Viro-immunological studies on the role of Epstein-Barr virus in the development of AIDS-related non-Hodgekin's lymphoma
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Chapter 6

Lack of differentiation of virus-specific CD8+ T cells into CD27− effector cells is associated with progression to clinical disease

submitted for publication
Lack of differentiation of virus specific CD8+ T cells into CD27− effector cells is associated with progression to clinical disease

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To investigate the differentiation status of CD8+ T cells most critical for efficient control of chronic viral infections, we studied CD27 expression on EBV and HIV-specific CD8+ T cells in the course of HIV-1 infection. Individuals co-infected with HIV and EBV persistently had low numbers of HIV specific CD27− T cells, in contrast to rising numbers of EBV-specific CD27+ CD8+ T cells. However, HIV-infected individuals developing EBV-associated AIDS-related non-Hodgkin lymphomas lacked EBV-specific CD27− CD8+ T cells. Higher numbers of HIV-specific CD27− CD8+ T cells were associated with delayed disease progression and virus-specific CD27+ T cells showed elevated IFNY production in response to viral peptides in vitro, indicative for strong effector function. Our data indicate that virus-specific CD27− T cells are important effector T cells in controlling chronic viral infections in humans and that lack of differentiation into CD27− effector T cells leads to disease progression.

Introduction

CD8+ MHC class I-restricted cytotoxic T-lymphocyte (CTL) responses are essential in controlling virus infections. 1−3 After exposure to virus, CD8+ T cells are activated to kill virus-infected cells or inhibit viral replication, which is achieved through diverse effector functions, such as cytolysis and cytokine release, most notably interferon gamma (IFNY). 4; 5 Naive CD8+ T cells encountering antigen undergo phenotypic and functional differentiation to effector cells, 6 and after viral clearance a fraction of the expanded effector cells remains as memory cells. 2 Naive cells express the CD45RA isoform and various costimulatory receptors such as CD27 and CD28. 7; 8 Activation of unprimed CD8+ CD45RA+CD27+ T cells results in loss of CD45RA expression and gain of CD45RO expression. 9 Subsequently, these primed CD45RO+CD27+ memory T cells irreversibly switch off CD27 expression when stimulated for prolonged periods. 10; 11 Eventually, CD45RO+CD27− T cells can revert to CD45RA+ T cells. 12; 13; 14; 15 On the basis of their direct cytolytic activity, granzyme and perforin expression 6 and high IFNY production 5 the CD27− cells are believed to represent effector cells. CD27−CD45RO+ cells exhibit memory characteristics with cytolytic activity only after restimulation. 6 The readily detectable effector functions of CD27− T cells would therefore predict these cells to be critical in control of virus replication.
In human viral infections such as Human Immunodeficiency Virus (HIV, the causative agent for AIDS), Epstein-Barr virus (EBV, a widespread persistent gamma herpesvirus), and Cytomegalovirus (CMV, a widespread betaherpesvirus), high levels of antigen specific CD8+ T cells are found.\(^{16-19}\) (van Baarle, submitted for publication) The majority of HIV-specific T cells have a memory phenotype (CD45RO+CD27+), while only minor populations of effector cells (CD27) were found.\(^{17}\) In contrast, higher levels of CMV-\(^{20}\) EBV-\(^{21}\) and Hepatitis C Virus-specific CD27 T cells were reported.\(^{22}\) Although apparently HIV-specific T cells initially control viral replication,\(^{23}\) HIV-specific T cell responses are lost during HIV-infection with progression to AIDS.\(^{24}\) Because during HIV-induced immunodeficiency, reactivation of EBV-infection can lead to uncontrolled lymphoproliferation,\(^{25}\) HIV-infected individuals are at high risk for EBV-positive AIDS-related Non-Hodgkin’s lymphomas (AIDS-NHL),\(^{26}\) which are thought to arise because of loss of EBV-specific T cell immunity.\(^{27}\) (van Baarle, submitted for publication)

HIV- and EBV-infection represent two different immunological conditions regarding CD4+ T cell help which is necessary to maintain functional CTL.\(^{28-29}\) HIV infection results in selective depletion of HIV-specific CD4+ T cells in the majority of the patients already during acute infection,\(^{30}\) although long term asymptomatics (LTA) may preserve these CD4+ T cells.\(^{31}\) EBV-specific CD4+ T cells, already generated before HIV-infection, probably are preserved for a longer period during chronic HIV-infection.

