Emergence of a CD4⁺CD28⁻ granzyme B⁺, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection

Ester M. M. van Leeuwen, Ester B. M. Remmerswaal, Mireille T. M. Vossen, Ajda T. Rowshani, Pauline M. E. Wertheim-van Dillen, Rene A.W. van Lier and Ineke J. M. ten Berge

Emergence of a CD4\(^+\)CD28\(^-\) granzyme B\(^+\), cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection

Ester M. M. van Leeuwen\(^1\), Ester B. M. Remmerswaal\(^2\), Mireille T. M. Vossen\(^2\)\(^3\), Ajda T. Rowshani\(^1\), Pauline M. E. Wertheim-van Dillen\(^4\), Rene A.W. van Lier\(^2\) and Ineke J. M. ten Berge\(^1\)

\(^1\)Department of Internal Medicine, Divisions of Nephrology and Clinical Immunology & Rheumatology, \(^2\)Department of Experimental Immunology, \(^3\)Emma Children’s Hospital and \(^4\)Department of Virology, Academic Medical Center, Amsterdam, The Netherlands

Abstract

Cytotoxic CD4\(^+\)CD28\(^-\) T cells form a rare subset in human peripheral blood. The presence of CD4\(^+\)CD28\(^-\) cells has been associated with chronic viral infections, but how these particular cells are generated is unknown. In this study, we show that in primary CMV infections, CD4\(^+\)CD28\(^-\) T cells emerge just after cessation of the viral load, indicating that infection with CMV triggers the formation of CD4\(^+\)CD28\(^-\) T cells. In line with this, we found these cells only in CMV-infected persons. CD4\(^+\)CD28\(^-\) cells had an antigen-primed phenotype and expressed the cytolytic molecules granzyme B and perforin. Importantly, CD4\(^+\)CD28\(^-\) cells were to a large extent CMV-specific because proliferation was only induced by CMV antigen, but not by recall antigens such as purified protein derivative or tetanus toxoid. CD4\(^+\)CD28\(^-\) cells only produced IFN-\(\gamma\) after stimulation with CMV antigen, whereas CD4\(^+\)CD28\(^-\) cells also produced IFN-\(\gamma\) in response to varicella zoster virus and purified protein derivative. Thus, CD4\(^+\)CD28\(^-\) T cells emerge as a consequence of CMV infection.

Introduction

Within the circulating human CD4\(^+\) T cell population, a subset of cytotoxic cells has recently been described\(^1\). These cells express the cytolytic molecules perforin and granzyme B (grB) and have no expression of the costimulatory molecules CD28 and CD27 or the chemokine receptor CCR7. CD4\(^+\)CD28\(^-\) T cells have been reported to be expanded in patients with rheumatoid arthritis (RA), especially in those with extra-articular inflammatory lesions and rheumatoid vasculitis\(^2\). CD4\(^+\)CD28\(^-\) T cells in RA have a limited TCR diversity, suggesting that they recognize only a few antigens\(^3\)\(^4\). Additionally, in patients with unstable angina, high numbers of CD4\(^+\)CD28\(^-\) IFN-\(\gamma\)-producing cells have been described, which were able to effectively lyse endothelial cells and thereby possibly contribute to plaque destabilization\(^5\)\(^6\). Finally, an increased
percentage of CD4⁺CD28⁻ T cells was found in the circulation of elderly individuals, but exceptions indicated that other factors besides age may be involved in the generation of these cells. The origin and specificity of CD4⁺CD28⁻ T cells is unknown. Because the percentage of CD4⁺CD28⁻ cells varied among individuals but was generally higher in HIV-infected individuals, Appay et al. suggest that the presence of these cells is related to chronic viral infections. In patients with RA and in the elderly, the expansion of CD4⁺CD28⁻ and CD8⁺CD28⁻ T cells has been described to be associated with CMV infection. Human CMV is a widespread member of the β-herpesvirus family that persists in the host in a latent state after primary infection. In healthy individuals, virus and host exist in a symbiotic equilibrium, such that infectious disease manifestations are hardly encountered. However, when the immune system is compromised, for example in HIV-infected individuals or in transplant recipients, CMV infection can lead to a number of disease symptoms. Although CD8⁺ T cells are believed to be most important in controlling CMV infection, CD4⁺ T cells also play a role in the defense. Previously, we showed that during primary CMV infection, virus-specific CD4⁺ T cells precede the appearance of both specific Abs and virus-specific CD8⁺ T cells in renal transplant recipients. In symptomatic primary CMV infection, CMV-specific IFN-γ-producing CD4⁺ T cells were delayed and only appeared after antiviral therapy, suggesting that CD4⁺ T cells are indispensable in protection against CMV disease. CMV infection exerts a profound effect on the CD8⁺ T cell pool that persists long after primary infection. In CMV carriers, increased percentages and absolute numbers of circulating cytolytic CD8⁺CD45RA⁻CD27⁻ T cells have been detected that were not observed after EBV or varicella-zoster virus (VZV) infection nor after vaccination with measles-mumps-rubella. The fact that CMV infection leaves a fingerprint within the CD8⁺ T cell compartment together with the observations that CD4⁺CD28⁻ T cells are predominantly found in CMV-infected individuals prompted us to analyze emergence and specificity of CD4⁺CD28⁻ T cells in primary CMV infection and during latency.

