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Immunosenescent CD57$^+$CD4$^+$ T-cells accumulate and contribute to interferon-γ responses in HIV patients responding stably to ART

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Abstract. HIV-infected individuals responding to antiretroviral therapy (ART) after severe CD4$^+$ T-cell depletion may retain low responses to recall antigens [eg: cytomegalovirus (CMV)] and altered expression of T-cell co-stimulatory molecules consistent with immunosenescence. We investigated the capacity of phenotypically senescent cells to generate cytokines in HIV patients receiving long-term ART ($n = 18$) and in healthy controls ($n = 10$). Memory T-cells were assessed by interferon (IFN)-γ ELISpot assay and flow cytometrically via IFN-γ or IL-2. Proportions of CD57$^{\text{bright}}$CD28$^{\text{null}}$ CD4$^+$ T-cells correlated with IFN-γ responses to CMV ($p = 0.009$) and anti-CD3 ($p = 0.002$) in HIV patients only. Proportions of CD57$^{\text{bright}}$CD28$^{\text{null}}$ CD8$^+$ T-cells and CD8$^+$ T-cell IFN-γ responses to CMV peptides correlated in controls but not HIV patients. IL-2 was predominantly produced by CD28$^+$T-cells from all donors, whereas IFN-γ was mostly produced by CD57$^+$ T-cells. The findings provide evidence of an accumulation of immunosenescent T-cells able to make IFN-γ. This may influence the pathogenesis of secondary viral infections in HIV patients receiving ART.

Keywords: CD57, HIV, immune activation, immunosenescence

1. Introduction

Many HIV patients beginning antiretroviral therapy (ART) with advanced disease experience persistent immune dysfunction despite long-term control of HIV replication and increased CD4$^+$ T-cell counts. This has been demonstrated using interferon (IFN)-γ ELISpot responses to index antigens such as cytomegalovirus (CMV). Poor responses correlate with an increased susceptibility to opportunistic infections [1] and faster HIV disease progression after cessation of ART [2]. Antigen-specific T-cell responses are lowest amongst patients with very low nadir CD4$^+$ T-cell counts prior to ART and do not correlate with current CD4$^+$ T-cell counts [3], suggesting ongoing immune dysfunction and/or irreversible damage to the immune system. As ART provides improved life expectancy for previously immunodeficient HIV patients, it becomes important to understand the capacity of their immune systems to respond to viral challenges.

Immunodeficient patients display reduced T-cell expression of the co-stimulatory molecule CD28 whilst gaining expression of CD57 and displaying reduced proliferative capacity [4,5]. Continued T-cell replication accelerates T-cell differentiation in HIV infection [6,7] and other chronic inflammatory diseases [8–10]. It is also a feature of normal aging of the immune system [11]. Several authors have speculated that HIV

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patients may progress to immunosenescence prematurely, but this has never been demonstrated in patients with a stable virological response to ART. Here we correlated the effector memory T-cell function (assessed using responses to CMV or a polyclonal stimulant) with T-cell expression of activation and co-stimulatory molecules associated with immunosenesence.

2. Materials and methods

Study groups comprised 18 male HIV-positive patients and 10 male healthy controls. Patients were identified from the HIV patient database of the Department of Clinical Immunology, Royal Perth Hospital (RPH) on the basis that they began ART with $CD4^+$ T-cell counts below 50/μL and had undetectable plasma HIV RNA levels ($<$ 50 copies/mL) for $>$ 6 months after $>$ 12 months on ART. ART comprised at least three antiretroviral drugs including a non-nucleoside reverse transcriptase inhibitor or protease inhibitor. All patients and controls were CMV seropositive based on detection of CMV-specific IgG (Department of Microbiology, RPH) and none had evidence of active infection (based on detection of CMV DNA using real-time PCR as described previously [12]). T-cell subsets were quantitated using whole blood stained with CYTO-STAT triCHROME$^{\mathrm{TM}}$ (Coulter, Miami, USA) using a Coulter EPICS-XL flow cytometer (Coulter, Miami, USA). Plasma HIV RNA levels were assayed by the Amplicor$^{\mathrm{TM}}$ method, version 1.5 (Roche Diagnostic Systems, Branchburg, USA). Informed consent was obtained from all participants and human experimentation guidelines of RPH and University of Western Australia were followed.