To define the role of CD27 T cells, characterised as effector cells,\(^{6}\) in human virus infections in relation to clinical outcome, we analysed HIV and EBV specific T cells in the course of HIV-1 infection. We studied the kinetics of virus-specific CD8+ T lymphocytes using MHC class I-peptide tetrameric complexes,\(^{32}\) and assessed their phenotype using CD45RO and CD27 monoclonal antibodies. Differentiation status of virus-specific CD8+ T cells was determined in both long-term asymptomatic (LTA) virus-carriers and HIV-infected individuals who progressed to AIDS-NHL or opportunistic infections (AIDS-OI). In addition, we studied the functional consequences of differentiation towards the CD27- phenotype in these individuals based on IFN\(\gamma\) production after antigen-stimulation.

**Methods**

**Study population**

This study was performed on participants of the Amsterdam Cohort studies on AIDS and HIV-1 infection. Peripheral blood mononuclear cells (PBMC) were cryopreserved according to a standard computerized freezing protocol. HIV-seropositive male individuals were selected according to duration of follow-up, availability of samples and HLA-type (HLA-A2 and/or B8).

In a cross-sectional analysis, 34 HIV-1-infected individuals were studied for the presence and phenotype of HIV-specific CD8+ T cells. (Table 1a) Furthermore, we analyzed longitudinal PBMC samples from 10 HIV-1 infected individuals for HIV-specific CD8+ T cells and 11 HIV-1 infected individuals for EBV-specific CD8+ T cells: Five of these individuals progressed to AIDS-related diffuse large
**Table 1a:** characteristics HIV-1 infected individuals in cross-sectional HIV study

<table>
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<th>n*</th>
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**Table 1b:** characteristics HIV-1 infected individuals in longitudinal HIV study

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**Table 1c:** characteristics HIV-1 infected individuals in longitudinal EBV study

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<td>87 (25-124)</td>
<td>100 (60-190)</td>
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<tr>
<td>AIDS-OI¶</td>
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<td>LTA‡</td>
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<td>39 (35-44)</td>
<td>128 (95-162)</td>
<td>340 (240-360)</td>
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</table>

* number of patients; † median age in years at HIV-1 seroconversion or first seropositive visit; ‡ median age at AIDS-diagnosis or for LTA last time point studied; ¶ median seropositive follow-up, from HIV-1 seroconversion or first seropositive visit until AIDS-diagnosis or last time point studied; § median CD4+ T cell numbers at AIDS-diagnosis or for LTA last time point studied; † patient group studied consisted of HIV-1 infected individuals who progressed to AIDS-related non-Hodgkin’s lymphoma (AIDS-NHL), AIDS with opportunistic infections (AIDS-OI) and Long-term asymptomatic individuals (LTA).

cell NHL, 3 progressed to AIDS (classification of the Centers for Disease Control 1993) with Opportunistic Infections (AIDS-OI) and 3 remained Long-term asymptomatic (LTA) with CD4+ T cells counts >500/mm³ during more than 8 years of asymptomatic follow-up. The earliest timepoint studied was between 1 to 2 years after HIV-seroconversion or study entry. The latest timepoint studied was around AIDS-diagnosis or the latest timepoint studied for LTA and was on average between 6 to 8 years after HIV-seroconversion or study entry. Characteristics of these HIV-1 infected individuals are in part published elsewhere 17 (Van Baarle and Kostense, submitted for publication) and summarized in Table 1b and c.

**Flow cytometry and Tetramer staining**

MHC class I tetramers complexed with EBV and HIV-peptides were produced as previously described. 32 The peptides used (synthesized by solid-phase methods using an automated multiple peptide synthesizer and Fmoc chemistry) were two immunodominant epitopes from EBV lytic cycle proteins, the HLA A2-restricted epitope GLCTLVAML (A2-GLC) from
BMLF-1 and the HLA B8-restricted epitope RAKFKQLL (B8-RAK) from BZLF-1, and one immunodominant epitope from the EBV latent antigen EBNA-3A, the HLA B8-restricted epitope FLRGRAYGL (B8-FLR). Peptides derived from p17 Gag and Pol (SLYNTVATL, ILKEPVHGVV respectively) were complexed with HLA-A2; p24 Gag and Nef peptides (EIYKRWII, FLKEKGGL) were refolded in HLA-B8 proteins. Biotinylated class I-peptide complexes were tetramerized by addition of allophycocyanin or phycoerythrin-conjugated streptavidin.

