Immune response to herpesvirus infections in immunocomprised children

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Chapter 2

Frequencies of circulating cytolytic, CD45RA⁺CD27⁻, CD8⁺ T lymphocytes depend on infection with CMV

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Abstract
Viral infections may cause serious disease unless the adaptive immune system is able to clear the viral agents through its effector arms. Recent identification and functional characterization of subpopulations of human CD8+ T cells has set the stage to study the correlation between the appearance of particular subsets and common viral infections during childhood, i.e., EBV, CMV, varicella-zoster virus (VZV), and the attenuated measles-mumps-rubella (MMR) vaccine strains. In a cohort of 220 healthy children, we analyzed lymphocytes and subpopulations of CD4+ and CD8+ T cells. The presence of the cytolytic CD45RA+CD27- subset of CD8+ T cells correlated to prior CMV infection as defined by seroconversion (P<0.0001). The number of this CD8+ T-cell subset remained stable during follow-up over 3 years in 40 children. The CD45RA+CD27- subset of CD8+ T cells first appeared during acute CMV infection and subsequently stabilized at an individual set-point defined by age and immunocompetence. The functional importance of these cells in CMV surveillance was reflected by their increased numbers in immunosuppressed pediatric kidney transplant patients. Preferential expansion of CD8+CD45RA+CD27- cytolytic T cells seems unique for CMV.

Introduction
In response to viral infection, naive CD8+, MHC class I-restricted, virus-specific T cells expand clonally and differentiate into effector cells that eliminate or neutralize the virus and memory cells that provide enhanced immunity on re-infection [1-3]. CD8+ T cells are the primary mechanism of the adaptive immune system to cope with the many viral diseases that are encountered during a lifetime, many of which occur during childhood. A better understanding of virus-induced CD8+ T cell development and functional properties of the respective CD8+ T cell subpopulations will thus be of great meaning to clinical diagnostics in viral disease.

Functionally distinct CD8+ T cell subsets can be distinguished by use of combinations of phenotypic markers such as CD45RA/CD45R0, the costimulatory receptors CD28 [4], CD27 [5], and the chemokine receptor CCR7 [6]. Naive CD8+ T cells express CD45RA (but not CD45R0) as well as CD27, CD28 and CCR7. Viral infection induces a massive expansion of specific CD8+ cells and rapid up-regulation of the cytolytic molecules perforin and granzyme B [7]. During acute infection these cells express CD45R0, CD38 and HLA-DR, CD27 and CD28, whereas later in infection CD27 and CD28 may become down-regulated [8]. In healthy individuals, two prevalent types of resting primed (memory) T cells can be discerned. CD45R0+CD28+CD27+ T cells that have the ability to secrete IL-2, IFN-γ, TNF-α, and IL-4 and contain virus-specific CTL precursors [9]. A second primed population can be characterized by CD45RA expression with concomitant absence of the costimulatory molecules CD27 and CD28 and of CCR7. This CD8+CD45RA+CD27- population can secrete IFN-γ and TNF-α but not IL-2 or IL-4, abundantly expresses CD95-ligand, perforin, granzyme B, and shows high cytolytic activity without in vitro prestimulation [10]. In healthy individuals, a high degree of variation in the subset composition of the circulating CD8+ T-cell population is observed. Although the way in which this variability is generated is unknown, recent studies have suggested that particular viruses may preferentially be associated with certain phenotypes of virus-specific T cells in latency [11].

