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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,$^{†}$ Mireille T. M. Vossen,$^{‡‡}$ Ajda T. Rowshani,* Pauline M. E. Wertheim-van Dillen,$^{§}$ René A. W. van Lier,$^{†}$ and Ineke J. ten Berge*$^{*}$

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 Ag-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-Ag, but not by recall Ags such as purified protein derivative; TT, tetanus toxoid; SEB, Staphylococcus aureus enterotoxin B.

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)$^2$ and have no expression of the co-stimulatory 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 Ags (3, 4). Additionally, in patients with unstable angina, high numbers of CD4$^+$CD28$^-$ IFN-γ-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 (7, 8).

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. (1) 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 (9–11). 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 (12). 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 (13, 14). 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 (14).

CMV infection exerts a profound effect on the CD8$^+$ T cell pool that persists long after primary infection (9, 15–17). 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 (18). 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 and 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 1 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 four renal transplant recipients who were CMV-seronegative before transplantation and who received a kidney from a CMV-seropositive donor (13). All patients gave written informed consent, and the study was approved by the local medical ethics committee.

**PBMCs**

Heparinized peripheral blood samples were collected, and PBMCs were isolated using standard density gradient centrifugation techniques and were 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 Ag and Epstein-Barr nuclear Ag by ELISA (Biotest, Dreieich, Germany).

**Immunofluorescent staining and flow cytometry**

PBMCs were washed in PBS containing 0.01% (w/v) NaN3 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, CD49f-FITC, CD58-PE, CD69-PE, anti-HLA-DR-FITC, anti-TCR-PE CD4-PerCP Cy5.5, CD4-allophycocyanin, CD25-PE, CD27-PE, CD28-PE, CD45RA-biotin, and CCR7-PE, (all from BD Pharmingen, San Diego, CA); CD11a-FITC, CD11b-FITC, CD11c-R-PE, and CD18-FITC (all from BD Biosciences). For the detection of intracellular cytokines and intracellular cytokines, cells were stained with 0.5 µg/ml of PPD (11.8 µg/ml; Satens Serum Institut, Copenhagen, Denmark), or tetanus toxoid (TT; 17.6 Lf/ml; RIVM, Borculo, The Netherlands), or VZV-Ag (20 µg/ml; Microbix Biosciences, Toronto, Canada), or EBV-viral capsid Ag and Epstein-Barr nuclear Ag by ELISA (Biotest, Dreieich, Germany).

**Statistical Analysis**

The two-tailed Mann-Whitney U test was used for analysis of differences between groups. Statistical 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 (1) and because prior CMV infection leaves a fingerprint in the CD8+ T cell pool (9, 15–18), 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 (13, 14). 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). 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− T 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, CD7 and CD45RA, or CD45R0 and CD45RA (data not shown).
Both groups (Fig. 2). The population of CD4 A population of cells lacking only CD27 expression was found in y A after primary CMV infection. show the appearance of CD28 H11002/H11001 after primary CMV infection. The numbers in the indicated quadrants represent the percentage within total CD4 in a primary CMV infection. The numbers in the indicated quadrants represent the percentage within total CD4 + T cells. The data shown are representative for one of four patients. 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 timepoint; wk 01, 11, 15, 18, and 46 indicate weeks after transplantation. Representative flow cytometric analysis of one of four patients. The upper graph shows the kinetics of the viral load (CMV DNA, copies/ml; f) during primary CMV infection. The lower graph shows the percentages of CD28 - grB + cells (left y-axis; A) and IFN-γ-producing cells upon CMV stimulation (right y-axis; B) within CD4 + T cells. The data shown are representative for one of four patients. 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 - T cells within CD4 + T cells was significantly higher in CMV-seropositive individuals (Fig. 3A). Whereas the percentage of 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 (8). In our study, however, the mean ages 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 Fig. 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 described for CD8 + T cells (18), CMV, and not other viruses, causes an increase in the percentage of circulating cytotoxic CD4 + T cells.