Materials & Methods

Subjects
Healthy CMV-seronegative (n=13) and -seropositive (n=15) healthy volunteers as well as CMV-seronegative (n=7) and -seropositive (n=26) renal transplant recipients (at least one year after transplantation) were included in this study. The renal transplant patients were treated with basic immunosuppressive therapy consisting of prednisolone, mycophenolate mofetil, and cyclosporine. In addition, we longitudinally studied 4 renal transplant recipients who were CMV seronegative before transplantation and who received a kidney from a CMV-seropositive donor. All patients gave written informed consent, and the study was approved by the local medical ethics committee.
Chapter 7

PBMCs

Heparinized peripheral blood samples were collected, and PBMCs were isolated using standard density gradient centrifugation techniques and subsequently cryopreserved until the day of analysis.

CMV-PCR, anti-CMV IgG, and anti-EBV IgG

Quantitative PCR was performed in EDTA whole blood samples as described before\(^19\). To determine CMV serostatus, anti-CMV IgG was measured in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL) according to the manufacturer's instructions. Measurements were calibrated relative to a standard serum. The EBV serostatus was investigated by determination of IgG to both EBV viral capsid antigen and Epstein-Barr nuclear antigen by ELISA (Biotest, Dreieich, Germany).

Immunofluorescent staining and flowcytometry

PBMCs were washed in PBS containing 0.01% (w/v) NaN\(_3\) and 0.5% (w/v) BSA. A total of 200,000 PBMCs were incubated with fluorescent-labeled conjugated mAbs (concentrations according to manufacturer's instructions) for 30 min at 4°C. For analysis of expression of surface markers, the following mAbs were used in different combinations: CD4-PerCP, CD4-PerCP Cy5.5, CD4-allophycocyanin, CD25-PE, CD27-PE, CD28-PE, CD45RA-FITC, CD45RO-PE, CD49d-PE, CD57-FITC, CD69-FITC, anti-HLA-DR-FITC, anti-TCR\(\gamma\delta\)-FITC, and Streptavidin-allophycocyanin (all from BD Biosciences, San Jose, CA); CD4-allophycocyanin, CD45RA-biotin, and CCR7-PE, (all from BD Pharmingen, San Diego, CA); CD11a-FITC, CD11b-FITC, CD11c-PE, and CD18-FITC (all from DAKO, Glostrup, Denmark); CD27-biotin, CD28-FITC, CD49f-FITC, and CD54 unlabeled (all from Sanquin, Amsterdam, The Netherlands); anti-CXCR3 (R&D Systemes, Minneapolis, MN); CD69- allophycocyanin (Caltaq Laboratories, Burlingame, CA); CD49e-FITC (Chemicon Europe, Chandlers Ford, U.K.); and anti-TCR -FITC (Intruchemie, Delfzijl, The Netherlands). To stain unlabeled monoclonals, goat anti-mouse Ig-R-PE (Southern Biotechnology Associates, Birmingham, AL) was used and for blocking normal mouse serum (Sanquin) was added before the rest of the staining was performed. Stainings for chemotactrant receptor of Th2 cells (CRTh2)-PE and CCR5-allophycocyanin (both from BD Biosciences) were performed on whole blood at room temperature followed by lysis of red cells with lysing solution (BD Biosciences). Cells were washed in PBS containing 0.01% (w/v) NaN\(_3\) and 0.5% (w/v) BSA and were analyzed using a FACSCalibur flow cytometer and Celisquest software (BD Biosciences).

Intracellular grB, perforin, and cytokine staining

For intracellular staining, cells were fixed in FACS lysing solution (BD Biosciences) and permeabilized (BD Biosciences) mAbs, according to manufacturer's instructions. Flowcytometric analysis was performed thereafter. For cytokine stainings, cells were first stimulated for 6 h at 37°C with CMV antigen (inactivated whole virus, 10 \(\mu\)l/ml; Microbix Biosystems, Toronto, Canada), VZV antigen (20 \(\mu\)l/ml; Microbix Biosystems), purified protein derivative (PPD; 11.8 \(\mu\)g/ml; Statens Serum Institute, Copenhagen, Denmark), or tetanus toxoid (TT; 17.6 Lf/ml; RIVM, Bilthoven, The Netherlands). As positive controls, cells were stimulated with PMA (1 ng/ml) / ionomycin (1 \(\mu\)g/ml) (Sigma-Aldrich, St. Louis, MO) or Staphylococcus aureus enterotoxin B (SEB; 2 \(\mu\)g/ml; ICN/Fluka, Buchs, Switzerland). All stimulations were performed in a final volume of 1 ml of RPMI 1640 (Life