Immunological assays utilised cryopreserved peripheral blood mononuclear cells (PBMC) with cell viability $>$ 95%. ELISpot assays utilised anti-IFN-γ antibodies (MabTech, Stockholm, Sweden) [13] and PBMC were cultured alone or with anti-CD3 (10ng/mL; MabTech), whole CMV sonicate (CMV strain AD169 grown in human fibroblasts), CEF control peptide pool (2 μg/mL; NIH AIDS Research and Reference Reagent Program), HLA-A*02 restricted CMV phosphoprotein 65 peptide (referred to as NLV peptide) corresponding to residues 495–503 or HLA-A*02 restricted CMV Intermediate Early 1 peptide (VLE peptide) corresponding to residues 316–324 (10 μg/mL; Proteomics International, Perth, Australia). Spots $>$ 10 units in size and $>$ 20 units in intensity were counted using an AID ELISpot Reader System (AID, Strassberg, Germany). Numbers of spots in unstimulated wells were subtracted from numbers in stimulated wells and adjusted per 2×10^5 PBMC. To evaluate T-cell phenotypes, PBMC were surface stained (15 minutes, room temperature) using conjugated monoclonal antibodies as follows: CD4-PC5 (13B8.2; Coulter Immunotech, Marseille, France), CD8-APC-Cy7 (SK1), CD28-PECy7 (CD28.2) or CD57-FITC (TB01) from eBioscience (San Diego, CA). For antigen stimulation, PBMC were washed and resuspended at 10^6 per mL alone or with anti-CD3 (10ng/mL; MabTech), whole CMV, NLV peptide or VLE peptide. Co-stimulatory antibodies α-CD28 and α-CD49d (BD Biosciences) were added at a final concentration of 1 μg/mL. Antigen stimulation was performed in polypropylene tubes for 6 hours with 10 μl Brefeldin A (BD Biosciences, San Jose, CA) added after 1 hour. PBMC were washed with cold 1% BSA/PBS and incubated with FcR blocking reagent (Miltenyi Biotec; 4°C, 20 minutes). Surface staining (15 minutes) utilized CD3-PerCP (SK7), CD4-FITC (RPA-T4), CD8-APC-Cy7 (SK1) from BD Biosciences and CD57-PE (TB03) from Miltenyi Biotec. Cells were permeabilised using Cytofix/Cytoperm$^{\mathrm{TM}}$ kits and intracellular staining (30 minutes) utilised IFN-γ-PCy7 (B27) and IL-2 APC (5344.111) from BD Biosciences. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences) within 4 hours using $>$ 100,000 events per tube and analysed using FlowJo software v7.2.2 (Tree Star, Ashland, OR). Statistical analyses were performed with Graphpad Prism 5.01 using Mann-Whitney tests for continuous variables and Spearman's Rank Correlation tests, with $p < 0.05$ accepted as a significant difference.

3. Results

HIV patients and healthy controls were comparable in age (Table 1). Patients displayed stable control of HIV replication for a median of 62 months with over four-fold increases in $CD4^+$ T-cell counts, but retained elevated $CD8^+$ T-cell counts at the time of study. IFN-γ responses to CMV lysate (mediated by $CD4^+$ T-cells) and anti-CD3 (mediated by $CD4^+$ and $CD8^+$ T-cells) were similar in patients and controls. $CD8^+$ T-cell mediated responses [CMV peptides (NLV and VLE) or CEF viral peptides] induced marginally higher responses in the patients, but no differences were significant.

