Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans

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Human cytomegalovirus (CMV) establishes persistent, usually asymptomatic, infection in healthy people. Because CMV infection is associated with the presence of lower proportions of peripheral naïve CD8+ T-cells and a higher fraction of late-differentiated CD8+ cells, commonly taken as biomarkers of age-associated compromised adaptive immunity (‘immunosenescence’), we asked whether chronic exposure to any persistent virus mediates these effects. Herpes simplex virus (HSV) is also a widespread herpesvirus that establishes lifelong persistence, but, unlike CMV, its impact on the distribution of T-cell subsets has not been established. Here, we analysed T-cell subsets in 93 healthy people aged 42–81 years infected or not infected with CMV and/or HSV. Individuals harbouring CMV were confirmed to possess lower frequencies of naïve CD8+ T-cells (defined as CD45RA+CCR7+CD27+CD28+) and greater proportions of late-differentiated effector memory (CD45RA−CCR7−CD27−CD28−) and so-called TEMRA (CD45RA−CCR7−CD27−CD28−) CD4 and CD8 subsets, independent of HSV seropositivity. In CMV-seronegative donors, HSV did not affect T-cell subset distribution significantly. We conclude that these hallmarks of age-associated alterations to immune signatures are indeed observed in the general population in people infected with CMV and not those infected with a different persistent herpesvirus.

INTRODUCTION

One common characteristic of infection with herpesviruses is their lifelong persistence in a latent state after primary infection of the host, and their capacity for periodic reactivation. Thus, the host immune system is repeatedly called upon to respond to and control infection. In the case of human cytomegalovirus (CMV), a high proportion of both CD4+ and CD8+ T-cells in the peripheral blood of asymptomatic infected individuals respond specifically to different antigens derived from this single virus (Sylwester et al., 2005). These findings illustrate the lifelong challenge faced by the immune system of an infected individual to keep this virus in check. As a result, the composition of the different T-cell subsets in infected individuals is tilted towards a lower frequency of naïve T-cells and accumulation of memory T-cells with a late-differentiated phenotype (Pawelec et al., 2009), commonly referred to in the literature as ‘senescent’. Based on findings from longitudinal studies in the very elderly and the impact of CMV on immune signatures, this virus has been suggested to be an important factor accelerating immunosenescence in infected individuals (Derhovanessian et al., 2009). Although little direct evidence is available, a reduced frequency of naïve T-cells and accumulations of ‘senescent’ T-cells filling the immunological space are assumed to be one reason behind the increased susceptibility of the elderly to not only novel, but also recurring infections. However, as not every elderly person is CMV-seropositive, it has also been hypothesized that other persistent sources of antigen,
e.g. Epstein–Barr virus (EBV), varicella-zoster virus (VZV) or herpes simplex virus (HSV), may contribute to a similar phenomenon in the elderly.

Similarly to CMV, clonal expansion of CD8+ T-cells carrying receptors for epitopes derived from another, even more frequent herpesvirus, EBV, has also been demonstrated in several studies, although at lower frequencies than CMV-specific T-cells (Colonna-Romano et al., 2007; Ouyang et al., 2003; Vescovini et al., 2004). These studies also showed that peripheral EBV-specific T-cells have a less-differentiated phenotype (Appay et al., 2002; Colonna-Romano et al., 2007; Vescovini et al., 2004). Thus, EBV seems to exert a similar, although lesser, effect on immune signatures to CMV. Accordingly, EBV seropositivity was not part of the ‘immune risk profile’ (IRP); a cluster of simple immune parameters associated with remaining survival time in the very elderly; Pawelec et al., 2009), whereas CMV seropositivity was one of the factors included in the IRP.