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To directly address this question, the relationship between CD8+ T-cell subset composition and common viral infections during childhood, i.e., EBV, CMV, varicella-zoster virus (VZV), and measles-mumps-rubella (MMR) vaccinations was studied. Both EBV and CMV are herpesviruses that infect the vast majority of humans, following a bimodal pattern of infection with peaks during early childhood and late adolescence, being defined to a large extent by socio-economic factors [12,13]. In the Netherlands, a cross-sectional serum study performed in 1991 had shown that 40% is EBV-seropositive at 10 years of age increasing to 98% at 30 years, while CMV immunity is detected in ~30% and ~50%, respectively [14]. Infection with these viruses early in life is most often asymptomatic, while infection later in life is a self-limiting disease more often associated with clinical symptoms [12]. On the other hand, chickenpox (varicella) is a common disease during childhood, caused by primary infection with VZV, a highly contagious herpesvirus with attack rates of >85% in susceptible contacts in the temperate climate under 7 years of age [15-17]. Varicella vaccine is not presently in use in The Netherlands. However, live attenuated measles, mumps, and rubella virus are used for routine MMR vaccination in The Netherlands with a coverage of >98% of the young children and high seroconversion rates regardless of intercurrent infections or fever [18-22]. Our analyses show that CMV is the main, if not only, factor responsible for the expansion of cytolytic CD8+CD45RA+CD27+ T cells.

Materials and Methods

Study design
Children and young adolescents under the age of 18 being routinely analyzed for pre- or peri-operative procedures were included in the study (n=220; control cohort; Table 1). We also included patients with a diagnosis of a (sub) acute viral infection and those tested or admitted for a suspected viral infection of the upper and lower respiratory, gastrointestinal, or urogenital tract (n=58). Those with an acute CMV infection were asked for consent to be followed-up with regular blood tests (n=16). In 10 families all members who underwent venipuncture for HLA-typing in a search for an HLA-identical family donor for a related sibling (not included in the pedigrees) were also tested for viral serology against various common viruses after written consent was obtained. Data for infants under the age of 6 months were not included to avoid the interference of (low titer) maternal Abs in our study. In each age category ~15-30% of the healthy controls were of African, Caribbean, or Mediterranean origin. The remainder were of Caucasian origin. Informed consent for the research purpose described was obtained from the parents of the children included in the studies as approved by the institutional medical ethical committee.

In addition, a pediatric cohort of 21 renal transplant patients was prospectively followed. Immunosuppressive medication consisted of prednisone (7.5 mg/m^2), cyclosporin (plasma trough range, 100-150 ng/ml), and either azathioprine (1-2 mg/kg), or mycophenolate mofetil (1.2 g/m^2/day in two doses).

Immunophenotyping of PBMC fractions from the blood from individuals was performed as described below. In the same blood samples, T cell proliferation tests were performed to define functional activity in vitro and thus exclude unforeseen immune dysfunction. Serology was performed in blood obtained at the same moment as the immune tests.

Laboratory tests for EBV, CMV, VZV and MMR serology
Specific IgM and IgG against the EBV-viral capsid Ag was determined by indirect immunofluorescence and Abs to Epstein-Barr nucleic acid by anticomplement immunofluorescence (Gull Laboratories, Salt Lake City, UT). Specific IgG Abs to CMV and rubellavirus were defined by Imx assays (Abbott Diagnostics, Amstelveen, The Netherlands). IgM to CMV and IgG to VZV were defined by VIDAS tests (Biomerieux, Lyon, France). Specific IgG to measles and mumps was defined by ELISA (Virotech, Russelheim, Germany). All tests were performed following the instructions of the manufacturers. Serology data for EBV, CMV, VZV, measles, mumps, and rubella in the control individuals are
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summarized in Table 1. The serological characteristics of the cohort are in agreement with previous studies [14].

Lymphocyte subsets and enumeration of cytolytic effector cells
The numbers of B cells (CD19+), T cell (CD2+, CD3+, CD4+, CD8+) subsets, and NK cells (CD2+/CD3+, CD16+, CD56+) were determined by standard FACSscan procedures with mAbs being produced by CLB (Amsterdam, The Netherlands). For determination of subpopulations within the CD8+ T cell fraction, FITC- and PerCP-conjugated CD3, CD8, and CD27 were all purchased from BD Biosciences (San Jose, CA). CD45RA-PE was obtained from Coulter Immunology (Hialeah, FL). Biotinylated CD27 was manufactured at CLB. Using these mAbs for triple staining, gating on CD8+ lymphocytes allowed further subtyping into naive resting cells (CD45RA+CD27+) and functional distinct subsets of primed cells, i.e., noncytolytic CD45RA+CD27+ (memory-type) and cytolytic CD45RA+CD27+ T cells (effector-type), as extensively characterized previously [6,7].

