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Elevated granzyme M-expressing lymphocytes during cytomegalovirus latency and reactivation after allogeneic stem cell transplantation

Niels Bovenschen^{a,b,*}, Sanne N.M. Spijkers^b, Annette C. Wensink^{a,b},
Ingrid M.M. Schellens^{b,c}, Robert van Domselaar^a, Debbie van Baarle^{b,c}

^a Department of Pathology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands

^b Laboratory for Translational Immunology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands

^c Department of Internal Medicine and Infectious Diseases, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

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Abstract Human cytomegalovirus (HCMV) reactivation can cause serious complications in allogeneic stem cell transplantation (SCT) patients. HCMV is controlled by cytotoxic lymphocytes that release antiviral granzymes. Recently, we have demonstrated that granzyme M (GrM) inhibits HCMV replication in vitro, however the physiological role of GrM and its cellular distribution during HCMV infection remains unknown. Here, we examined GrM expression in lymphocyte populations during HCMV infection. The percentage of GrM-expressing effector-memory CD4⁺ T-cells was higher in HCMV latently-infected healthy individuals compared to that of uninfected individuals. SCT recipients had higher percentages of GrM-expressing CD4⁺ T, CD8⁺ T, $\gamma\delta$ T, and NKT cells. Despite lower total T-cell numbers, HCMV reactivation in SCT patients specifically associated with higher percentages of GrM-expressing CD4⁺ (total and central-memory) T-cells. GrM was elevated in plasma during HCMV reactivation, pointing to extracellular perforin-independent functions of GrM. We conclude that GrM may be important in regulating HCMV latency and reactivation in SCT patients.

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Abbreviations: HCMV, human cytomegalovirus; SCT, stem cell transplantation; GrM, granzyme M; GrB, granzyme B.

* Corresponding author at: Department of Pathology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Fax: +31 88 7569593.

E-mail address: n.bovenschen@umcutrecht.nl (N. Bovenschen).

1. Introduction

Human cytomegalovirus (HCMV) is a widespread β -herpesvirus that causes lifelong asymptomatic infections in humans [1,2]. However, HCMV infections can lead to severe disease in immunocompromised individuals. Particularly, reactivation

from latency can cause severe complications in allogeneic hematopoietic stem cell transplant (SCT) recipients due to an impaired immune system caused by conditioning regimens and T-cell depletion therapy in combination with immunosuppressive drugs [3,4]. Differences in T-cell reconstitution and the antiviral immune response of individual patients likely discriminate between efficient immunological control of HCMV infections and reactivation.

HCMV infections are generally controlled by cytotoxic lymphocytes, including cytotoxic T lymphocytes, NK cells, $\gamma\delta$ T-cells, and NKT-cells [1]. This is illustrated by strong virus-specific CD4⁺ and/or CD8⁺ T-cell responses that are directed to a broad range of viral epitopes with marked hierarchies of immunodominance [5–7]. Cytotoxic lymphocytes exert their antiviral functions predominantly through releasing interferon- γ (IFN- γ) and the granule-exocytosis pathway [8–11]. The latter pathway is characterized by release of the pore-forming protein perforin and a family of homologous serine proteases, called granzymes. Perforin allows the entry of granzymes into the target cell where granzymes can mediate their antiviral effects by cleaving host cell or viral proteins resulting in clearance of the virus or blockade of its replication. In humans, five granzymes (GrA, GrB, GrH, GrK, and GrM) have been identified with distinct substrate specificities and antiviral pathways [12]. Besides pro-apoptotic effects of granzymes, we have recently shown that GrM can also inhibit HCMV replication in the absence of cell death *in vitro*, mediated through cleavage of viral protein pp71 and host cell heterogeneous nuclear ribonucleoprotein K [13,14].

It has been demonstrated that GrB-positive HCMV-specific CD8⁺ and CD4⁺ (effector) T lymphocytes emerge after primary HCMV infection and are maintained during latency, suggesting a role for GrB in HCMV infections *in vivo* [15–19]. Although GrM-deficient mice are more susceptible to murine CMV infections [20], the physiological relevance of GrM as well as its (cellular) distribution during HCMV infections in humans remains unknown. This knowledge is of importance to further uncover the effector immune response against HCMV. In the present study, we addressed the question how GrM expression is distributed among cytotoxic lymphocyte subsets and in plasma during HCMV latency and reactivation *in vivo*. To this end, we used HCMV-(un)infected healthy individuals as well as patients that received allogeneic SCT, which are ideal to discriminate between HCMV latency and reactivation. We conclude that GrM is expressed by lymphocyte populations that play a role in HCMV immunity, suggesting that GrM is important in regulating HCMV latency in healthy individuals and in controlling HCMV reactivation in SCT patients. Furthermore, GrM levels were elevated in plasma during HCMV reactivation, pointing to a previously unrecognized extracellular role of GrM in controlling HCMV infection.