112
CD4+CD28+ T cells are induced by cytomegalovirus

Technologies, Rockville, MD) containing 10% heat-inactivated FCS and 1 μg/ml (final concentration) VLA-4 mAb (CD49d; BD Biosciences). For the final 5 h of culture, brefeldin A (Sigma-Aldrich) was added to the culture in a final concentration of 10 μg/ml. After culture, the same intracellular staining procedure described above was performed using anti-IFN-γ-FITC, anti-TNF-α-FITC, anti-IL-2-FITC, and anti-IL-4-PE (all from BD Biosciences).

**CFSE labelling**

PBMCs were resuspended in PBS at a final concentration of 5-10 x 10^6 cells/ml. PBMCs were labelled with 0.5 μM CFSE (final concentration; Molecular Probes, Eugene, OR) in PBS for 8-10 min, shaking at 37°C. Cells were washed and subsequently resuspended in IMDM supplemented with 10% human pool serum (BioWhittaker, Verviers, Belgium), antibiotics, and 3.57 x 10^-4 % (w/v) 2-ME (Merck, West Point, PA) (culture medium).

**Proliferation assays**

PBMCs from CMV-seropositive donors were sorted by FACSaria (BD Biosciences) into a CD4+CD28+ population, a CD4+CD28-CD45RA+ population, and a CD4+CD28-CD45RA- (naive) population. These sorted cells were labeled with CFSE and left in culture medium for 4 days. Cells were cultured in the presence of autologous PBMCs that were, before irradiation, cultured for 5 h with CMV antigen, PPD, or TT or in just medium. All stimulations were performed in both the absence and presence of IL-2 (50 U/ml; Biotest). Control stimulations consisted of medium only, IL-2 only, autologous irradiated PBMCs only, or autologous irradiated PBMCs with IL-2. As a positive control, cells were stimulated with PHA (Life Technologies) for 3 days.

**Amplification of CDR3 regions**

cDNA was synthesized from RNA from equal amounts of sorted CD4+CD28+ and CD4+CD28- cells. PCR was performed as described previously in single TCRBV PCRs with a TCRBC primer labeled with a fluorescent dye (Life Technologies; CAG GCA CAC CAG TGT GGC-FAM). CDR3 size distributions were visualized with the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Statistical Analysis**

The two-tailed Mann-Whitney U test was used for analysis of differences between groups. Statistically significance was indicated by p values < 0.05.

**Results**

**CD4+CD28- grB-expressing cells appear in peripheral blood after primary CMV infection**

Because the presence of cytotoxic CD4+CD28- T cells in the circulation seems to be associated with chronic viral infections and because prior CMV infection leaves a fingerprint in the CD8+ T cell pool, we investigated the involvement of CD4+CD28- cells in CMV infection. Therefore, we longitudinally studied CMV-seronegative recipients of a CMV-seropositive kidney graft who experienced a primary CMV infection. As described before, the first sign of specific immunity to CMV is the appearance of IFN-γ-producing CD4+ T cells in the circulation around the peak of the viral load, followed by specific Abs and CMV-specific CD8+ T cells. Interestingly, we found that only after
cessation of the viral load an increase was observed in CD4⁺CD28⁻ T cells expressing the cytolytic molecule grB (Fig. 1A). The percentage of CD4⁺CD28⁻grB⁺ T cells continued to increase long after the viral load became undetectable (Fig. 1B).

Figure 1: CD4⁺CD28⁻grB⁺ T cells appear in the peripheral blood after primary CMV infection.

(A) Dot plots gated on total CD4⁺ T cells show the appearance of CD28⁻grB⁺ cells after cessation of the viral load in a primary CMV infection. The numbers in the indicated quadrants represent the percentage within total CD4⁺ T cells. Pre Tx indicates pretransplantation time point; wk 01, 11, 15, 18, 46 indicate weeks after transplantation. Representative flow cytometric analysis of one of four patients is shown. (B) The upper graph shows the kinetics of the viral load (CMV DNA, copies/ml; ■) during primary CMV infection. The lower graph shows the percentages of CD28⁻grB⁺ cells (left y-axis; ▲) and IFN-γ-producing cells upon CMV stimulation (right y-axis; ●) within CD4⁺ T cells. The data shown are representative for one of four patients.