<table>
<thead>
<tr>
<th>Age category</th>
<th>CMV (%)</th>
<th>EBV (%)</th>
<th>VZV (%)</th>
<th>MMR (compl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5-1 year (n=19)</td>
<td>36.5</td>
<td>15.3</td>
<td>5.2</td>
<td>0</td>
</tr>
<tr>
<td>1-2 year (n=17)</td>
<td>19.4</td>
<td>11.8</td>
<td>54.7</td>
<td>52.9</td>
</tr>
<tr>
<td>2-3 year (n=16)</td>
<td>25.2</td>
<td>31.2</td>
<td>59.0</td>
<td>81.3</td>
</tr>
<tr>
<td>3-4 year (n=12)</td>
<td>23.3</td>
<td>30.0</td>
<td>66.7</td>
<td>91.7</td>
</tr>
<tr>
<td>4-5 year (n=16)</td>
<td>25.0</td>
<td>43.8</td>
<td>90.2</td>
<td>93.8</td>
</tr>
<tr>
<td>5-6 year (n=15)</td>
<td>33.3</td>
<td>73.3</td>
<td>86.6</td>
<td>93.3</td>
</tr>
<tr>
<td>6-7 year (n=10)</td>
<td>35.4</td>
<td>60.0</td>
<td>93.4</td>
<td>99.8</td>
</tr>
<tr>
<td>7-8 year (n=13)</td>
<td>53.8</td>
<td>76.9</td>
<td>92.3</td>
<td>96.8</td>
</tr>
<tr>
<td>8-10 year (n=19)</td>
<td>52.6</td>
<td>57.9</td>
<td>94.7</td>
<td>100</td>
</tr>
<tr>
<td>10-12 year (n=14)</td>
<td>35.8</td>
<td>57.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>12-14 year (n=28)</td>
<td>64.2</td>
<td>71.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>14-16 year (n=19)</td>
<td>73.6</td>
<td>68.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>16-18 year (n=22)</td>
<td>68.8</td>
<td>70.0</td>
<td>91.2</td>
<td>100</td>
</tr>
<tr>
<td>&gt;18 year (n=28)</td>
<td>74.5</td>
<td>76.8</td>
<td>88.7</td>
<td>93.4</td>
</tr>
</tbody>
</table>

* The first age category comprises controls from 6-12 months only, to exclude interference of maternal Ab as much as possible. The categories consist of 2-year groups from the age of 8 years, apart from the category >18 years.

* The number tested per age category was always >80% of the total number of controls included in each category.

* MMR compl., complete seroconversion for all three MMR vaccine components, determined as IgG positivity above the standard cut-off levels for the three different components' test Ag. Incomplete MMR seroconversion against one or two viral Ag was detected in 8% of the cases; these were considered negative in the calculations shown.

Generation of HLA-A2.1/CMVpp65(NLVPVMATV) and HLA-B7/CMVpp65(TPRVTGGGAM) tetrameric complexes
Tetrameric complexes were generated essentially as described by Altman et al. [23]. In brief, purified HLA-A2.1 and HLA-B7 heavy chains and β2 microglobulin were synthesized using a prokaryotic expression system (pET; Novagen, Milwaukee, WI). The heavy chains were modified by deletion of the transmembrane/cytosolic tail and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site. The HLA-A2.1 binding CMV pp65-derived peptide NLVPVMATV and the HLA-B7 binding CMV pp65-derived peptide TPRVTGGGAM were used for refolding. The refolded products were isolated using HPLC, biotinylated by BirA (expressed using the pET expression system, purified using cobalt beads (Clontech, Palo Alto, CA)) in the presence of biotin (Molecular Probes, Eugene, OR), ATP (Sigma-Aldrich, St. Louis, MO) and MgCl2. The biotinylated products were separated from free biotin by gel filtration using HPLC [24]. Streptavidin-PE or streptavidin-allophycocyanin conjugate (Molecular Probes) was added in a 1/4 molar ratio.
Immunofluorescent staining and flow cytometry