As expected, $CD4^+$ T-cells expressing high levels of CD57 did not express CD28 (Fig. 1A). $CD57^{\mathrm{bright}}$ $CD28^{\mathrm{null}}$ $CD4^+$ T-cells were more abundant in patients

Table 1
IFN-γ responses are similar in HIV patients and control donors, but proportions of immunosenescent T-cells are higher in patients

	Patients ($n = 18$)	Controls ($n = 10$)	p^{b}
Age	51 (42–67)[a]	49 (32–59)	0.16
Current $CD4^+$ T-cells/μL	601 (209–1152)	796 (494–1260)	0.20
Current $CD8^+$ T-cells/μL	941 (234–1840)	414 (208–735)	0.002
Nadir $CD4^+$ T-cells/μL	21 (0–48)	NA	–
Months on ART	103 (38–111)	NA	–
Months HIV RNA $<$ 50 copies/mL	62 (31–103)	NA	–
IFN-γ responses assessed by ELISpot (per 200,000 cells)			
$\alpha-$CD3	2451 (973–5082)	2583 (1255–4758)	0.98
CMV lysate	205 (19–850)	202 (63–1106)	0.35
CEF control peptide pool	609 (0–1788)	445 (0–1146)	0.46
NLV peptide	635 (0–1444)	311 (0–902)	0.09
VLE peptide	328 (0–1805)	66 (0–466)	0.15
T-cells with a senescent or effector phenotype (as a % of $CD4^+$ or $CD8^+$ T-cells)			
$CD57^{\mathrm{bright}}CD28^{\mathrm{null}}$ $CD4^+$ T-cells (%)	8.7 (0.01–69)	2.4 (0.01–18)	0.03
$CD57^{\mathrm{dim}}CD28^+CD4^+$T-cells (%)	3.2 (0.1–8.7)	2.1 (0.08–4.1)	0.006
$CD57^{\mathrm{bright}}CD28^{\mathrm{null}}$ $CD8^+$ T-cells (%)	31 (6.2–52)	35 (4.0–76)	0.3
$CD57^{\mathrm{dim}}CD28^+$ $CD8^+$ T-cells (%)	4.6 (1.6–13)	4.9 (0.8–13)	0.6

[a]median (range); [b]Mann-Whitney test.

than controls (Table 1) and correlated with IFN-γ responses to CMV (Fig. 1B) and anti-CD3 ($r = 0.77$, $p = 0.002$) in HIV patients. In non-HIV controls, proportions of $CD57^{\mathrm{bright}}CD28^{\mathrm{null}}CD4^+$ T-cells did not correlate with IFN-γ responses to CMV ($r = 0.45$, $p = 0.19$) or anti-CD3 ($r = 0.37$, $p = 0.29$).

$CD4^+$ T-cells with low level expression of CD57 expressed CD28 (denoted $CD57^{\mathrm{dim}}CD28^+$ T-cells, Fig. 1A). Proportions of $CD57^{\mathrm{dim}}CD28^+CD4^+$T-cells were higher in patients than controls (Table 1), but did not correlate with $CD4^+$ T-cell IFN-γ responses to CMV (Fig. 1C) or anti-CD3 ($r = 0.08$, $p = 0.74$) in HIV patients or controls ($r = 0.09$, $p = 0.8$ and $r = -0.4$, $p = 0.3$; data not shown).

Analysis of CD57 and CD28 expression by $CD8^+$ T-cells also defined $CD57^{\mathrm{bright}}$ $CD28^{\mathrm{null}}$ and $CD57^{\mathrm{dim}}$ $CD28^+$ populations (data not shown). These were present in similar proportions in PBMC from patients and controls (Table 1). The proportion of $CD8^+$ T-cells that were $CD57^{\mathrm{bright}}$ $CD28^{\mathrm{null}}$ correlated with IFN-γ responses to the CEF peptide pool ($r = 0.72$, $p = 0.02$) and anti-CD3 ($r = 0.68$, $p = 0.03$) in controls and were moderately associated with IFN-γ responses to the NLV/VLE (CMV) peptides ($r = 0.46$, $p = 0.09$). In contrast, neither subset correlated with $CD8^+$ T-cell IFN-γ responses to the CEF peptide pool, anti-CD3 or the NLV and VLE peptides in the HIV patients ($p > 0.3$ for all correlations).