The increase in CD4⁺CD28 grB⁺ T cells during primary CMV infection was not seen in renal transplant recipients who remained seronegative for CMV, excluding that either the allograft itself or initiation of immunosuppressive drugs caused the appearance of cytotoxic CD4⁺ T cells (data not shown). The presence of CD4⁺CD28 grB⁺ cells was also not affected by episodes of acute allograft rejection (data not shown). This important finding indicates that CMV infection is the key factor that causes the large increase in the percentage of cytotoxic CD4⁺CD28 grB⁺ T cells. CD4⁺CD28 grB⁺ T cells will be referred to as CD4⁺CD28⁻ cells in the following paragraphs.

The percentage of circulating CD4⁺CD28⁻ cells is highly increased in CMV-seropositive individuals
We next investigated whether the observed change in the composition of the CD4+ T cell compartment lasted during CMV latency. When comparing CMV-seropositive and CMV-seronegative renal transplant recipients, no clear differences were observed in the distribution of CD4+ T cell subsets defined by CD27 and CD45RA, CCR7 and CD45RA or CD45R0 and CD45RA (data not shown). However, only in CMV-seropositive patients a clearly distinguishable population of CD4+CD28-CD27- cells was found (Fig. 2). A population of cells lacking only CD27 expression was found in both groups (Fig. 2). The population of CD4+ T cells expressing grB was also larger in CMV-seropositive individuals, supporting the association between lack of CD28 and the expression of cytolytic molecules (Fig. 2).

Also, in cohorts of both renal transplant recipients and healthy individuals the percentage of CD28-CD27- cells within CD4+ T cells was significantly higher in CMV-seropositive individuals (Fig. 3A). Whereas the percentage CD4+CD28-CD27- T cells in CMV-seronegative individuals was always below 0.5%, these percentages ranged in CMV-seropositive healthy individuals from 0.7% to 6.2% and in renal transplant recipients from 0.9% to 61.4% (Fig. 3A). The presence of CD28- T cells has been associated with age. In our study, however, the mean age of the CMV-seronegative and CMV-seropositive groups did not differ (32.8 vs 32.5 for the healthy individuals and 47.6 vs 48.7 for the renal transplant patients, respectively; ns). Despite a large variation in percentages of CD4+CD28-CD27- T cells in CMV-seropositive renal transplant recipients, the median was significantly higher than in seropositive healthy individuals (p=0.0006; Fig. 3A). This is not due to the difference in age because no relation was found between the percentage of CD4+CD28-CD27- cells and age (data not shown).

To investigate a possible effect of other persistent viruses such as EBV on the presence of CD4+CD28-CD27- cells, four groups were discerned in the healthy individuals tested, based on EBV and CMV serostatus. As shown in Figure 3B, the percentage of CD4+CD28-CD27- T cells was significantly higher in peripheral blood of CMV-seropositive individuals, independent of their EBV serostatus. This indicates that, as

Figure 2: During latency, CD4+ T cells in CMV-seropositive individuals are CD28-CD27- and express grB. Representative flow cytometric analysis is shown of one of five CMV-seronegative and five CMV-seropositive renal transplant recipients, respectively (at least 1 year after transplantation). Left plots are gated on total CD4+ T cells; right plots are gated on total lymphocytes. The numbers represent the quadrant percentages of cells within CD4+ T cells. "CMV-" indicates CMV-seronegative individual, "CMV+" indicates a CMV-seropositive individual.
Chapter 7

described for CD8*T cells, CMV, and not other viruses, causes an increase in the percentage of circulating cytotoxic CD4*T cells.

![Graph A](image1.png)

**Figure 3:** Percentages of CD4*CD28*CD27* T cells are significantly higher in CMV-seropositive individuals. Percentages of CD28*CD27* cells within total CD4*T cells are shown. (A) "Healthy" indicates healthy individuals, and "RTx" indicates renal transplant recipients at least one year after transplantation. "CMV -" indicates CMV-seronegative individuals (n=13 healthy and n=7 RTx individuals), and "CMV +" indicates CMV-seropositive individuals (n=15 healthy and n=26 RTx individuals). (B) Healthy individuals were divided into four groups according to CMV and EBV serostatus. "-" indicates seronegative, "+" indicates seropositive for CMV and/or EBV. n=7 CMV-/EBV-, n=6 CMV-/EBV+, n=2 CMV+/EBV- and n=13 CMV+/EBV+.

**CD4*CD28** T cells have the phenotype of cytotoxic antigen-experienced cells but are not recently activated

To gain more insight in the role of CD4*CD28* T cells in CMV infection, we further analyzed the phenotype of these cells using different surface markers to classify T cells. As shown in Figure 4A, CD4*CD28* T cells may be classified as effector memory type cells: they did not express the costimulatory receptor CD27 but uniformly expressed CD57, CD45R0 and not CD45RA. CD4*CD28* cells expressed the cytolytic molecules grB and perforin (Fig. 4A), suggesting cytotoxic potential. Furthermore, CD4*CD28* T cells expressed LFA-1 α- and β-chain (CD11a and CD18), macrophage adhesion molecule 1 (CD11b), ICAM-1 (CD54), and VLA 4-6 (CD49d-f) and did not express CD11c. All CD4*CD28* T cells were TCR αβ-positive and did not express TCR γδ (data not shown). CD4*CD28* T cells appeared not to be recently activated because CD69, CD25, CD38, and HLA-DR were not expressed (Fig 4B).
CD4-CD28 T cells are induced by cytomegalovirus