Freshly isolated or thawed PBMCs were resuspended in RPMI containing 10% FCS and antibiotics. PBMCs (n=200,000) were incubated with fluorescent label-conjugated mAbs (concentrations in accordance with manufacturer's instructions) and an appropriate concentration of tetrameric complexes in a small volume for 30 min at 4°C, protected from light. Cells were washed in PBS containing 0.01% (w/v) NaN₃ and 0.5% (w/v) BSA. Analyses consisted of allophycocyanin-conjugated tetramers and CD8-PerCP in combinations with CD45RA-FITC and CD27-PE (all BD Biosciences). Analysis of cells for the expression of cell surface markers was performed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Statistics

Student's t test and ANOVA were applied for normally distributed data, and the Wilcoxon two-sample test and the Kruskal-Wallis test were used for nonnormally distributed data. Fisher's exact test was used for statistical analysis in the cohorts of controls and patients.

Results

Frequencies of cytolytic CD8⁺CD45RA⁻CD27⁻ T cells correlate to prior CMV infection

Cohorts of patients are often compared with historic and/or presumably healthy controls, disregarding the potential impact of their infectious history. An additional problem arises because of the differences in lymphocyte numbers and lymphocyte subset distribution depending on age [25,26]. We performed an extensive immunophenotyping of PBMCs from healthy children to define normal levels of predefined CD4⁺ and CD8⁺ T-cell subpopulations in various age cohorts. In most of the children, serology (IgM and IgG) against EBV, CMV, VZV, and MMR was simultaneously performed. In this way, we had the opportunity to analyze the relation between viral infections and immunization in the past, on the one hand, and the number of T cell subsets, on the other.

Table 2 Correlation coefficients of seropositivity for various viruses with the CD8⁺CD45RA⁺CD27⁻ T cells in all healthy controls tested

<table>
<thead>
<tr>
<th>Cellular immune parameter</th>
<th>Antiviral IgG Ab (seropositivity)</th>
<th>CMV (n=214)</th>
<th>EBV (n=198)</th>
<th>VZV (n=176)</th>
<th>MMR (compl) (n=188)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of CD8⁺CD45RA⁺CD27⁻ T cells of total CD8⁺ T cells</td>
<td>0.780</td>
<td>0.140</td>
<td>-0.072</td>
<td>-0.068</td>
<td></td>
</tr>
<tr>
<td>(P&lt; 0.0001)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abs. no. of CD8⁺CD45RA⁺CD27⁻ T cells</td>
<td>0.697</td>
<td>0.102</td>
<td>-0.134</td>
<td>-0.146</td>
<td></td>
</tr>
<tr>
<td>(P&lt; 0.01)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Abs. no. of CD8⁺CD45RA⁺CD27⁻ T cells divided by total no. of CD3⁺ T cells</td>
<td>0.728</td>
<td>0.144</td>
<td>-0.055</td>
<td>-0.060</td>
<td></td>
</tr>
<tr>
<td>(P&lt; 0.005)</td>
<td></td>
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</tbody>
</table>

* Serum responses were positive or negative according to the standard cut-off values described in "Materials and Methods" for the various serology tests applied in accordance with the manufacturer's instructions. The qualitative seroreactivity was correlated to the quantitative number of cytotoxic T cells. The CD45RA⁺CD27⁻ cells were considered as the percentage of total CD8⁺ T cells, as an absolute cell number (percentage x 0.01 x absolute CD8⁺ T cell count x 1000/ml), or as this absolute CD8⁺CD45RA⁺CD27⁻ cell count divided by the absolute number of total CD3⁺ T cells.

b See note 3 of the legend of Table 1. Incomplete MMR seroconversion against 1 or 2 viral antigens was detected in 8% of the cases; these were considered negative in the calculations shown. When the partially incomplete seroconversion samples were considered seropositive instead, the result was still not significant in the calculation of the correlation coefficient (not shown).