Four colour fluorescence analysis was performed. Briefly, PBMC were thawed and 1.5 x 10^6 cells were stained in with appropriate MHC class I tetramers and fluorochrome conjugated antibodies anti-CD8, anti-CD45RO, anti-CD27, for subset distribution analysis; anti-CD8, CD27 and anti-CD28 to further subdivide the CD27+ population. After staining, cells were washed with PBA and fixed in PBS/1% paraformaldehyde, (PFA), and at least 250000 events were acquired using a FACSCalibur flow cytometer (Becton Dickinson). Lymphocytes were gated by forward and sideward scatter. Data were analyzed using the software program CELL Quest (Becton Dickinson).

**Intracellular IFNγ staining after antigen specific stimulation**

Two million PBMC/ml were either stimulated with 1µg EBV (RAK) or HIV (Nef)-peptide, used in the tetrameric complexes, or PMA/ionomycin (positive control) or not stimulated (medium alone as negative control) at 37°C for 4 hours in the presence of 3µM monensin (Kostense, Ogg, submitted for publication). This stimulation was sufficient to induce IFNγ production in the majority of potential cells. Stronger stimulation protocols (10µg peptide/ml for 6hours) did not substantially increase the number of IFNγ producing cells. After incubation, cells were washed and stained in PBS supplemented with 0.5% (v/v) bovine serum albumin (PBA) for 15 minutes with HLA-B8-RAK tetramers (PE) in case no intracellular IFNγ was stained. For phenotypic analysis, cells were also stained with PerCP conjugated Mab CD8 (Becton Dickinson, San José, California, USA), anti-CD27 (FITC) and anti-CD45RO (APC) (BD). After membrane staining, cells were washed with PBA and fixed with 4% paraformaldehyde, permeabilised (Permeabilisation kit, BD) and stained intracellularly with IFNγ-PE (BD) for 30 minutes at 4°C. At least 200,000 events in the lymphocyte were acquired using a FACSCalibur flow cytometer (Becton Dickinson).

**ELIspot assay for single cell IFNγ-release**

IFNγ producing antigen-specific T cells were enumerated using IFNγ specific ELIspot assays as previously described 33. 96-well nylon-backed plates (Nunc, Roskilde, Denmark) were coated overnight with 50 µl of 15 µg/ml of anti-IFNγ mAb, 1-DIK (MABTECH, Stockholm, Sweden) in 0.1 M carbonate/bicarbonate buffer pH 9.6. After 6 wash steps with culture medium (RPMI1640, Gibco BRL, Life Technologies, Breda, The Netherlands) to remove unbound antibody, plates were blocked for 1 h with RPMI 1640 supplemented with 10% FCS. Subsequently, PBMC were added in triplicate wells at 1 x 10^5.
cells/well in case of HLA B8-restricted responses or 2 x 10^6 cells/well in case of HLA A2-restricted responses in the absence or presence of 2 μM peptide. As a positive control to test the capacity of PBMC to produce IFNγ in general, Phytohemoagglutinin (PHA) (Murex Diagnostics, Dartford, UK) was added. Cultures were incubated overnight at 37°C in 5% CO2. The next day, cells were removed by washing with PBS/0.05% Tween 20 and the second biotinylated anti-IFNγ mAb, 7-B6-1 biotin (MABTECH), was added at 1 μg/ml in PBS and left for 3 h at room temperature, followed by streptavidin-conjugated alkaline phosphatase (MABTECH) for an additional 2 h. Individual cytokine-producing cells were detected as dark purple spots after a 10-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT, Sigma, St. Louis, Missouri, USA). Reactions were stopped by extensive washing in water. Nylon membranes were dried and spots were counted after computerized visualization by a scanner (Hewlett Packard Company, Baise, Idaho, USA). The number of specific T cell responders per 10^6 PBMC was calculated after subtracting negative control values. Because the percentage of dead cells and the percentage of CD8+ T cells was assessed in the same samples, the number of specific T cell responders/10^6 living CD8+ T cells could be calculated. This assay was very reproducible when performed on multiple samples from EBV-positive donors, detecting as low as 1 positive cell per 1x10^5 PBMC (0.001%).