Figure 4: CD4-CD28 T cells have a primed but not recently activated phenotype. (A) Phenotype of CD4+CD28 and CD4+CD28- cells in relation to subset markers CD27, grB, perforin, CD57, CD45R0 and CD45RA. (B) Phenotype of CD4+CD28- and CD4+CD28+ cells in relation to activation markers CD69, CD25, CD38 and HLA-DR. (C) Phenotype of CD4+CD28- and CD4+CD28+ cells in relation to chemokine receptors CCR7, CRTh2, CCR5 and CXCR3. All dot plots are gated on total CD4+ T cells. Representative flow cytometric analysis from four donors is shown, CXCR3 staining is shown from three different donors selected from a total of eight donors.

Concerning the expression of chemokine receptors, CD4+CD28- T cells did not express CCR7, which again shows that these cells have a memory phenotype (Fig. 4C). CRTh2 was not expressed, whereas most cells were CCR5+, indicating a Th1 phenotype. Remarkably, the expression of the inducible chemokine receptor CXCR3 on CD4+CD28- T cells was highly variable (0-100%) among different donors, as shown for three donors in Figure 4C. The data from the phenotypic analysis of CD4+CD28- T cells are summarized in Table I.
Chapter 7

To investigate the clonality of CD4⁺CD28⁻ cells, the TCR Vβ repertoire of sorted CD4⁺CD28⁻ T cells was determined and compared with that of CD4⁺CD28⁺ T cells. The CD4⁺CD28⁻ T cell subset showed a skewed distribution of Vβ subfamilies compared with CD4⁺CD28⁺ T cells (data not shown), indicating that CD4⁺CD28⁻ T cells have clonally expanded upon antigen exposure.

**Table I: Expression of phenotypic markers on CD4⁺CD28⁻ cells**

<table>
<thead>
<tr>
<th>marker</th>
<th>CD4⁺ CD28</th>
<th>marker</th>
<th>CD4⁺ CD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD27</td>
<td>−</td>
<td>CD11a</td>
<td>+</td>
</tr>
<tr>
<td>CCR7</td>
<td>−</td>
<td>CD11b</td>
<td>+</td>
</tr>
<tr>
<td>CD57</td>
<td>+</td>
<td>CD11c</td>
<td>−</td>
</tr>
<tr>
<td>CD45R0</td>
<td>+</td>
<td>CD18</td>
<td>+</td>
</tr>
<tr>
<td>CD45RA</td>
<td>−</td>
<td>CD54</td>
<td>+</td>
</tr>
<tr>
<td>granz. B</td>
<td>+</td>
<td>CD25</td>
<td>−</td>
</tr>
<tr>
<td>perforin</td>
<td>+</td>
<td>CD38</td>
<td>−</td>
</tr>
<tr>
<td>CD49d</td>
<td>+</td>
<td>CD69</td>
<td>−</td>
</tr>
<tr>
<td>CD49e</td>
<td>+</td>
<td>HLA-DR</td>
<td>−</td>
</tr>
<tr>
<td>CD49f</td>
<td>+</td>
<td>CXCR3</td>
<td>+/-</td>
</tr>
<tr>
<td>CRTh2</td>
<td>−</td>
<td>CCR5</td>
<td>+</td>
</tr>
</tbody>
</table>

**CD4⁺CD28⁻ T cells proliferate and produce cytokines upon CMV stimulation**

To test whether CD4⁺CD28⁻ T cells are CMV-specific, CD4⁺ T cells from a CMV-seropositive donor were sorted into CD28⁻, CD28⁺CD45RA⁻, and CD28⁺CD45RA⁺ populations and were stimulated with CMV antigen, PPD, or TT in the absence or presence of IL-2. Stimulation with IL-2 alone or with irradiated autologous PBMCs alone did not induce proliferation of the sorted cell populations (Fig. 5). After CMV antigen stimulation, CD4⁺CD28⁻ cells proliferated, and this was enhanced by addition of IL-2 (Fig. 5). Upon each division, CD28 was up-regulated on CD4⁺CD28⁻ cells. Stimulation with PPD and TT did not induce proliferation of CD4⁺CD28⁻ cells, not even when IL-2 was added. CD28⁺CD45RA⁻ cells from the same donor did proliferate upon stimulation with CMV and PPD. When IL-2 was added, CD28⁺CD45RA⁻ cells proliferated in response to irradiated autologous PBMCs plus IL-2 without antigen, but this was enhanced when CMV, PPD, or TT was added. CD28⁺CD45RA⁻ naive cells did not proliferate under any stimulatory condition (Fig. 5). As described before, CD4⁺CD28⁺, but not CD4⁺CD28⁻ cells, proliferated poorly upon PHA stimulation (data not shown). CMV specificity was corroborated by antigen-induced cytokine production analysis. After stimulation of PBMCs from a CMV-seropositive donor with CMV antigen, only CD4⁺CD28⁺ T cells produced IFN-γ, whereas upon PMA / ionomycin or SEB stimulation, both CD28⁻ and CD28⁺ CD4⁺ T cells produced this cytokine (Fig. 6A and data not shown). The percentage of cytokine-producing cells upon PMA / ionomycin stimulation
within CD4^+CD28^- cells was lower than within CD4^+CD28^+ cells, which can be explained by the presence of naive CD4^+ cells in this fraction, which are not so efficient in producing cytokines^{28}. 