2. Methods

2.1. Patient and transplantation characteristics

Forty patients receiving allogeneic SCT were prospectively followed up for 12 weeks after SCT and the characteristics have previously been summarized [21]. For this study, we used a selection of 31 of these 40 patients based on the

availability of patient material (Table 1), which were weekly analyzed for HCMV-reactivation and lymphocyte subsets. The underlying hematological disease consisted for the majority of acute lymphatic leukemia, acute myeloid leukemia, multiple myeloma and non-Hodgkin's lymphoma. Patients received an allogeneic SCT from either a related ($n = 9$) or an unrelated ($n = 22$) donor. The stem cell source was mostly peripheral blood, and for most patients a nonmyeloablative conditioning regimen was used. *In vivo* T-cell depletion consisting of ATG was added to the conditioning regimen for patients receiving grafts from unrelated or HLA-mismatched donors. Whole blood samples were routinely drawn weekly from all patients to determine HCMV DNA loads. Plasma was removed for HCMV determination. Whole blood was used to determine absolute CD4⁺ and CD8⁺ T-cell counts and the leftover blood was used to isolate peripheral blood mononuclear cells (PBMCs). PBMCs stored in liquid nitrogen were used for this study. In total, 14 of 31 patients had evidence of a HCMV reactivation. Of those patients, 6 had a minor reactivation (denoted as +; peak viral load between 50 and 1000 copies/mL) and 8 had a major reactivation (denoted as ++; peak viral load > 1000 copies/mL). Written informed consent was obtained from all patients, in accordance with the Declaration of Helsinki (Medical ethical committee of the UMCU no. 05174).

2.2. HCMV status and monitoring

HCMV monitoring was based on a real-time TaqMan HCMV DNA PCR assay in ethylenediaminetetraacetic acid (EDTA)-treated plasma, which was prospectively performed weekly for all patients until 4 months after transplantation. Patients were treated preemptively with valganciclovir (900 mg twice

Table 1 Patient characteristics.

Characteristic	All	HCMV reactivation
No of patients	31	14 (45.2)
Sex		
Male	17	8 (47.1)
Female	14	6 (42.9)
Median age, years (range)	51.6 (21.6–65.9)	54.4 (23.8–65.9)
Stem cell source		
Cord blood	3	2 (33.3)
Peripheral blood	27	11 (40.7)
Bone marrow	1	1 (100)
Donor		
Related	9	3 (33.3)
Unrelated	22	11 (50.0)
HLA mismatch		
Yes	8	5 (62.5)
No	23	9 (39.1)
HCMV serological status (R/D)		
R ⁺ /D ⁺	10	8 (80.0)
R ⁺ /D [−]	12	5 (41.7)
R [−] /D ⁺	2	1 (50.0)
R [−] /D [−]	7	0 (0)

Note. Data are no. (%) of patients, unless otherwise indicated. HCMV, human cytomegalovirus; R/D, recipient/donor.

daily) when the HCMV DNA load exceeded 500 copies/mL. Valaciclovir was given to all patients prophylactically (500 mg twice daily). Viral reactivation and/or infection was defined as an HCMV load exceeding the detection limit of 50 copies/mL in plasma [21]. Because no plasma or serum was available from healthy controls, HCMV status was determined by analysis of the presence of a specific T-cell response by IFN- γ Elispot assays and 12 day expansion assays. IFN- γ Elispot assays were performed as previously described [22]. Twelve day expansion assays were performed as previously described [23], except cells were stimulated with HCMV peptide pools.