HSV is another member of the family Herpesviridae with a widespread distribution. The rate of infection differs for HSV types 1 (HSV-1) and 2 (HSV-2). HSV-1 has a 60–80% seroprevalence worldwide in the adult population, comparable to that of CMV, whereas HSV-2 seroprevalence varies markedly from country to country, from as low as 7% to as high as 80% (Smith & Robinson, 2002). Primary infection is followed by lifelong latency of the virus in trigeminal (HSV-1) and sacral (HSV-2) ganglions, respectively. Reactivation occurs spontaneously and is not always accompanied by clinical manifestation (Mark et al., 2008). Thus, intermittent exposure of the host immune system to the viral antigens and the necessity for long-term immunosurveillance is also required to contain this virus. Studies from murine models and the increased severity and persistence of recurrent herpes in patients with acquired immunodeficiency syndrome (AIDS) reflects a key role of T-cells, especially CD4+ T-cells, in maintaining latency (Cunningham et al., 2006). To the best of our knowledge, the impact of HSV infection on the distribution of different peripheral T-cell subsets has not been investigated in humans. In HSV-infected mice, a recent study demonstrated the accumulation of late-differentiated memory CD8+ T-cells (lacking CD62L, CD127 and CD27), similar to that seen for CMV (Lang et al., 2009), suggesting that HSV may be similar to CMV in driving the T-cell compartment towards a more late-differentiated phenotype. Whether this is also true in humans is not yet known. Another open question is whether infection with multiple agents has additive effects on immune signatures. To address these questions, we have analysed the seroprevalence of HSV, EBV and VZV, as well as that of CMV, in a representative population of middle-aged Caucasian donors. We have shown previously in the same population that infection with CMV is associated with a marked reduction of peripheral naïve T-cells and accumulation of late-differentiated CD8+ T-cells (Derhovanessian et al., 2010). The essentially ubiquitous presence of EBV (98%) and VZV (99%) in this cohort did not allow us to determine their effects on immune signatures, but we report here that infection with HSV did not have any impact on the distribution of the different CD4+ and CD8+ T-cell subsets, whether or not there was co-infection with CMV.

RESULTS

Seroprevalence of herpesviruses in the population analysed

Table 1 shows the seroprevalence of CMV and HSV infection according to gender and age of the study cohort. Seropositivity for CMV was detected in 53% of the subjects, with no difference according to gender and no significant increase with age, although the highest proportion of infected individuals was found in the third quartile, but this then decreased again in the fourth quartile. HSV infection was detected in 69% of the subjects, with slightly fewer men than women infected. Unlike CMV, the seroprevalence of HSV did increase with age from 42 to 81 years (for trend, P=0.02; Table 1). This result remained statistically significant after adjusting for gender (P=0.03). Latent infection with EBV and VZV was detected in 98 and 100% of subjects, respectively.

More than one-third (38%) of subjects were co-infected with CMV and HSV. The prevalence of double or single infections with these viruses did not differ between men and women. There was an age-dependent decline in the percentage of individuals not infected with either of the viruses (P=0.04; Table 1). However, the infection rate with one or both of the viruses was quite constant across all age groups.

HSV infection and the distribution of different CD8+ T-cell subsets in CMV-seropositive and -seronegative subjects

Next, we performed detailed immune phenotyping of the 100 subjects using polychromatic flow cytometry. Data from 93 subjects were evaluable. Considering the large impact of CMV on the constellation of different T-cell subsets and in order to distinguish the effect of HSV from that of CMV, the individuals were grouped according to their CMV and HSV serostatus and the frequencies of different T-cell subsets were compared. The demographic characteristics of the four groups are shown in Table 2.

In the CD8+ T-cell population, a significantly lower percentage of naive (CD45RA+CCR7+CD27+CD28+) central memory (CD45RA-CCR7+CD27+CD28+) and effector memory 1 (CD45RA-CCR7-CD27+CD28+) subsets was observed in CMV-seropositive subjects (Fig. 1a, upper panel). However, the percentage of these subsets carrying both CD27 and CD28 costimulatory receptors did not differ between HSV-seronegative and -seropositive individuals (Fig. 1a, upper panel). Reciprocally, there was a
significant accumulation of late-differentiated T-cell subsets lacking CD27 and CD28 in individuals infected with CMV (Fig. 1a, lower panel). In contrast, a latent infection with HSV did not have any impact on the percentage of effector memory 3 (CD45RA-CCR7-CD27-CD28+) or effector (CD45RA+CCR7-CD27-CD28+) subsets. Due to the great inter-individual variation, we determined the impact of CMV and/or HSV infection at an individual level and calculated the ratio between the percentage of naïve and late-differentiated effector subsets for each individual [naïve/effector (N/E) ratio]. In line with our findings at the population level, CMV-infected subjects had significantly lower N/E ratios than CMV-seronegative subjects. This was independent of HSV infection, which had no significant impact on this parameter, although the inter-individual variability was higher in the HSV-seropositive group (Fig. 1b). Finally, we determined the level of expression of the differentiation markers CD57 and KLRG-1, known to be upregulated on very late-differentiated T-cells, and often referred to in the literature as markers of ‘senescence’. There was a highly significant accumulation of T-cells carrying these receptors in CMV-seropositive individuals, whereas a latent infection with HSV did not influence their expression (Fig. 1c). A similar trend was observed when absolute numbers rather than percentages of cells belonging to the different subsets were analysed (Fig. 2). Although the frequency of early-differentiated subsets carrying both CD27 and CD28 did not differ between CMV-seropositive and -seronegative individuals (Fig. 2a, upper panel), a latent infection with CMV, but not HSV, was associated with higher numbers of late-differentiated effector memory and effector subsets (Fig. 2a, lower panel) as well as T-cells carrying CD57 and KLRG-1 (Fig. 2b).