To test whether cytokine production was in general restricted to CD4^+CD28^- cells or whether this was specific for CMV, we performed the assay with PBMCs from a CMV-
seropositive donor who had recently been in contact with a child experiencing varicella (chickenpox), in which case it is possible to measure VZV-specific CD4⁺ T cells by IFN-γ production. Only CD4⁺CD28⁺ T cells produced IFN-γ upon VZV stimulation, in contrast to the CMV-induced IFN-γ production by CD4⁺CD28⁻ cells (Fig. 6B). In cells from a CMV-seropositive donor who reacted to PPD and TT, IFN-γ production after stimulation with CMV was mostly restricted to CD28⁻ cells, whereas only CD28⁺ cells produced this cytokine upon PPD stimulation (Fig. 6C). Stimulation with TT did not result in any IFN-γ production (data not shown). These data indicate that CD4⁺CD28⁻ are especially CMV specific, whereas CMV-reactive cells are also present within CD4⁺CD28⁻ cells. Concerning the production of other cytokines by CD4⁺CD28⁻ cells, we found that TNFa, like IFN-γ, was produced after stimulation with CMV, but not with VZV or PPD. IL-4 was not produced at all, whereas only low amounts of IL-2 were produced by CD4⁺CD28⁻ cells after stimulation with PMA / ionomycin or SEB (data not shown).

Discussion

In this study, we show that the percentage of CD4⁺CD28⁻CD27⁻ grB-expressing T cells in the circulation largely increases after primary CMV infection. Previously, we have demonstrated the emergence of CMV-specific, IFN-γ-producing CD4⁺ T cells shortly after first appearance of CMV DNA in peripheral blood. These cells were in cell cycle and showed the features of recently activated cells. In contrast, cytotoxic CD4⁺CD28⁻ T cells appeared in the circulation only after cessation of viral replication and were detectable in much higher frequencies in CMV-seropositive individuals during latency. The very low percentages (<0.5%) of CD4⁺CD28⁻ cells in CMV-seronegative individuals might be the result of sterile CMV infections or they could be induced by infections with other pathogens. The percentages of cytotoxic CD4⁺CD28⁻ T cells were higher in CMV-seropositive renal transplant recipients than in healthy individuals, which corresponds with the higher percentages of CMV-specific, effector-type T cells during immunosuppression. Within the group of CMV-seropositive renal transplant recipients, a dichotomy is observed. We related the percentages of CD4⁺CD28⁻ cells to different parameters like prior primary CMV infection after transplantation, number of rejections, age, and the development of chronic rejection, but none of these seemed to explain the division in two groups. This contrasts a recent publication which states that patients with chronic kidney graft rejection have higher percentages of CD4⁺CD28⁻ cells. We could demonstrate CMV specificity of a considerable portion of CD4⁺CD28⁻ cells because both proliferation and cytokine production were induced by CMV stimulation and not by stimulation with other antigens such as PPD or VZV. This is in line with recent data for other antigens because in patients with chronic beryllium disease, CD4⁺CD28⁻ cells from peripheral blood did not produce IFN-γ when stimulated with beryllium (BeSO₄), whereas CD4⁺CD28⁻ cells did.
CD4⁺CD28⁺ T cells are induced by cytomegalovirus

The increase in CD4⁺CD28⁻ cells in the peripheral blood compartment is only after the viral load became undetectable.