Statistical analysis

To compare the ratio CD27− effector/CD27+ memory between subgroups of HIV-infected individuals, Mann-Whitney tests were performed, using the software program SPSS 7.5 (SPSS Inc., Chicago, Illinois, USA). To test the relation between several variables and the number of CD27− effector T cells, repeated measurements analyses were performed after cube root transformation of all variables. In analyses, we corrected for dependency between observations within a person assuming a compound symmetry (CS) structure using the software program SAS/STAT (SAS Institute Inc. Cary, USA).

Results

EBV- and HIV-specific T cells in asymptomatic virus-carriers are predominantly of memory (CD45RO+CD27+) phenotype

To study the phenotype of EBV and HIV-specific CD8+ T cells in healthy virus carriers with stable clinical status, we stained EBV-specific CD8+ T cells in an HIV− EBV-seropositive individual and HIV-specific CD8+ T lymphocytes in an HIV-seropositive individual using tetrameric HLA-EBV- or HIV-peptide complexes and phenotypic markers. Many HIV-specific CD8+ T cells in a long-term asymptomatic (LTA) HIV carrier were directed against Gag. (fig 1a, left panel) Despite persistent active viral-replication (+/- 100,000 viral RNA copies/ml) which would imply a drive of HIV-specific T cells into the effector phenotype 10; 11, costaining with CD8, CD45RO and CD27 revealed that almost all the HIV (B8-Gag)-specific CD8+ T cells were of the CD27− memory (CD45RO+CD27−) phenotype (fig. 1a, right panel).
Most EBV-specific CD8+ T cells in a HLA-B8 long-term HIV- EBV carrier were directed against the lytic epitope RAK and the majority was of the CD27+ memory phenotype (figure 1b). However, in contrast to HIV-specific T cells, a substantial proportion of the EBV-specific CD8+ T cells could also be found in the CD45RO+CD27- effector subpopulation. These data indicate that virus-specific CD27- CD8+ T cells are present in individuals who control their virus (EBV) infection, but are infrequent in HIV-infected individuals.

Lack of differentiation of HIV-specific but not of EBV-specific T cells to CD27- effector T cells in the course of HIV-infection

To investigate whether HIV-specific CD27- effectors T cells do accumulate after a longer period of HIV-infection, we investigated changes in the phenotype of both HIV- and EBV-specific CD8+ T cells in different HIV-1 infected individuals in the course of HIV-1 infection. As shown in figure 2, early in HIV infection the majority of the HIV-specific T cells (80-100%), had a CD45RO+CD27+ (memory) phenotype (figure 2 # 57 and # 232, first time point). Despite the persistently higher viral RNA load (>100,000 RNA copies/ml serum) patient # 232 maintained lower numbers of CD27- effector T cells (<10%) in the course of infection than patient # 57 (20%) whose viral load remained low (1000 copies/ml) for over 130 months. (fig.2a and b, left panel) In contrast to these HIV-specific T cells, a substantial proportion (30-50%) of the EBV-specific T cells was CD27- already early in HIV-infection in the same patients (fig.2: # 57 and # 232, first time point), and EBV-specific T cells further accumulated in the CD27- effector population in the course of HIV-1 infection (fig.2: # 57 and # 232). Interestingly, after initiation of HAART, also the HIV-specific CD8+ T cells in patient #232 were enriched in the CD27- effector T cell population. (fig. 2) In addition, costaining with CD28 revealed that both CD27- and CD27+ T cells were CD28- (data not shown).