2.3. GrM measurements and absolute T-cell count determination

Frozen PBMCs were thawed in RPMI supplemented with 20% fetal calf serum (FCS) and pen-strep, washed in RPMI supplemented with 10% FCS and pen-strep, counted, and washed in PBS supplemented with 0.5% bovine serum albumin (BSA) and 0.1% sodiumazide. 1×10^6 cells were used for flowcytometric assessment of GrM and GrB expression within different lymphocyte subsets. Cells were incubated with one of three cell-surface marker combinations to determine GrM and GrB protein levels in different cell subsets; 1. CD3-PerCP, CD56-APC (Biolegend), CD8-AmCyan (V500) and CD16-Pacific Blue (BD); 2. CD3-PerCP, CD8-V500, CD27-PE-Cy7 (eF780) (eBioscience) and CD45RO-APC-Cy7 (BD); 3. CD3-Pacific Blue (eF450) (eBioscience), CD8-V500, TCR $\gamma\delta$ -APC (BD) and TCR V δ 2-PerCP (Biolegend). Cells were subsequently permeabilized and lysed for intracellular granzyme staining. After permeabilization, cells were incubated with Alexa Fluor 488-conjugated anti-GrM (clone 4B2G4), generated as described previously [24], and GrB-PE (Sanquin). Samples were measured on an LSR II FACS machine and analyzed using FACSdiva software. In almost all samples at least 200,000 events were acquired. Intracellular perforin staining was performed as previously described [21]. Absolute T-cell numbers per milliliter of whole blood were determined as previously described [21]. GrM plasma levels were measured by enzyme-linked immunosorbent assay according to manufacturers' instructions (USCN Life Science Inc.).

2.4. Statistical analysis

Median percentages of granzyme-positive cells were determined and all data were considered non-Gaussian distributed. The Mann–Whitney U test was used to compare granzyme protein levels between individual cell subsets. Spearman was used to calculate correlations. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of granzyme protein levels in lymphocyte subsets

To assess the role of HCMV latency and reactivation on GrM and GrB protein levels in different lymphocyte populations, we identified $\alpha\beta$ T cells, $\gamma\delta$ T cells and NKT cells within the

CD3⁺ lymphocyte population by flow cytometry. $\alpha\beta$ T cells were first divided into CD4⁺ (CD3⁺CD8⁻) and CD8⁺ (CD3⁺CD8⁺) T cells, and further subdivided into the naive (T_N) (CD27⁺CD45RO⁻), effector (T_{EFF}) (CD27⁻CD45RO⁻), effector memory (T_{EM}) (CD27⁺CD45RO⁺), and central memory (T_{CM}) (CD27⁻CD45RO⁺) T cell phenotypic subsets (see Supplementary Fig. 1A). $\gamma\delta$ T cell subsets (CD3⁺V δ 2⁻ and CD3⁺V δ 2⁺) and NKT cells (CD3⁺CD8⁺CD56⁺) were also identified (see Supplementary Figs. 1B–C). Within the CD3⁻ lymphocyte population, NK cells were defined as CD3⁻CD16⁺CD56⁺ (see Supplementary Fig. 1B). In each lymphocyte subset, intracellular GrM and GrB protein levels were analyzed (see Supplementary Figs. 1D–F).

3.2. Higher percentages of GrM- and GrB-positive effector memory CD4⁺ T-cells in HCMV latently-infected healthy individuals

To examine the long-term effects of HCMV infection on GrM and GrB levels in lymphocytes, we assessed granzyme protein levels in lymphocyte populations of 31 HCMV latently-infected healthy individuals and 7 uninfected healthy individuals. No significant differences in the percentage of GrM- and GrB-positive cells could be observed within total CD4⁺ (Fig. 1A) and total CD8⁺ (Fig. 1B) T-cell populations between both groups. When phenotypic subsets within the CD4⁺ and CD8⁺ T-cell compartments were analyzed, however, we observed significantly higher percentages of GrM- and GrB-positive CD4⁺ T_{EM} -cells (Fig. 1A). Percentages of granzyme-positive cells within CD8⁺ T-cell subsets did not differ between latently-infected and uninfected individuals (Fig. 1B), nor were there any differences observed within the NK and NKT-cell populations (Fig. 1C), or $\gamma\delta$ T-cell subsets (Fig. 1D). These data point to a protective role of GrM- and GrB-positive CD4⁺ T_{EM} cells during HCMV latency.