Absolute numbers of CD3⁺ lymphocytes, CD3⁻ (CD16⁺CD56⁺) NK cells, CD4⁺CD45RA⁺ and CD4⁺CD45R0⁺ T cells did not show a correlation of any kind with the serology findings against the viruses tested. However, a highly significant correlation was found between the
percentage as well as the absolute number of circulating CD8+CD45RA+CD27- cytolytic T cells and CMV seropositivity in healthy individuals (Table 2). Representative histograms of a CMV-seronegative and an age-matched CMV-seropositive individual (left and right panels, respectively) are shown in Fig. 1.

**Figure 1 Extensive immunophenotyping of CD8+ T cells according to CD45RA and CD27 subsets in two healthy controls, 8 years of age.** The left dot plot represents CMV IgG-seronegative individuals (A), and the right dot plot represents CMV IgG-seropositive individuals (B). Both controls were positive for EBV, VZV, measles, mumps, and rubella IgG Abs. The subpopulation of CD8+CD45RA+CD27- T cells (upper left quadrant) was detectable in CMV-seropositive individuals, but was almost absent in CMV-seronegative controls. Dot plots are gated on CD8+ T cells. The data shown are representative for >200 controls (see text).

As expected, the CMV-seropositive and CMV-seronegative population differed in age (Table 3), but not in gender or racial background (data not shown). Moreover, the percentage and absolute number of CD45RA+CD27- cells within the CD8+ T cell fraction were different between the two groups. To avoid the impact of background staining variability in routine immunophenotyping procedures, the cut-off for a positive result in the number of CD8+CD45RA+CD27- T cells was set at 0.02x10^6/ml blood as a minimum. Absolute cell counts above that number strongly correlate to prior CMV infection (odds ratio, 229.5; 95% confidence interval: 69.6-757.2; see Table 3). When arbitrarily set at 0.01x10^6 cells/ml, absolute numbers of cytolytic CD8+ T cells remained highly predictive (odds ratio, 81.2; 95% confidence interval, 18.9-348.7). The number of CD8+CD45RA+CD27- T cells remained surprisingly constant over a period of 2-4 years in a series of 40 controls who were checked at a regular base for other than infectious reasons (data not shown).

**Table 3 CMV status in relation to the number of CD8+CD45RA+CD27- T cells**

<table>
<thead>
<tr>
<th></th>
<th>CMV-positive (n=106)^a</th>
<th>CMV-negative (n=100)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in (months) ± SD</td>
<td>105 ± 63.9 (8.7 years)^b</td>
<td>75 ± 60.7 (6.2 years)^b</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>% CD8+CD45RA+CD27- T cells of total CD8+ T cell number ± SD</td>
<td>21.6 ± 19.0</td>
<td>1.3 ± 0.98</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Abs. no. of CD8+CD45RA+CD27- T cells (10^6/ml; mean ± SD)</td>
<td>0.0847 ± 0.0071</td>
<td>0.0041 ± 0.0040</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>No. of patients with &gt;0.02x10^6 CD8+ cytolytic T cells/ml</td>
<td>102</td>
<td>10</td>
<td>Odds ratio: 229.5</td>
</tr>
</tbody>
</table>

^a Serum responses were positive or negative according to the standard cut-off values described in Materials and Methods for the CMV serology test.

^b Mean in years is in parentheses.
CD8*CD45RA*CD27− T cells rise during acute CMV infection

Children without a known immune disorder of any kind who came to our attention because of acute CMV disease (n=16) were studied. From two patients, blood samples were available from a time point before the start of CMV disease (Fig. 2 B and C). Acute disease was defined by positive cultures, CMV IgM serology, and, when performed, the presence of CMV DNA in whole blood as detected by a quantitative PCR technique [27]. A progressive rise in CD8*CD45RA*CD27− T cell numbers was observed starting within 3-4 weeks after diagnosis, which is in agreement with our recent observations in adult patients receiving allo-kidney grafts. Within 2 months a plateau was reached, with stable numbers of CD8*CD45RA*CD27− T cells for the following months of follow-up (Fig. 2 A-C). Although in principle unselected, some bias in patient inclusion because of the presence of clinical symptoms of acute CMV disease at presentation cannot be excluded at this point. The variety of symptoms was diverse; some patients were admitted, while others were followed at the out-patient care unit.

