are associated with defects in apoptotic pathways. *J Immunol* 2000; **165**:6301–7.

23 Warrington KJ, Takemura S, Goronzy JJ, Weyand CM. CD4$^+$, CD28$^-$ T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. *Arthritis Rheum* 2001; **44**:13–20.

24 Betjes MG, Huisman M, Weimar W, Litjens NH. Expansion of cytolytic CD4$^+$ CD28$^-$ T cells in end-stage renal disease. *Kidney Int* 2008; **74**:760–7.

25 Cooper AC, Breen CP, Vyas B, Ochola J, Kemeny DM, Macdougall IC. Poor response to recombinant erythropoietin is associated with loss of T-lymphocyte CD28 expression and altered interleukin-10 production. *Nephrol Dial Transplant* 2003; **18**:133–40.

26 Pawlik A, Florczak M, Masiuk M, Dutkiewicz G, Machalinski B, Rozanski J, Domanski L, Gawronska-Szklarz B. The expansion of CD4$^+$CD28$^-$ T cells in patients with chronic kidney graft rejection. *Transplant Proc* 2003; **35**:2902–4.

27 Brossart P, Bevan MJ. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* 1997; **90**:1594–9.

28 Suto R, Srivastava PK. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 1995; **269**:1585–8.

29 Lipsker D, Ziylan U, Spehner D *et al.* Heat shock proteins 70 and 60 share common receptors which are expressed on human monocyte-derived but not epidermal dendritic cells. *Eur J Immunol* 2002; **32**:322–32.

30 Yen JH, Moore BE, Nakajima T, Scholl D, Schaid DJ, Weyand CM, Goronzy JJ. Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis. *J Exp Med* 2001; **193**:1159–67.

31 Recio-Mayoral A, Banerjee D, Streather C, Kaski JC. Endothelial dysfunction, inflammation and atherosclerosis in chronic kidney disease – a cross-sectional study of predialysis, dialysis and kidney-transplantation patients. *Atherosclerosis* 2011; **216**:446–51.

32 Yavuz O, Bicik Z, Cinar Y, Guney Y, Guler S. The effect of different dialysis membranes on oxidative stress and selenium status. *Clin Chim Acta* 2004; **346**:153–60.