As proportions of $CD57^{\mathrm{bright}}CD28^{\mathrm{null}}CD4^+$ T-cells correlated with $CD4^+$ T-cell IFN-γ responses in HIV patients, we assessed whether CD57 defined cells capable of IFN-γ production. Overall, IFN-γ production by $CD4^+$ T-cells was predominantly from $CD57^+$ cells in HIV patients ($p = 0.04$ when compared with $CD57^-$ cells). The trend was similar in controls but not significantly different (Fig. 1D). IL-2 was produced predominantly by $CD57^-$ $CD4^+$ T-cells (Fig. 1E). IFN-γ production by $CD8^+$ T-cells was predominantly from the $CD57^+$ subset in both patients and controls ($p = 0.0002$ and $p = 0.01$, respectively; Fig. 1F), whilst more IL-2 was produced by $CD57^-CD8^+$ T-cells ($p = 0.0002$ and $p = 0.002$; Fig. 1G). Similar trends were observed when IFN-γ and IL-2 production by $CD57^+$ $CD4^+$ or $CD8^+$ T-cells was assessed in response to the CMV antigen, or the CEF, NLV and VLE peptides (data not shown).

4. Discussion

In the patients selected for this study, IFN-γ responses to CMV lysate, CMV peptides or polyclonal stimuli were similar to uninfected donors. This allowed us to examine whether responses that appear to have "recovered" on ART reflect activation of similar T-cell populations in HIV patients and controls. We found that the HIV patients had increased proportions of circulating $CD57^{\mathrm{bright}}CD28^{\mathrm{null}}$ cells in the $CD4^+$ T-cell population when compared to healthy controls. This was not evident amongst $CD8^+$ T-cells, but the absolute numbers of $CD8^+$ T-cells were higher in patients.

$CD57^{\mathrm{bright}}CD28^{\mathrm{null}}$ T-cells represent highly differentiated effector memory T-cells ($CD45RA^-$ $CCR7^-$)