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

To gain more insight into 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 Fig. 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, CD45RO, and not CD45RA.

CD4 +CD28 - T cells expressed the cytolytic molecules grB and perforin (Fig. 4A), suggesting cytotoxic potential (1). 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–I) and did not express CD11c (data not shown). 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). 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 Fig. 4C. The data from the phenotypic analysis of CD4⁺CD28⁻ T cells are summarized in Table I.

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 had clonally expanded upon Ag exposure (22–25).

**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

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**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 1 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 and “+” indicates seropositive for CMV and/or EBV. n = 7 CMV −/EBV −, n = 6 CMV −/EBV +, n = 2 CMV +/EBV −, and n = 13 CMV +/EBV +.

**FIGURE 4.** CD4⁺CD28⁻ T cells have a primed but not recently activated phenotype. All dot plots are gated on total CD4⁺ T cells. 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. Representative flow cytometric analysis from four donors is shown; CXCR3 staining is shown from three different donors selected from a total of eight donors.
CD4\(^+\)CD28\(^-\) T cells are induced by CMV

Table 1. Expression of phenotypic markers on CD4\(^+\)CD28\(^-\) cells

<table>
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<tr>
<th>Marker</th>
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<td>CD27</td>
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<td>CCR7</td>
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<td>Perforin</td>
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<td>CD49d</td>
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<td>CD49e</td>
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<td>CD49f</td>
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<td>CRT/H2</td>
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CMV specificity was corroborated by Ag-induced cytokine production analysis. After stimulation of PBMCs from a CMV-seropositive donor with CMV Ag, only CD4\(^+\)CD28\(^-\) cells produced IFN-\(\gamma\), 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-\(\gamma\) production (29). Only CD4\(^+\)CD28\(^+\) T cells produced IFN-\(\gamma\) upon VZV stimulation, in contrast with the CMV-induced IFN-\(\gamma\) production by CD4\(^+\)CD28\(^+\) cells (Fig. 6B). In cells from a CMV-seropositive donor who reacted to PPD and TT, IFN-\(\gamma\) 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-\(\gamma\) 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 TNF-\(\alpha\), like IFN-\(\gamma\), 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-\(\gamma\)-producing CD4\(^+\) T cells

FIGURE 5. CD4\(^+\)CD28\(^-\) cells proliferate upon CMV Ag stimulation but do not proliferate upon stimulation by PPD or TT. CD28\(^-\) are sorted CD4\(^+\)CD28\(^-\) cells, CD28\(^+\)CD45RA\(^-\) are sorted CD4\(^+\)CD28\(^-\)CD45RA\(^-\) cells, and CD28\(^+\)CD45RA\(^-\) are sorted CD4\(^+\)CD28\(^+\)CD45RA\(^+\) naive cells. Dot plots show CFSE profiles of the sorted cell populations after 4 days of stimulation with medium or irradiated autologous PBMCs unlaoded or loaded with CMV Ag, PPD, or TT. The left panel shows stimulations without IL-2 and the right panel shows them with IL-2. For clarity, only 33% of dots are shown from the two CD28\(^+\) populations; numbers indicate percentage of divided cells. Flowcytometric analysis is displayed from one CMV-seropositive donor who was known to show a proliferative response upon PPD stimulation. Experiments in which proliferation of CD4\(^+\)CD28\(^-\) cells upon CMV stimulation were tested have been performed in four different individuals and gave similar results.

stimulated with CMV Ag, 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 Ag 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 Ag (26), 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 (27), CD4\(^+\)CD28\(^-\) but not CD4\(^+\)CD28\(^+\) cells, proliferated poorly upon PHA stimulation (data not shown).