Overall, early in HIV-1 infection (within 2 years after study entry or HIV-seroconversion) the majority (40-95%) of the HIV- (n=9) and EBV-specific CD8+ T

Figure 1. Phenotypic analysis of HIV and EBV specific CD8+ T cells in asymptomatic virus carriers.
Distribution over the CD45RO+CD27+, CD45RO+CD27-, CD45RO+CD27+ and CD45RO+CD27- subsets (right panels) of tetramer* CD8+ T cells (left panels) for a. HIV-specific T cells in an HIV-positive individual and b. EBV-specific T cells in an HIV-negative EBV-positive individual.
Lack of CD27- effector CD8+ T cells associated with disease progression

Figure 2. Longitudinal changes in CD45RO and CD27 expression on virus specific T cells. Percentage CD45RO⁺CD27⁺, CD45RO⁺CD27⁺, CD45RO⁺CD27⁺ and CD45RO⁺CD27⁺ HIV- and EBV-specific T cells as determined by FACS analysis in the course of HIV-1 infection in a. one long-term asymptomatic HIV-carrier and EBV-specific CD8⁺ T cells in one AIDS-NHL patient and b. one slow progressor to AIDS (opportunistic infection).

cells (n=11) had a memory phenotype. In the course of HIV-1 infection the percentage memory T cells specific for HIV remained stable, whereas many EBV specific memory T cells lost CD27 expression and differentiated to the effector population. This is illustrated by the stable and increased ratio of CD27⁻ effector/CD27⁺ memory T cells for HIV- and EBV- specific T cells respectively (figure 3A). At a late time point in HIV-1 infection (between 6 and 8 years after study entry or HIV-seroconversion) the ratio effector/memory was significantly higher (R=4) for EBV-specific CD8⁺ T cells compared to HIV-specific CD8⁺ T cells (R=0.5) (p<0.014, Mann-Whitney test).

Lack of differentiation to EBV-specific CD27⁻ effector T cells in AIDS-NHL patients

In contrast to the observed increase in the ratio CD27⁻ effector/ CD27⁺ memory for EBV-specific CD8⁺ T cells in an HIV-infected LTA (# 57) and a progressor to AIDS-OI (# 232), however, the majority of the EBV-specific cells remained of the memory phenotype in the course of HIV-1 infection in an individual developing EBV-related AIDS-NHL. (fig.2a; # 118). Therefore, we compared AIDS-NHL patients (n=5), who had a likelihood of defective EBV-specific immunity, with HIV-1 infected individuals who either
Figure 3. Phenotype of HIV and EBV-specific T cells in the course of different groups of HIV-infected individuals

Ratio CD27 effector/CD27+ memory CD8+ T cells in HIV-infected individuals early (within 2 years after study entry or seroconversion) and late (between 6 to 8 years after entry or seroconversion) in HIV-1 infection for a. HIV- and EBV-specific T cells and b. EBV-specific T cells in three different groups of HIV-infected individuals: Long-term asymptomatic individuals (LTA), progressors to AIDS with opportunistic infections (PROG) and progressors to AIDS-NHL (NHL).

progressed to AIDS with opportunistic infections (PROG/AIDS-OI, n=3) or remained clinically stable (LTA, n=3). As shown in figure 3b, in AIDS-NHL patients EBV-specific T cells persistently were of the memory phenotype, whereas an increase in the effector/memory ratio was observed for all epitopes in LTA. The effector/memory ratio in LTA was significantly higher (median 1.48) at a late time point than in AIDS-NHL patients (median 0.43) (p<0.034, Mann-Whitney test). Progressors to opportunistic infections (AIDS-OI) also showed a trend towards higher percentages of EBV-specific effector T cells (median ratio CD27−/CD27+ = 0.80), but no statistical difference was found with the ratio CD27−/CD27+ in AIDS-NHL patients at a late time point. Thus, in AIDS-NHL patients, who were thought to have failing immune control of EBV infection, we observed the same lack of differentiation to the CD27− effector phenotype for EBV-specific T cells as for HIV-specific T cells, which also fail to control HIV-infection.

Abundance of HIV-specific CD27− T cells is associated with slower progression to AIDS.