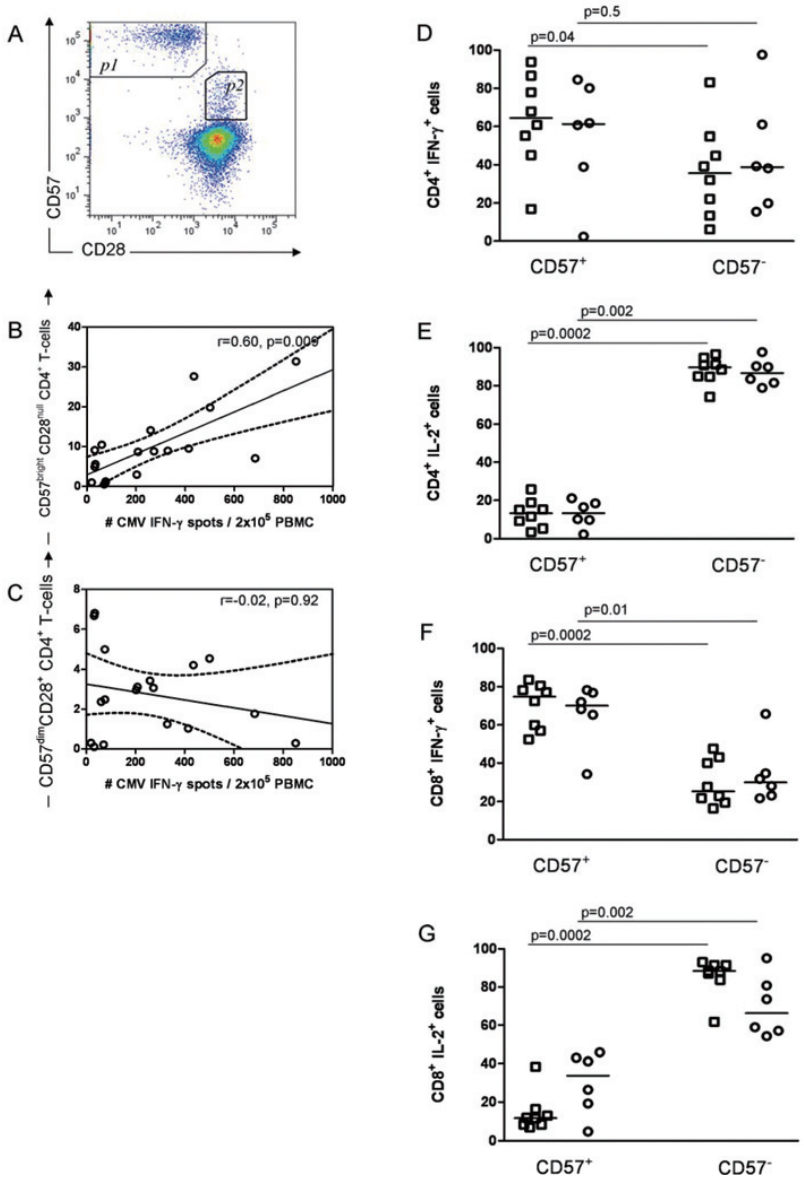

Fig. 1. Bright (p1) and dim (p2) expression of CD57 defined two populations of CD4 T-cells. (**B**) In HIV patients, the proportion of $CD4^+$ T-cells with the $CD57^{\mathrm{bright}}$ phenotype correlated with $CD4^+$ T-cell IFN-γ responses to CMV. (**C**) The proportion of $CD4^+$ T-cells with the $CD57^{\mathrm{dim}}$ phenotype was not correlated with IFN-γ responses to CMV. (**D**) Following culture of PBMC with α-CD3, IFN-γ was more frequently produced by $CD57^+$ T-cells in HIV patients () whilst $CD57^+$ and $CD57^-$ cells from controls produced IFN-γ (◯). (**E**) IL-2 was produced predominantly by $CD57^-$ $CD4^+$ T-cells in patients () and controls (◯). In both patients () and controls (◯), (**F**) IFN-γ was produced predominantly by $CD57^+CD8^+$ T-cells, whilst (**G**) IL-2 arose largely from $CD57^-CD8^+$ T-cells.

that have lost expression of CD27 and CD28, and/or terminally differentiated ($CD45RA^+$ $CCR7^-$) effector memory cells [6]. $CD27^-$ $CD28^-$ cells accumulate with age at the expense of the $CD27^+$ $CD28^+$ effector memory cells. This loss of co-stimulatory molecules could compromise the re-activation of memory cells [6]. Our analysis of IFN-γ and IL-2 production by $CD57^+$ $CD4^+$ T-cells stimulated with anti-CD3 demonstrated that such cells can be stimulated and produce IFN-γ but little IL-2. This population was proportionately larger in HIV patients responding stably to ART.

The accumulation of $CD57^{\mathrm{bright}}CD28^{\mathrm{null}}$ $CD4^+$ T-cells in the circulation of HIV patients receiv-

ing long-term ART is similar to findings in patients with autoimmune diseases, such as rheumatoid disease and multiple sclerosis [8–10]. It is hypothesised that continuous immune stimulation expands populations of terminally differentiated effector memory T-cells with characteristics of immunologically senescent T-cells [9], generating premature aging of the immune system. $CD28^{null}CD4^{+}$T-cells may contribute to early onset atherosclerotic vascular disease in patients with rheumatoid disease [14]. Moreover the anti-inflammatory effects of statin therapy in patients with unstable angina include a reduction of the frequency of circulating $CD28^{null}CD4^{+}$T-cells [15]. These findings are pertinent to HIV patients receiving long-term ART as they display an increased risk of atherosclerotic vascular disease [16].

The activation of $CD4^{+}$ T-cells (assessed by HLA-DR expression) correlates with CD57 expression in HIV patients receiving long-term effective ART [3], driving differentiation towards a senescent phenotype. Immune activation may reflect ongoing HIV replication in 'reservoirs', such as the gut-associated lymphoid tissue [17], and/or continued translocation of bacterial products across the gut wall [18]. Otherwise healthy CMV-seropositive donors and HIV patients may also exhibit large clonal expansions of cells with limited antigen specificity, which may contribute to the population of immunosenescent $CD28^{null}$ T-cells [19,20]. This may not be unique to CMV as other chronic viral infections promote expansion of T-cells with a limited TCR repertoire [21].

In summary, it is likely that most effector memory T-cells producing IFN-γ in response to CMV antigens in previously immunodeficient HIV patients stably responding to ART are immunosenescent $CD57^{bright}$ $CD28^{null}$ T-cells accumulating in response to persistent immune activation. Immunosenescent T-cells may have altered function *in vivo* and may contribute to non-AIDS complications such as atherosclerotic vascular disease. This warrants further study.

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