FIGURE 6. CD28\(^+\)CD45RA\(^-\)CD45RO\(^+\)CD27\(^+\)CD57\(^+\) cells are sorted CD4\(^+\)CD27\(^+\) cells. Stimulation with CMV, PPD, or TT is possible to measure VZV-specific CD4\(^+\) T cells by IFN-\(\gamma\) production (29). Only CD4\(^+\)CD28\(^+\) T cells produced IFN-\(\gamma\) upon VZV stimulation, in contrast with the CMV-induced IFN-\(\gamma\) production by CD4\(^+\)CD28\(^-\) cells (Fig. 6B). In cells from a CMV-seropositive donor who reacted to PPD and TT, IFN-\(\gamma\) 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-\(\gamma\) 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.
shortly after first appearance of CMV DNA in peripheral blood. These cells were in cell cycle and showed the features of recently activated cells (13). In contrast, cytotoxic \( \text{CD}4^{+}\text{CD}28^{-} \) 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 \( \text{CD}4^{+}\text{CD}28^{-} \) 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 \( \text{CD}4^{+}\text{CD}28^{-} \) 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 (18, 30–32). Within the group of CMV-seropositive renal transplant recipients, a dichotomy is observed. We related the percentages of \( \text{CD}4^{+}\text{CD}28^{-} \) 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 the two groups. This contrasts with a recent publication that states that patients with chronic kidney graft rejection have higher frequencies of \( \text{CD}4^{+}\text{CD}28^{-} \) cells (33). We could demonstrate CMV specificity of a considerable portion of \( \text{CD}4^{+}\text{CD}28^{-} \) cells because both proliferation and cytokine production were induced by CMV stimulation and not by stimulation with other Ags such as PPD or VZV. This is in line with recent data for other Ags because in patients with chronic beryllium disease, \( \text{CD}4^{+}\text{CD}28^{-} \) cells from peripheral blood did not produce IFN-\( \gamma \) when stimulated with beryllium (BeSO\(_4\)), whereas \( \text{CD}4^{+}\text{CD}28^{-} \) cells did (34).

The increase in \( \text{CD}4^{+}\text{CD}28^{-} \) cells in the peripheral blood compartment is only after the viral load became undetectable. This might be explained by entry of \( \text{CD}4^{+}\text{CD}28^{-} \) 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 \( \text{CD}4^{+}\text{CD}28^{-} \) 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 (14). 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 (28). 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 (14). 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 and Ref. 35). 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 (7, 36), 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 CD8+ T cell clones after anti-CD3 stimulation (37). In addition, it was recently shown that CD4+CD28− T cells can become CD28+ after stimulation with anti-CD3 in combination with IL-12 (38). CD28-B7 interactions provide important costimulatory signals for T cell activation. CD8+ T cells cannot be stimulated anymore via this pathway, which could mean that these cells can function independently of costimulation. However, Park et al. (39) showed that CD4+CD28− cells proliferate better in the presence of accessory cells, suggesting that CD28− T cells are not necessarily costimulation-independent but could receive signals via molecules other than CD28. Recently, 4-1BB ligand has been shown to costimulate CD28− T cells (40), 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. (41), CD4+CD8+ 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+CD8+ 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 (42, 43). 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 (14). 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 (44).

Although CD4+CD28− T cells proliferate and secrete IFN-γ 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 would 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 (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− T cells have been found in patients with RA and with cardiovascular diseases (2, 3, 5, 6). This raises the question of whether CMV infection plays a role in these diseases. Also, in RA high frequencies of CD8− T cells were associated with CMV seropositivity (9). 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 (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 respond specifically to CMV and possibly cross-reacts with other Ags. CMV infection has also been associated with the development of cardiovascular diseases, as described in several papers (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 (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 Ref. 52).

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 chemoattractant 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 Ags (53). At the site of infection, the T cells present will produce inflammatory cytokines and will 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− T cells in RA and cardiovascular diseases and the data from this paper showing that CD4+CD28− cells emerge as a consequence of CMV infection.

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