**HSV infection and the distribution of different CD4+ T-cell subsets in CMV-seropositive and -seronegative subjects**

In the CD4+ population, a latent infection with CMV or HSV did not have any impact on the percentage or absolute numbers of naïve, central memory and effector memory T-cells double-positive for CD27 and CD28 (data not shown). However, in CMV-seropositive subjects, CD45RA-CCR7-CD27-CD28- and CD45RA+CCR7-CD27-CD28- cells were present within the CD4+ subset, which were absent in CMV-seronegative individuals (Fig. 3a, b). A latent infection with HSV did not have any impact on the percentage or number of these subsets (Figs 3b and 4a). When the N/E ratio for CD4+ T-cells was calculated, a highly significant difference was documented between CMV-seropositive and -seronegative individuals, but again independently of HSV infection status (Fig. 3c). Similar to the CD8+ population, infection with CMV, but not HSV, was associated with accumulation of T-cells carrying CD57, whereas the expression level of KLRG-1 was not affected by CMV or HSV (Figs 3d and 4b).

**Correlation analysis between IgG titres and the distribution of different T-cell subsets**

Next, we sought to determine whether the levels of IgG antibodies against CMV, HSV and EBV correlated with the distribution of T-cell subsets analysed above. In the case of CMV, we observed a statistically significant difference between the percentage and absolute number of late-differentiated effector memory and effector subsets, as well as in the level of CD57 expression in the CD4 compartment in seropositive individuals with higher-than-median titres of

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**Table 1. Prevalence (%) of latent infection with CMV and HSV**

Individuals were grouped according to gender and increasing age. Data from 100 individuals (for CMV) and 93 individuals (for HSV) are summarized.

<table>
<thead>
<tr>
<th>Serostatus</th>
<th>All Gender</th>
<th>1st (42–55 years)</th>
<th>2nd (56–61 years)</th>
<th>3rd (62–66 years)</th>
<th>4th (67–81 years)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=100</td>
<td>n=61 Male</td>
<td>n=39 Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV+</td>
<td>53.0</td>
<td>53.8</td>
<td>52.4</td>
<td>0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV+</td>
<td>69.4</td>
<td>61.5</td>
<td>72.9</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV+HSV-</td>
<td>16.3</td>
<td>20.5</td>
<td>13.5</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV+HSV+</td>
<td>14.3</td>
<td>15.4</td>
<td>13.5</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV-HSV+</td>
<td>31.6</td>
<td>25.6</td>
<td>35.6</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV+HSV+</td>
<td>37.8</td>
<td>38.5</td>
<td>37.3</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV+</td>
<td>48.0</td>
<td>48.0</td>
<td>64.0</td>
<td>52.0</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>HSV+</td>
<td>62.5</td>
<td>56.0</td>
<td>70.8</td>
<td>88.0</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>CMV+HSV-</td>
<td>20.8</td>
<td>28.0</td>
<td>12.5</td>
<td>4.0</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>CMV+HSV+</td>
<td>16.7</td>
<td>16.0</td>
<td>16.7</td>
<td>8.0</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>CMV+</td>
<td>33.3</td>
<td>24.0</td>
<td>25.0</td>
<td>44.0</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>HSV+</td>
<td>29.2</td>
<td>32.0</td>
<td>45.8</td>
<td>44.0</td>
<td>0.18</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 2. Characteristics of the subjects tested for different T-cell subsets**