![Graphs showing CD8+ T cell and CMV DNA load over time](image)

**Figure 2** Increase in CD8*CD45RA*CD27− T cells after primary CMV infection, as shown for three separate individuals. The absolute numbers (10⁶/ml) of CD8+ T cells (●) and CD8*CD45RA*CD27− T cells (▲) are compared with the CMV DNA concentration expressed as copies per milliliter of whole blood (●). Patient A was an infant treated with gancyclovir because of respiratory failure due to CMV pneumonitis; patients B and C were an untreated infant and young child with symptoms of vomiting and a temporary growth failure due to CMV-hepatitis. Time zero represents retrospectively defined blood samples, once collected and preserved for other reasons. Data are representative for 16 patients in total.

To investigate the phenotype of CD8+ T cells with confirmed CMV specificity, studies with CMV-specific tetramers were performed. On 50 blindly selected healthy individuals in our study, HLA-genotyping was performed regardless of the CMV serology or number of CD8*CD45RA*CD27− T cells. From these blindly selected individuals, 23 were CMV
seropositive, and 27 CMV seronegative at that time. HLA-A2 and/or HLA-B7 were present upon HLA-typing in seven individuals with prior CMV infection, as defined by positive anti-CMV IgG titers. False-positive findings of CMV-specific tetramer binding to HLA-A2 and/or -B7+ T cells were not observed in the absence of prior CMV infection (0 of 22), or vice versa in the case of prior CMV-infection but absence of the correct HLA-A2 and/or B7-haplotype (0 of 16). In agreement with previous findings [28,29], tetramer-positive, CMV-specific cells were never detected in the naive (CD45RA+CD27bright) subset of CD8+ T cells (Fig. 3 A and B). Enrichment of CMV peptide-specific T cell was particularly observed in the CD27- pool of CD8+ T cells as well as in the CD27null fraction (Fig. 3B) [8,11]. The percentage of tetramer-positive T cells ranged from 0.8-4.9 % of total CD8+ T cells. It is likely that the Ag specificity within the pre-activated CD27- cytolytic cell fraction is much broader than to the single CMV peptide tested. Coinciding with the increase in pre-activated CD8+CD45RA+CD27- T cells, we observed a rapid appearance of the CMV-specific CD8+ T cells in an HLA-A2+ child with acute CMV hepatitis. These CMV-tetramer positive CD8+ T cells progressively increased before stabilization over a period of 12 months of follow-up (Fig. 3C).

Figure 3 Tetramer staining of CMV peptide-specific CD8+ T cells. Tetramer-positive cells were detected in CD8+ T cells as a distinct set of T cells (A), primarily detected within the CD27-dull or -negative CD8+ T cell subset (B) (total CD8+ T cells plotted in gray, and CMV-specific CD8+ T cells plotted in black). The time course in acute CMV disease is shown for a 2-year-old HLA-A2+ girl tested for CMV-specific T cells and number of CD8+CD45RA+CD27- T cells. (C) The absolute numbers (10^6/ml) of CD8+ T cells (a) and CD8+CD45RA+CD27- T cells (c) are compared with the percentage of tetramer-positive (tet+) CD8+ T cells (▲).

Individual set-point in the formation of CD8+CD45RA+CD27-, cytolytic T cells: family segregation?
A genetic correlation with the absolute number of CD4+ and CD8+ T cells has been previously documented [30]. The number at which a person's CD8+ cytolytic T cells stabilize was investigated. Ten family pedigrees were tested for a pattern of segregation and gene inheritance (HLA class I and class II typing). As shown for three representative families (Fig. 4, A-C), the presence of CD8+CD45RA+CD27- T cells was unrelated to any of the viruses tested other than CMV, without a pattern of parental HLA haplotype inheritance (data not shown). Offspring from parents with high numbers of these cytolytic cells did not all show high numbers (Fig. 4B). Vice versa, children with high CD8+CD45RA+CD27- T cell numbers can have parents with low cytolytic T cell numbers (Fig. 4C). In some families one of the parents (mostly fathers (Fig. 4B and C); rarely mothers (Fig. 4A)) was still CMV seronegative and did not have cytotoxic CD8+ T cells, whereas his/her partner and children showed positive reactivity.
Individual set-point of CD8⁺CD45RA⁺CD27⁻ T cells: a matter of immune competence?