3.3. Higher percentages of granzyme-positive CD4⁺ T, CD8⁺ T, NKT, and $\gamma\delta$ T-cells in SCT patients

GrM and GrB protein levels were measured weekly in lymphocytes of SCT patients after transplantation ($n = 31$) and median percentages of granzyme-positive lymphocytes throughout a 12 week follow-up period were determined in order to avoid fluctuations based on variable onset of reactivation and T-cell reconstitution. Percentages of both GrM- and GrB-positive cells were higher within the CD4⁺ T-cell (Fig. 2A), CD8⁺ T-cell (Fig. 2B) and $\gamma\delta$ T-cell (Fig. 2E) populations of SCT patients compared to healthy individuals. Within the NKT-cell population, only the percentage of GrM-positive, but not GrB-positive, cells was higher in SCT patients compared to healthy individuals (Fig. 2D). No differences were observed within the NK cell population between SCT patients and healthy individuals (Fig. 2C). In all lymphocyte populations, there was a significant correlation between the percentages of GrM- and GrB-positive lymphocytes. These data indicate that shortly after SCT transplantation percentages of granzyme-positive cells within the CD4⁺ T-cell, CD8⁺ T-cell, NKT-cell, and $\gamma\delta$ T-cell compartments are increased.

Figure 1 GrM and GrB expression in lymphocytes during HCMV latency. Median percentages of GrM- and GrB-positive cells were determined in lymphocyte subsets of HCMV latently-infected ($n = 31$, +) and uninfected ($n = 7$, -) healthy individuals. (A) Median percentages of GrM- and GrB-positive T-cells in total CD4⁺ and CD8⁺ T-cells, (B) CD4⁺ and CD8⁺ T-cell phenotypic subsets, (C) NK and NKT-cells, and (D) $\gamma\delta$ T-cell subsets. In box plots, the horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers above and below the box mark the range. (T_{CM}, central memory T-cells; T_{EM}, effector memory T-cells; T_{EFF}, effector T-cells; T_N, naive T-cells) * $p < 0.05$, ** $p < 0.01$.

3.4. GrM- and GrB-positive lymphocytes are associated with HCMV reactivation

To determine whether GrM and GrB are associated with HCMV reactivation, median numbers of total cells and granzyme-positive cells per lymphocyte subset throughout follow-up were determined and correlated to HCMV reactivation. Absolute numbers of CD4⁺ T-cells were significantly lower in SCT patients

with minor (+) and major (++) HCMV reactivation compared to patients with no reactivation (-) (Fig. 3A), and as a result there was a significant lower number of total GrM-positive CD4⁺ T-cells (Fig. 3B). We further analyzed differences in the percentages of granzyme-expressing lymphocytes throughout follow-up and correlated this to minor or major HCMV reactivation (Fig. 3C). Interestingly, the percentage of GrM-positive CD4⁺ T-cells was significantly higher in patients with

Figure 2 Correlation between GrM and GrB expression in lymphocyte subsets of healthy individuals and SCT patients. GrM and GrB expression was analyzed in lymphocyte subsets of both healthy donors ($n = 38$) and SCT patients ($n = 31$) throughout 12 week follow-up. Mean percentages of granzyme-positive cells are depicted in both box plots and scatter plots. In scatter plots, the median percentages of GrM-positive cells were plotted against the median percentages of GrB-positive cells for (A) CD4⁺ T-cells (healthy donors, $r = 0.6884$, $p < 0.0001$; SCT patients, $r = 0.8725$, $p < 0.0001$), (B) CD8⁺ T-cells (healthy donors, $r = 0.7255$, $p < 0.0001$; SCT patients, $r = 0.6668$, $p < 0.0001$), (C) NK cells (healthy donors, $r = 0.3734$, $p = 0.0209$; SCT patients, $r = 0.3180$, $p = 0.0813$), (D) NKT-cells (healthy donors, $r = 0.6115$, $p < 0.0001$; SCT patients, $r = 0.07210$, $p = 0.7050$), and (E) γδT-cells (healthy donors, $r = 0.6921$, $p < 0.0001$; SCT patients, $r = 0.7048$, $p < 0.0001$). In box plots, the horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers above and below the box mark the range. ** $p < 0.01$, *** $p < 0.001$.

major HCMV reactivation compared to patients without reactivation. There were no differences in the percentage of GrM-positive CD8⁺ T-cells between patients with or without HCMV reactivation. Unlike GrM, the percentage of GrB-positive CD8⁺ T-cells was significantly higher in SCT

patients with major HCMV reactivation compared to patients without reactivation. Analysis of the T-cell phenotypic subsets showed that the higher percentage of GrM-positive CD4⁺ T-cells in SCT patients with major HCMV reactivation is mostly reflected in the central memory CD4⁺