Although the majority of HIV-specific T cells expressed CD27, in some patients
higher numbers of HIV-specific CD27− effector cells were observed. To investigate the impact of CD27− HIV-specific T cells on disease progression, we correlated the proportion of HIV-tetramer+ cells lacking CD27 expression with months of AIDS free follow up in a cross-sectional analysis including 33 HLA-A2 or -B8 participants of the Amsterdam Cohort. High percentages of HIV-specific CD27− cells correlated with delayed disease progression (R=0.47, p<0.006). (Figure 4a) These data indicate that CD27− CD8+ T cells are more efficient than HIV-specific T cells with other phenotypes, in particular CD27+ memory T cells, in controlling HIV-infection and delaying disease progression.

CD27− effector T cells are high IFNγ producers

Since CD27− T cells seem to be protective and it was reported that CD27− effector T cells exert strong effector functions, such as high IFNγ, granzyme B production and cytotoxicity 6, we tested the relation between the number of CD27− T cells and the number of IFNγ producing T cells, used as a read-out for effector function. We selected those HIV-1 infected individuals in whom an accumulation of CD27− T cells was observed (3 progressors to OI and 3 LTA). Using regression analyses (mixed linear model) the number of EBV-specific CD27− T cells measured at all time points (n=34) was positively correlated with the number of EBV-specific CD8+ T cells producing IFNγ (β = 1.2), which was highly significant in multivariate (p<0.001) analyses controlling for EBV load and the number of CD4+ T cells. (fig. 4b) Similar results were obtained for HIV-specific CD27+ T cells (Kostense et al. submitted for publication).

Figure 4. Impact of CD27 T cells on HIV infection and IFNγ production.
A. Correlation between percentage HIV-specific CD27 T cells, as determined by FACS-analysis, and AIDS free survival in 34 HIV-infected individuals. B. Correlation between numbers of EBV-specific CD27+ T cells (cube root transformed), as determined by FACS-analysis, and numbers of EBV-specific IFNγ producing T cells (cube root transformed), as determined by elispot assay after in vitro stimulation with peptide, in 6 HIV-infected individuals.
To investigated whether the overall increase in IFNγ producing antigen-specific T cells was indeed caused by an increase in the percentage of virus specific effector T cells, we studied simultaneously the distribution of virus-specific T cells defined by tetramer-staining and by IFNγ production, using intracellular IFNγ staining after stimulation with the specific viral peptide, over the different T cell subsets. As shown in figure 5, for a healthy EBV-positive individual with controlled viraemia, the distribution of EBV-specific T cells detected by tetramer-staining (3.3% of CD8) (fig. 5a, left panel) or IFNγ staining (1.6% of CD8) (fig.5b, left panel) was similar over the different T cell subsets with most of the EBV-specific T cells in the CD45RO⁺CD27⁺ memory (58% by tetramer-staining and 54% by IFNγ staining) and a substantial proportion in the CD45RO⁺CD27⁻ effector (35% tetramer⁺ and 37% IFNγ⁺) subset.

In a long-term asymptomatic HIV-infected individual, however, EBV-specific T cells as detected by tetramer-staining showed a different distribution over the T cell subsets than as assessed by IFNγ-staining. (figure 6) At an early time point in HIV-infection (left panels), the tetramer⁺ T cells resided in the CD45RO⁺CD27⁺ memory (58%), CD45RO⁺CD27⁻ effector (28%) and CD45RO⁻CD27⁻ effector (5%) subset (fig 6a), whereas only 28% of the IFNγ⁺ EBV-specific T cells resided in the memory, 32% in the CD45RO⁺ effector and 28% in the CD45RO⁻ effector subset (fig. 6b), showing that IFNγ producing cells were enriched in the CD27⁻ subset compared to tetramer binding cells. Also, the shift from a memory phenotype (CD45RO⁺CD27⁺) to a more effector phenotype (CD45RO⁺CD27⁻) in the course of HIV-1 infection (right panels) resulted in a total increase in the number of IFNγ producing T cells from 0.16% to 0.30%. (fig. 6b) These IFNγ producing T cells resided mainly in the effector T cell population (61%). Similar results were obtained when HIV-specific CD8⁺ T cells were studied after initiation of HAART, which led to increased percentages of B8-Nef-specific CD27⁻ T cells and percentage IFNγ production (data not shown).