Figure 6: CD4⁺CD28⁺ cells produce IFN-γ upon CMV antigen stimulation, but not upon stimulation by VZV or PPD. All dot plots are gated on CD4⁺CD28⁺ cells (CD28⁺) or CD4⁺CD28⁻ cells (CD28⁻). Dot plots show IFN-γ production vs CD69 expression of CD28⁺ or CD28⁻ CD4⁺ T cells after stimulation with medium, CMV antigen, VZV antigen or PPD, or PMA/ionomycin as a positive control. The number of events shown from the CD4⁺CD28⁺ cells is adapted to the number of events from the CD4⁺CD28⁻ cells (except for VZV and PPD). Numbers indicate percentages of CD69⁺IFN-γ⁺ cells (upper right) within CD4⁺CD28⁺ or CD4⁺CD28⁻ cells. (A) Cells from a CMV-seropositive donor. (B) Cells from a CMV-sero⁺ donor after recent contact with a VZV-infected child. (C) Cells from a CMV-sero⁺ donor who reacted to PPD stimulation.
This might be explained by entry of CD4⁺CD28⁻ cells into the circulation from the infected tissues only once the acute infection is over. Apart from this redistribution effect, differentiation of the cells also can cause the appearance of CD4⁺CD28⁻ cells.

As described for CD8⁺ T cells in primary CMV infection, cells change their phenotype during differentiation. T cells lose the expression of CD28 (and CD27 and CCR7) but this is a slow process that even continues long after the antigenic load has become undetectable. Thus, the period between start of the infection and appearance of CD4⁺CD28⁻ cells may reflect the time needed to acquire the effector phenotype.

The regulation of CD28 and CD27 expression on T cells is not completely understood. Naive cells express both costimulatory molecules, and during differentiation, expression can be lost simultaneously with acquisition of effector functions. However, it seems that the order of changes in phenotype is not similar for CD8⁺ and CD4⁺ T cells. Differentiating CD8⁺ T cells first lose expression of CD28 and only in a later phase they lose CD27, thus CD8⁺CD27⁻ cells always have lost CD28 expression. For CD4⁺ T cells it seems to be the opposite: all CD28⁻ cells are CD27⁻ but not vice versa, indicating that during differentiation CD4⁺ T cells first lose expression of CD27 (this paper), CD4⁺CD28⁻ cells are not commonly seen and, as we show in this study, infection with CMV is the major factor causing this differentiation step of CD4⁺ T cells. In contrast with reports describing that the CD28⁻ phenotype is stable and that CD28 expression cannot be restored, CD28 was clearly up-regulated on CD4⁺CD28⁻ T cells that proliferated after CMV stimulation. This is in line with previous data showing re-expression of CD28 in CD28⁻ T cell clones after anti-CD3 stimulation. In addition, it was recently shown that CD4⁺CD28⁻ T cells can become CD28⁺ after stimulation with anti-CD3 in combination with IL-12. CD28-B7 interactions provide important costimulatory signals for T cell activation. CD28⁻ T cells cannot be stimulated anymore via this pathway, which could mean that these cells can function independently from costimulation. However, Park et al. showed that CD4⁺CD28⁻ cells proliferated better in the presence of accessory cells, suggesting that CD28⁻ T cells are not necessarily costimulation-independent but could receive signals via other molecules than CD28. Recently, 4-1BB ligand has been shown to costimulate CD28⁻ T cells, and we found that cytokine production by CD4⁺CD28⁻ cells can be enhanced by adding an Ab against VLA-4 (CD49d; data not shown). In a paper by Suni et al., CD4⁺CD8dim T cells were described to be enriched for CMV-specific cells. Indeed, we found that a small percentage (0-20%) of CD4⁺CD28⁻ T cells expressed low levels of CD8, whereas this was hardly seen in CD4⁺CD28⁺ T cells. Thus, CD4⁺CD8dim T cells probably represent a subpopulation of CD4⁺CD28⁻ T cells.

What exact function cytotoxic CD4⁺CD28⁻ T cells have in controlling CMV infection is not clear yet. One of the immune-evasion strategies of CMV is to reduce MHC class I expression and thereby impede CD8⁺ T cell immune surveillance. Therefore, the role of CD4⁺ T cells and their recognition of MHC class II may be critical for activating the immune system and sustaining the balance between virus and host immunity during latency. This is in agreement with the need for CMV-specific CD4⁺ T cells to protect
against CMV disease\textsuperscript{44}. Another possible function of cytotoxic CD4\(^+\)CD28\(^-\) T cells might
be a role in a negative feedback loop, namely eliminating APCs to dampen the immune
response as described for cytotoxic CD8\(^+\) T cells\textsuperscript{44}.

Although CD4\(^+\)CD28\(^-\) T cells proliferate and secrete IFN-\(\gamma\) only upon CMV stimulation,
not all CD4\(^+\)CD28\(^-\) T cells responded in these in vitro assays, which may seem
contradictory in relation to the finding that CD4\(^+\)CD28\(^-\) cells only appear after CMV
infection. Several explanations can be raised for this paradox. First, the CD4\(^+\)CD28\(^-\) T
cells were generated in vivo upon infection with CMV and it could well be that the
peptides presented during infection and where they are presented are not exactly the
same as during the experimental CMV stimulation. Second, the in vitro stimulation is
performed with an inactivated laboratory CMV strain, which may induce a different
immune response than the primary infection with one of the different natural CMV
strains. Finally, it is well known that CMV is able to encode a range of gene products and
uses several mechanisms to manipulate the host immune system\textsuperscript{42,43}. Part of the
CD4\(^+\)CD28\(^-\) T cells therefore could be inhibited in their response to CMV or,
alternatively, could be directed not against the virus itself but against molecules induced
by CMV infection.