Figure 3 Association between granzyme expression in lymphocyte subsets and HCMV reactivations in SCT patients. In total, 14 of 31 patients had evidence of a HCMV reactivation. Of those patients, 6 had a minor reactivation (peak viral load between 50 and 1000 copies/mL) and 8 had a major reactivation (peak viral load > 1000 copies/mL). GrM and GrB expression was analyzed in lymphocyte subsets of SCT patients that experienced major ($n = 8$, ++), minor ($n = 6$, +), or no ($n = 17$, -) HCMV reactivation throughout 12 week follow-up period. (A) Median absolute numbers of total CD4⁺ and CD8⁺ T-cells or (B) GrM- and GrB-positive CD4⁺ and CD8⁺ T-cells. (C) Median percentages of GrM- and GrB-positive cells within the total CD4⁺ or CD8⁺ T-cell population. (D) Median percentages of GrM- and GrB-positive cells within CD4⁺ T-cell phenotypic subsets. (E) Median percentages of GrM- and GrB-positive cells within CD8⁺ T-cell phenotypic subsets. (F) Median percentages of NK cells within the total CD3⁺ lymphocyte population, and NKT and $\gamma\delta$ T-cells within the total CD3⁺ lymphocyte population. (G) Median percentages of GrM- and GrB-positive cells within the total NK, NKT and $\gamma\delta$ T-cell population. In box plots, the horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers above and below the box mark the range. (T_{CM}, central memory T-cells; T_{EM}, effector memory T-cells; T_{EFF}, effector T-cells; T_N, naive T-cells) * $p < 0.05$, ** $p < 0.01$.

T-cell pool (Fig. 3D). In addition, percentages of GrB-expressing central and CD4⁺ T_{EM} cells, and CD8⁺ T_{EM} cells were higher in SCT patients with major HCMV reactivation (Figs. 3D,E). Analysis of NK, NKT and $\gamma\delta$ T-cells showed no significant differences in the percentages of total cells (Fig. 3F), but the percentage of GrM-positive NK cells was lower in SCT patients with major HCMV reactivation compared to patients without reactivation (Fig. 3G). Altogether, these data show that not the absolute numbers but rather the percentages of granzyme-positive cells of several lymphocyte populations are associated with HCMV reactivation. Whereas GrB associates with CD4⁺ and CD8⁺

T-cell responses, GrM associates with CD4⁺ T-cells and NK cell responses towards HCMV reactivation.

3.5. Distinct CD4⁺ and CD8⁺ T-cell responses in SCT patients with HCMV reactivation

In our previous analysis, we assessed the median total numbers or percentages of granzyme-positive lymphocytes throughout the 12 week follow-up period. However, when we analyzed patients on an individual basis, distinct immune response patterns could be observed. Fig. 4 shows representative

graphs of individual patients. On the one hand, an increase in GrM- and GrB-positive CD4⁺ and CD8⁺ T-cells coincided with an increase in HCMV viral load (Fig. 4A) in 8 of the 14 reactivating patients. This could indicate that the increase in granzyme-positive CD4⁺ and CD8⁺ T-cells leads to control of HCMV reactivation. On the other hand, granzyme-positive CD4⁺ and CD8⁺ T-cells were inversely correlated to HCMV viral load in 4 other reactivating patients, characterized by decreased percentages of GrM- and GrB-positive CD4⁺ and CD8⁺ T-cells when HCMV viral load is increasing (Fig. 4B). This may suggest that either HCMV reactivation leads to suppression of T-cell responses or that a decrease in T-cell responses causes HCMV reactivation. As HCMV DNA load decreases later on when GrM- and GrB-positive T-cells increase, the latter may be more likely. Notably, the kinetics of GrM-expressing T lymphocytes coincided with the kinetics of GrB-positive, and to a lesser extent, perforin-expressing T lymphocytes. These data demonstrate the diversity and complexity of human T-cell responses during HCMV reactivation in SCT patients.