![Figure 5. Distribution of IFNγ producing virus specific T cells over the CD27⁺ memory and CD27⁻ effector populations in clinically stable HIV individuals.](image-url)

The distribution of a. the total number of EBV-specific, as assessed by tetramer staining (left panel), and b. IFNγ-producing EBV-specific CD8⁺ T cells, as assessed by intracellular IFNγ staining after in vitro stimulation with peptide (left panel), over the CD27⁺ and CD27⁻ T cells (right panel) in an HIV-negative individual.
Figure 6. Distribution of IFNγ producing virus specific T cells over the CD27+ memory and CD27- effector populations in a long-term asymptomatic HIV-infected individual. The distribution of a. the total number of EBV-specific (left panels), as assessed by tetramer staining and b. the number of IFNγ-producing EBV-specific CD8+ T cells (left panels), as assessed by intracellular IFNγ staining after in vitro stimulation with peptide, over the CD27+ and CD27- T cells (right panels) in an HIV-positive long-term asymptomatic individual at an early and late time point in HIV-infection. FACS staining for CD8, CD45RO, CD27 and tetramer or IFNγ after peptide stimulation was performed as described in Methods.

Discussion

In this study we investigated the phenotype of HIV- and EBV-specific CD8+ T cells in the course of HIV-1 infection to evaluate the role of CD27- effector CD8+ T cells in human viral infections in relation to clinical outcome. Using HLA-tetrameric complexes containing HIV and EBV peptides, we measured the number of HIV- and EBV-specific T cells and analysed the distribution over the CD27+ "memory" and CD27- "effector" T cell subsets. We showed that in long-term asymptomatic virus carriers, the majority of virus (both HIV and EBV)-specific T cells resided in the memory population, although EBV-specific T cells contained higher percentages CD27- T cells than HIV-specific T cells. In the course of HIV-1 infection, the majority of the HIV-specific CD8+ T cells remained of the memory phenotype, whereas EBV-specific CD8+ T cells differentiated into CD27- effector cells in most individuals. Likewise, in AIDS-NHL patients, EBV-specific CD8+ T cells remained of the memory phenotype in the course of HIV-1 infection, despite high levels of EBV load in PBMC, which was comparable to EBV load in LTA and other progressors (van Baarle, submitted for publication). In contrast, in LTA and
patients that progressed to opportunistic infections, EBV-specific CD8\(^+\) T cells were enriched in the CD27\(^-\) effector population. Finally we showed that differentiation of virus-specific CD8\(^+\) memory T cells into CD27\(^-\) effector T cells resulted in higher numbers of T cells producing IFN\(\gamma\). In case HIV-specific CD27\(^+\) memory T cells did differentiate into CD27\(^-\) effector cells to appreciable levels, it was associated with delayed progression to AIDS.

The finding that in healthy individuals the majority of the EBV-specific T cells had a memory phenotype is in concordance with studies showing that after acute viral infection, memory T cells are maintained at a certain level in balance with the viral load.\(^{34,35}\) Re-infection or reactivation would again induce cells with an effector phenotype with higher antiviral and cytolytic capacity. However, in HIV-infection, we did not observe higher numbers of CD27\(^-\) effector T cells, despite the persistent high viral replication. It has been shown that CD8\(^+\)CD45RO\(^-\)CD27\(^-\) cells are induced by antigen and have gone through extensive rounds of division, reflected by their skewed V\(\beta\) repertoire and shortened telomeres.\(^{15}\) However, in AIDS-NHL patients, who have similar EBV loads in comparison to other progressors to AIDS and HIV-infected LTA (D. van Baarle, submitted for publication), EBV-specific CD8\(^+\) T cells apparently did not differentiate to the CD27\(^-\) effector stage. Thus, in AIDS-NHL patients we observed the same lack of differentiation into CD27\(^-\) effector cells specific for EBV as seen for HIV-specific T cells. Interestingly, both these clinical conditions are characterised by eventual loss of immune control of virus replication.

Defective maturation of HIV-specific T cells was recently suggested to occur in HIV-infected individuals, based on lower perforin levels in mainly CD27\(^+\) T cells.\(^{36}\) Our paper extends on this suggestion, in that we performed a longitudinal follow-up study with a clinical end-point, which allowed us to draw conclusions with regard to disease progression. Our study shows, that there is actual biological relevance for differentiation into CD27\(^-\) T cells and that it occurs over time and thereby provides new insights into the importance of virus-specific CD27\(^+\) effector T cells in protection from disease. In addition, we show that impaired maturation of CD8\(^+\) T cells is not specific for HIV but also happens in others conditions with failing anti-viral immune control. Impaired differentiation of EBV specific CD8\(^+\) T cells also occurs and leads to development of EBV-associated AIDS-related Non-Hodgkin’s Lymphoma.