The set-point of CD8⁺CD45RA⁺CD27⁻ T cells was only weakly related to age, whereas clinical symptoms during CMV infection, prior viral infections, racial background, and sex did not show any relation to the set-point (data not shown). When the two groups most separated in age were compared, a more direct age effect seemed apparent. Young children (age, 0-4...
years; n=23) and young adolescents (age, 15-18; n=23) differed significantly in number and percentage of CD8$^+$ cytolytic T cells, also when the increased absolute number of CD8$^+$ T cells at young age was taken into account (Table 4).

Immunosuppressed renal transplant patients who were infected with CMV after transplantation had significantly higher CD8$^+$CD45RA$^-$CD27$^-$ T cell numbers than those who were CMV IgG-positive before transplantation or non-immunosuppressed, age-matched controls (for whom we do not know at which age CMV was contracted; Fig. 5).

<table>
<thead>
<tr>
<th>Table 4 Age in relation to CD8$^+$CD45RA$^-$CD27$^-$ T cells in CMV-positive controls$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td>(n=23)</td>
</tr>
<tr>
<td>Age 0-4 Years</td>
</tr>
<tr>
<td>Age 15-18 Years</td>
</tr>
<tr>
<td><strong>P-Value</strong></td>
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</tbody>
</table>

$^a$ Serum responses were positive or negative according to the standard cut-off values described in Materials and Methods for the CMV serology test.

Discussion

Extensive T cell phenotyping has become common practice as a relevant immuno-diagnostic tool. Previously, our group ascribed direct ex vivo cytotoxicity to a defined subset of CD8$^+$CD45RA$^-$CD27$^-$ T cells, which, without prior activation, express not only perforin and granzyme, but also CD95 ligand [5,10]. We tested >200 healthy children for T cell subset distributions in relation to the serologic proof of acute or past infection with CMV, EBV, or VZV or immunization with attenuated MMR strains. The presence of cytolytic CD8$^+$CD45RA$^-$CD27$^-$ T cells strongly correlated to CMV infection (p < 0.000001), but importantly not with other viruses (Table 2).

The role of CMV in the selective expansion of particular CD8$^+$ T cell subsets had been suggested [18,31,32]; however, further phenotypic (apart from CD57) and functional characterization is lacking [31,32]. Moreover, the studies were performed in small cohorts of patients without long-term follow-up. It is unclear whether CD8$^+$CD57$^+$ T cells represent a unique and homogeneous subset with distinct functions. Using CD8$^+$ T cells isolated from patients at various times after allogeneic bone marrow transplantation, high numbers of CD8$^+$CD57$^+$ T cells correlated to diminished killing of CMV-infected targets [33]. Such a reduced cytotoxic capacity would support a functional relationship between immune competence (age, immunosuppression) and the number of CD8$^+$CD45RA$^-$CD27$^-$ cytolytic T cells (Fig. 5), of which most coexpress CD57 [9]. In allogeneic transplants CD8$^+$CD57$^+$ T effector cells did not produce any TNF-α or IFN-γ after anti-CD3 stimulation [33], whereas isolated CD8$^+$CD45RA$^-$CD27$^-$ T cells from healthy controls did [5]. Moreover, the vast majority of resting CD8$^+$ T cells capable of rapid induction of TNF-α and IFN-γ synthesis in response to CMV peptides were found in a subset with intermediate to high expression of CD57, low CD27, and reversal of the classical memory CD45RO to the CD45RA phenotype [34]. This subpopulation probably includes the fully differentiated effector-type cells responsible for long term suppression of the virus and avoidance of disease from CMV.
reactivation. Using tetramer staining we found that these cells are generated at a relatively short time (2 months) after primary CMV infection in children (Fig. 3C). As we now demonstrate, CMV infection is the major trigger for the selective expansion of the CD8⁺CD45RA⁺CD27⁻ T cell subset to an individual set-point at which the number of these CD8⁺ T cells stabilizes for the following years (Fig. 2 and 3). Of the various factors studied (such as race, sex, inheritance, coinfections (prior EBV, VZV, or MMR)), young age or immunosuppression at the onset of acute CMV infection determines to a large extent this individual set-point. An independent genetic impact on the number of T cells has been suggested [30]. Yet, an inherited set-point for CD8⁺ cytolytic T cells after CMV infection was not supported by a study in 10 families, although the confounding factor age at onset of CMV infection was unknown for the families tested.