3.6. GrM but not GrB correlates with perforin expression in CD8⁺ T-cells in SCT patients

Antiviral functions of granzymes can be both dependent and independent of perforin [8]. Recently, we have demonstrated

that perforin expression in CD8⁺ T-cells correlates with HCMV reactivation in SCT patients [21]. Here, we showed that granzyme-positive CD8⁺ T-cells also associated with HCMV reactivation (Fig. 2). Therefore, we analyzed the correlation between granzyme-positive and perforin-positive CD8⁺ T-cells. There was no significant correlation between the percentages of GrM-positive CD8⁺ T-cells and the percentage of perforin-positive CD8⁺ T-cells when all SCT patients were analyzed (Fig. 5A). However, when SCT recipients were divided into patients with or without HCMV reactivation, there was a positive correlation between the percentage of GrM-positive and perforin-positive CD8⁺ T-cells in SCT patients without HCMV reactivation and a negative correlation in patients with reactivation. There were no significant correlations between the percentages of GrB- and perforin-positive CD8⁺ T-cells in SCT patients (Fig. 5B). These data suggest that GrM released by CD8⁺ T-cells during HCMV reactivation may predominantly exert its antiviral effects in a perforin-independent manner.

3.7. Plasma GrM is elevated during HCMV reactivation

If GrM has perforin-independent extracellular functions, one would expect GrM levels in plasma to be elevated. Therefore, we measured plasma GrM in three individual

Figure 4 Longitudinal analysis of granzyme expression in lymphocytes of individual SCT patients with HCMV reactivation. GrM and GrB expression was analyzed in CD4⁺ and CD8⁺ T-cells of SCT patients with HCMV reactivation ($n = 14$) throughout 12 week follow-up period. Perforin expression in CD8⁺ T-cells was analyzed previously [21]. Of note, perforin expression was not measured in CD4⁺ T-cells. (A) Representative graphs of a SCT patient with increased percentages of granzyme-expressing T-cells that coincided with an increase in HCMV load. (B) Representative graphs of a SCT patient with decreased percentages of granzyme-expressing T-cells that coincided with an increase in HCMV load.

SCT patients with HCMV reactivation (Fig. 6). Interestingly, the plasma GrM peak coincided with plasma HCMV load (Fig. 6). This suggests that either the intracellular GrM pool is released from these T-cells making them less positive for intracellular GrM and/or that GrM is secreted by NK(T) cells, $\gamma\delta$ T-cells, or other cell types. These data indicate that GrM plasma levels are elevated during HCMV reactivation in SCT patients and may point to an extracellular antiviral function of GrM.

4. Discussion

HCMV infection leaves a fingerprint in the total T-cell pool characterized by high numbers of GrB-expressing CD4⁺ and CD8⁺ T_{EM} cells during latency in HCMV-seropositive healthy individuals [16,25,26]. In this study, we confirmed the higher numbers of GrB-positive CD4⁺ T_{EM} cells during HCMV latency (Fig. 1) [16]. In addition, we showed for the first time that the percentage of GrM-positive CD4⁺ T_{EM} cells was higher in HCMV latently-infected compared to uninfected healthy individuals (Fig. 1). Thus, our data indicate that primary HCMV infection and/or latency increases the frequency of

both circulating GrM- and GrB-positive CD4⁺ T-cells. The importance of CD4⁺ T-cell responses during HCMV latency is supported by several studies. HCMV latently-infected cells recruit and counteract CD4⁺ T-cell responses through expression of the viral IL-10-encoding gene *UL111A* and other immunosuppressive factors [27,28]. During latency, dominant HCMV-specific GrB-expressing CD4⁺ T-cell clones emerge that are poorly represented in the acute phase of HCMV infections and have immediate cytotoxic capacity towards HCMV antigen-loaded target cells [16,17,29]. Also, it has been shown that young children have impaired HCMV-specific CD4⁺ T-cell responses, predominantly reduced HCMV-specific CD4⁺ T_{EM} cell responses, but normal HCMV-specific CD8⁺ T-cell responses, which coincided with persistent HCMV replication [30]. Finally, HCMV-specific IFN- γ -producing CD4⁺ T-cells were shown to contribute to protection from HCMV disease in HIV-infected patients [31]. In this perspective, circulating granzyme-expressing (effector memory) CD4⁺ T-cells may contribute to the maintenance of HCMV latency.