Our data on the accumulation of virus specific CTL in the CD27\(^-\) CD8\(^+\) population in diverse clinical settings provides evidence that these virus-specific effector T cells could be critical for controlling chronic active viral infections characterized by active viral replication. This is compatible with the finding that these T cells may have augmented antiviral effector function compared to T memory cells. We indeed observed a correlation between CD27\(^-\) effector T cells and IFN\(\gamma\) production. In addition, differentiation of virus-specific CD8\(^+\) memory T cells into CD27\(^-\) effector T cells leads to higher numbers of T cells producing IFN\(\gamma\). Furthermore, in a previous study we observed that B8-Nef-specific CD8\(^+\) T cells were associated
Lack of CD27-effector CD8+ T cells associated with disease progression

with slower progression to AIDS, comprised a higher proportion of CD27- effector T cells than B8-Gag specific T cells and also produced more IFNγ (Kostense, Ogg et al. submitted). These observations are consistent with our previous results showing sustained Elispot IFNγ production in asymptomatic HIV-infected individuals, but decreasing IFNγ production in AIDS-NHL-patients. (van Baarle, submitted for publication) In addition, previous studies have shown that CD27- T cells contain more granzyme B and perforin and exert strong direct cytolytic activity. Furthermore, both mouse and human studies have shown a higher number of IFNγ producing T cells in the effector T cell subset than in the memory T cell population. Moreover, also more IFNγ is produced by effector T cells on a per cell basis. Interestingly, according to these functional aspects the CD27- effector population described here seems to resemble the recently described functional phenotype of CCR7- effector memory T cells.

As shown previously, CD4+ T cell numbers indeed correlated with the number of EBV-specific IFNγ producing T cells, suggesting a role for CD4+ T cells in maintaining functional capacity of CD8+ T cells (Van Baarle, submitted for publication). The expression of CD27 has been shown to decrease after interaction with its specific ligand, CD70. In addition, CD70 expression is upregulated by T helper 1 cytokines. Therefore, it could be that helper T cells, directly or indirectly, contribute to this differentiation process of CD8+ T cells.

T helper dependence of functional LCMV-specific CTL in mouse models and CMV and HIV-specific CTL in humans has been reported. Progressive loss of CTL in the absence of adequate helper cell function has been demonstrated for several murine viral infections and in case of adoptively transferred virus-specific CTL in humans.

In the natural course of HIV-1 infection, it has been shown that patients progressing to AIDS lose CD8+ CTL when functional HIV-specific CD4+ T cells disappear. It has been reported that in HIV-infection, most HIV-specific CD4+ T cells may be irreversibly lost during acute infection, probably due to selective deletion of activated CD4+ T cells. This could explain why HIV-specific CD8+ T cells do not efficiently differentiate to CD27- effector T cells, despite high HIV viraemia. Indeed, we observed significant numbers of CD27- effector cells only in LTA (this study) individuals with relatively stable clinical course and modest viral loads, known to have measurable HIV-specific CD4+ T helper activity. In most HIV-infected patients these helper responses seem to
be largely absent. 28-31 In contrast, for EBV-specific T cell responses, it is likely that CD4+ T cells were present before HIV seroconversion, and these cells may decrease more gradually because of lower EBV viral load and viral dynamics. Initially, when EBV-specific CD4+ T cells are still present, differentiation to CD27− effectors can occur. However, when EBV-specific CD4+ T cells are lost, which might be the case in AIDS-NHL patients, also in these individuals no functional differentiation to EBV-specific CD27− T cells will occur. In conclusion, our data indicate a critical role for CD27− effector cells in control of chronically active viral infections, by virtue of high effector function. Differentiation to these CD27− effector T cells may be dependent on virus-specific CD4+ T cells and warrants further study into the molecular mechanism of CD8+ T cell differentiation and its dependence on CD4+ T helper cells.

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