Enhanced numbers of CD4\(^+\)CD28\(^-\) cells have been found in patients with RA and with
cardiovascular diseases\textsuperscript{5,3,5,6}. This raises the question of whether CMV infection plays a
role in these diseases. Also, in RA high frequencies of CD28\(^-\) T cells were associated
with CMV seropositivity\textsuperscript{5}. Furthermore, CMV DNA has been detected in synovial tissue
and fluid of arthritis patients, and subpopulations of CD8\(^+\) synovial fluid mononuclear
cells showed CMV specificity\textsuperscript{45,46}. Thinking of the association among T cells, RA, and
CMV, it could be that part of the CD4\(^+\)CD28\(^-\) T cells in patients with RA responds
specifically to CMV and possibly cross-reacts to other antigens. CMV infection has also
been associated with the development of cardiovascular diseases, as described by
several papers\textsuperscript{47-50}. Interestingly, it was recently described that the increased levels in
CD8\(^+\)CD28\(^-\) T cells in patients with coronary artery disease were mainly determined by
CMV seropositivity\textsuperscript{51}. Although data are not conclusive yet, numerous studies also
suggest an influence of CMV infection in triggering chronic rejection of different organ
grafts in humans as well as in experimental animal models (reviewed in\textsuperscript{55}).

So, how are CMV and effector-type T cells involved in the tissue damage occurring in
RA, cardiovascular diseases, and graft rejection? CMV establishes latency in various cell
types, including myeloid lineage cells but also endothelial cells. Inflammatory processes
caused by different stimuli may cause reactivation of CMV in the endothelial cells, which
could directly lead to vascular pathology. Apart from the direct effects from CMV, the
tissue damage in the different diseases can also result from immunopathology. Once
there is a reactivation of CMV and virus is produced in endothelial cells, this will attract
cytotoxic CD4\(^+\) and CD8\(^+\) T cells, which are induced in high numbers by CMV infection.
The migration of T cells to the site of infection could be mediated by the inducible
chemokine receptor CXCR3, which we found to be expressed on variable percentages
(up to 100\%) of CD4\(^+\)CD28\(^-\) T cells (Fig. 4C). In addition, it has been described that
upon CMV infection, expression of MHC class II molecules on endothelial cells is
induced, which means that CD4⁺ T cells also can recognize the presented antigens. At
the site of infection, the T cells present will produce inflammatory cytokines and have
high levels of grB and perforin, which can cause tissue damage to the endothelial cells
possibly resulting in an increase in atherosclerotic, vasculitic, or extra-articular
rheumatoid lesions. Altogether, this provides a link between the presence of CD4⁺CD28⁻
cells in RA and cardiovascular diseases and the data from this paper showing that
CD4⁺CD28⁻ cells emerge as a consequence of CMV infection.

Acknowledgments

We thank the patients and the healthy volunteers for their blood donations, Berend
Hooibrink (Department of Cell Biology and Histology, Academic Medical Center,
Amsterdam, The Netherlands) for sorting the different cell populations, technicians from
the Department of Clinical Virology for performing CMV PCRs and CMV and EBV
serology, and Drs Eric Eldering and Martijn A. Nolte (both from Laboratory for
Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands) for
critical reading of the manuscript.

References

Smith D, McMichael AJ, Cooper DA, Rowland-Jones SL, Kelleher AD. Characterization of
2. Martens PB, Goronzy JJ, Schaid D, Weyand CM. Expansion of unusual CD4+ T cells in
3. Schmidt D, Goronzy JJ, Weyand CM. CD4+ CD7-CD28- T Cells are expanded in
rheumatoid arthritis and are characterized by autoreactivity. J.Clin.Invest 1996;97:2027-
2037.
4. Schmidt D, Martens PB, Weyand CM, Goronzy JJ. The repertoire of CD4+CD28- T cells in
Perturbation of the T-cell repertoire in patients with unstable angina. Circulation
1999;100:2135-2139.
cell-mediated lysis of endothelial cells in acute coronary syndromes. Circulation
2002;105:570-575.
7. Vallejo AN, Nestel AR, Schirmer M, Weyand CM, Goronzy JJ. Aging-related deficiency of
CD28 expression in CD4+ T cells is associated with the loss of gene-specific nuclear factor
CD4+CD28 T cells are induced by cytomegalovirus


CD4^+CD28^− T cells are induced by cytomegalovirus


Chapter 7

chemokine expression correlates with the acceleration of chronic rejection in rat heart transplants. J.Virol. 2003;77:2182-2194.