Reactivation of HCMV from latency can cause serious disease in immunocompromised patients, for instance following allogeneic SCT [3,4]. We showed that HCMV reactivation in

Figure 5 Correlation between granzyme and perforin expression in CD8⁺ T-cells of SCT patients. GrM and GrB expression was analyzed in CD8⁺ T-cells of all SCT patients ($n = 31$), SCT patients without HCMV reactivation ($n = 17$), or patients with HCMV reactivation ($n = 14$) throughout 12 week follow-up period. Perforin expression was analyzed previously [21]. (A) The median percentages of GrM-positive CD8⁺ T-cells were plotted against the median percentages of perforin-positive CD8⁺ T-cells (All patients, $r = 0.2119$, $p = 0.2525$; No reactivation, $r = 0.5129$, $p = 0.0353$, HCMV reactivation, $r = -0.6057$, $p = 0.0217$). (B) The median percentages of GrB-positive CD8⁺ T-cells were plotted against the median percentages of perforin-positive CD8⁺ T-cells (All patients, $r = 0.2454$, $p = 0.1833$; No reactivation, $r = 0.2589$, $p = 0.3157$, HCMV reactivation, $r = -0.1980$, $p = 0.4974$).

Figure 6 Plasma GrM is elevated during HCMV reactivation. (A–C) Three representative SCT patients with HCMV reactivation throughout 12 week follow-up period were measured for intracellular GrM expression in CD4⁺ and CD8⁺ T-cells, perforin expression in CD8⁺ T-cells, HCMV load, and plasma levels of GrM (pg/mL).

SCT patients associated with lower numbers of CD4⁺ and CD8⁺ T-cells (Fig. 3), confirming that T-cell responses are important in preventing HCMV reactivation [1]. Interestingly, percentages of granzyme-positive T-cells were associated with HCMV reactivation. Although absolute numbers of both CD4⁺ and CD8⁺ T-cells were markedly lower in SCT patients with HCMV reactivation, the percentage of GrM-positive (total and central memory) CD4⁺ T-cells was higher (Fig. 3). Our observation that GrM-expressing central memory CD4⁺ T-cells are associated with HCMV reactivation is remarkable, since it is believed that these cells have no effector functions [17,32,33]. Even though there are no noteworthy differences in the distribution of CD4⁺ T-cell phenotypic subsets (Fig. 3), it could indicate antigen-driven stimulation and differentiation of central memory CD4⁺ T-cells into effector memory and/or effector CD4⁺ T-cells in response to major HCMV reactivation. Alternatively, GrM may play an extracellular perforin-independent immuno-regulatory role following cellular secretion. This is consistent with our observations that there was a negative correlation in patients with detectable HCMV reactivation between GrM- and perforin-positive CD8⁺ T-cells (Fig. 5), and that GrM levels in plasma were elevated during HCMV reactivation in 3 out of 3 patients that were measured (Fig. 6). Higher percentages of GrB-positive CD4⁺ and CD8⁺ T-cells in SCT patients with HCMV reactivation (Fig. 3) is in agreement with previous studies that identified the emergence of GrB-positive HCMV-specific CD4⁺ and CD8⁺ T_{EM} cells after primary HCMV infection in renal transplant recipients [15,16,19]. Interestingly, percentages of GrM-positive NK cells

were lower in SCT patients with HCMV reactivation (Fig. 3). Altogether, these associations could point to a two-step model in which 1) HCMV reactivation is triggered by inadequate NK, CD4⁺ and CD8⁺ T-cell responses in the initial phase, and 2) secondary T-cell responses with increased granzyme-positive CD4⁺ and CD8⁺ T-cells exert antiviral activities to control HCMV infection. However, longitudinal analysis of individual SCT patients showed that T-cell responses greatly differ between patients (Fig. 4), which is most likely the result of various variable transplantation-related factors. This complicates the establishment of a general model for initiation and immune-regulated control of HCMV reactivation in SCT patients and emphasizes the complexity of the interplay between the host immune response and virus.

We showed differences in the median percentages of granzyme-expressing cells within the total CD4⁺ and CD8⁺ T-cell population as well as in the phenotypic subsets between SCT patients with and without HCMV reactivation. Since we analyzed total T-cell populations, differences could be more pronounced within the HCMV-specific T-cell compartment. Unfortunately, analysis of HCMV-specific T-cells in large patient cohorts is complicated by, among others, the high variety of HLA-specific HCMV epitopes between individuals [5–7]. Studying immune responses during HCMV reactivation in SCT patients provides valuable information to our understanding how the immune system controls HCMV infections and might lead to new antiviral therapies. Cellular immuno-therapy is a promising approach to treat HCMV reactivation in

SCT patients [34]. Adoptive transfer of donor-derived HCMV-specific T-cells has already been proven to be a safe and effective treatment for HCMV infection [35–40]. It would be worthwhile to investigate whether increasing granzyme protein levels within these HCMV-specific T-cells could provide improved protection to HCMV reactivation.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2013.11.005>.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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