![Diagram](image)

**Figure 5** Individual set-points of stable CD8⁺CD45RA⁺CD27⁻ T cell numbers (10⁶/ml) in pediatric kidney transplant patients depend on the moment of primary CMV infection: prior to or after transplantation. Significance is indicated when P<0.05. Left, Age-matched controls (seropositive, n=28; seronegative, n=23); right, recipients of a kidney transplant (Tx, n=21; seronegative, n=6; infected under immunosuppression, n=8; pre-Tx seropositive CMV reactivation, n=7). Reactivation of CMV was defined as renewed IgM-seropositivity, positive cultures, and/or a detectable CMV DNA load in whole blood while negative in the past.

Speculations can be made about development of the cytotoxic CD8⁺ T cell subset. Although CD8⁺CD45RA⁺CD27⁻ T cells are detected in all CMV-infected individuals, their numbers increase in clinical situations of immunosuppression (Fig. 5) [11]. In immunosuppressed patients, CMV reactivation could still occur under relatively normal conditions (here evaluated by CD4⁺ T cell number and proliferation tests) during follow-up, allowing adaptive responses of the CD8⁺ T cell subsets to increased levels of viral antigen. Possibly CMV antigen not only induces further expansion of virus-specific cells, but also their development into CD8⁺CD45RA⁺CD27⁻ T cells [28]. The bulk of fully differentiated CD8⁺ T effector-type cells may, in fact, be different in specificity or binding avidity from the most potent cytotoxic T cells generated during CMV infection. CMV immunity in humans is focussed on two immunodominant proteins, i.e., pp65 and, to a lesser extent, IE1 [35]. Using the pp65 peptide-containing tetramer to detect CMV-specific T cells, we found that most of the CMV-tetramer positive cells (50-60%) were present in the CD8⁺CD45RA⁺CD27⁻ T cell fraction,
representing <10% of the total CD8*CD45RA*CD27* T cells (data not shown). Confirming data from adults [11], EBV-tetramer-positive cells (80-90%) predominated in CD45R0*CD27* (memory-type) CD8* T cell fraction (not shown). The restriction of EBV- and HIV-derived peptide-specific CD8* T cells in adult patients to this type may very well represent a normal phenomenon [29].

The use of tetramers has, in just 4 years, transformed our understanding of the immune responses against HIV, human T lymphotropic virus 1, hepatitis B and C virus, CMV, and EBV, and holds promise in a number of areas where quantitative analysis of the antiviral response in terms of both number and function is critical. Nevertheless, the technique remains limited in terms of suitability of the HLA background as well as availability of reagents to cover a more complete spectrum of protein-derived peptides involved in immunity. These techniques, however, leave open the question by which mechanism or protein(s) CMV induces the expansion of CD8*CD45RA*CD27* T cells.

In conclusion, we show that not only may CMV-specific cells preferentially have a CD8*CD45RA*CD27* phenotype, but, importantly, CMV is the predominant factor that determines the presence of these T cells in peripheral blood. Age at onset of CMV infection or, in more general terms, immunocompetence determines to a great extent the level or set-point at which an individual's number of cytolytic cells stabilizes. The mechanism by which CMV induces a large number of uniquely expanded CD8* cytolytic T cells warrants further study as an intriguing facet of the interplay between CMV and the immune system.

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