<table>
<thead>
<tr>
<th>CMV+HSV- (n=15)</th>
<th>CMV+HSV+ (n=31)</th>
<th>CMV+HSV- (n=13)</th>
<th>CMV+HSV+ (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, n (%)</td>
<td>8 (53.3)</td>
<td>21 (67.7)</td>
<td>7 (53.8)</td>
</tr>
<tr>
<td>Age (years), mean (sd)</td>
<td>58 (5.9)</td>
<td>62 (8.9)</td>
<td>60 (8.8)</td>
</tr>
</tbody>
</table>
Fig. 1. Impact of CMV and HSV infection on the distribution of CD8+ T-cell subsets. Different naïve and memory subsets were characterized according to surface expression of CD45RA, CCR7, CD27 and CD28 following widely accepted models (Koch et al., 2008; Romero et al., 2007). Individuals were grouped according to latent infection with CMV and/or HSV into four groups. (a) Frequency from total CD8+ T cells of naïve (CD45RA+CCR7+CD27+CD28+), central memory (CD45RA+CCR7+CD27+CD28+) and effector memory 1 (EM1, CD45RA+CCR7+CD27+CD28+) subsets (upper panel) and late-differentiated effector memory 3 (EM3, CD45RA+CCR7−CD27−CD28−) and effector (E, CD45RA−CCR7−CD27−CD28−) subsets in individuals grouped according to latent infection with CMV and/or HSV. (b) For each individual, the ratio between naïve (N) and effector (E) cells was calculated and compared between different groups. (c) Frequency of CD8+ T-cells carrying the markers of late-differentiation CD57 or KLRG-1. CMV−HSV−, n=15; CMV+HSV+, n=31; CMV+HSV−, n=13; CMV+HSV+, n=34.
anti-CMV IgG compared with seropositive individuals with low antibody levels (Fig. 5a). This was not the case for CD8\(^+\) T-cells (Fig. 5b). We then grouped the subjects according to the presence or absence of CMV infection and the level of IgG antibodies against HSV and EBV. Unlike CMV, the level of anti-HSV antibody did not have any impact on the percentage or absolute number of late-differentiated CD4\(^+\) T-cells (Fig. 6a). The same was true when individuals were grouped according to anti-EBV IgG titres (Fig. 6b). There was no significant correlation between antibody titres for any of the three viruses and the distribution of early differentiated T-cells or the level of KRLG-1 expression in either the CD4 or CD8 subset (data not shown).

**DISCUSSION**

In this study we have analysed the impact of HSV infection on the distribution and absolute counts of different CD4\(^+\) and CD8\(^+\) T-cell subsets in peripheral blood of subjects of
the general population aged 42–81 years. Primary infection with HSV-1 usually occurs in childhood, commonly reaching 40% of the population by the age of 15 years, before increasing in a linear fashion with age to 60–80% of older adults in most populations (Smith & Robinson, 2002). HSV-2 prevalence, although greatly variable from
country to country, is also strongly associated with age, increasing from negligible levels in children younger than 12 years to as high as 80% in some populations (Smith & Robinson, 2002). In our cohort, there was also an increase in the prevalence of HSV, as well as a decrease in the percentage of individuals negative for both CMV and HSV, in older age groups.

Considering the strong impact of CMV on the distribution of different T-cell subsets (Derhovanessian et al., 2009), we analysed the association between HSV serostatus and these parameters in the presence or absence of an accompanying latent CMV infection. Our data demonstrate that, unlike CMV, a latent infection with HSV is not associated significantly with altered immune signatures in middle-aged to elderly individuals. One explanation for this could be related to the type of cell that serves as a reservoir for each of these viruses during latency. In the case of CMV, among others, these are probably the cells of the myeloid lineage, including monocytes and immature dendritic cells, allowing direct encounter of viral antigens with T-cells (Crough & Khanna, 2009). In contrast, latent HSV resides in neurons, which are less accessible to the immune system. However, repeated reactivation of the virus in immunosuppressed individuals, such as patients with AIDS (Siegal et al., 1981), and data from murine models (Cunningham et al., 2006), imply a role for the immune system in keeping the virus in a latent state in neurons. On the other hand, rapid clearance of the virus after reactivation in immunocompetent hosts (Mark et al., 2008), as well as ample evidence from studies in human herpetic lesions (Cunningham et al., 2006), suggests the involvement of both CD4+ and CD8+ T-cells in clearance of the virus from these lesions. Reactivation of HSV results in its transport to the original infecting dermatome and into the stratified squamous epithelium or skin (Cunningham et al., 2006). Skin-residing Langerhans cells, as well as monocyte-derived dendritic cells, express receptors for HSV and can thus activate CD8+ T-cell responses in lymph nodes upon primary infection or reactivation of the virus in the skin (Bosnjak et al., 2005). In the case of Langerhans cells, at least in murine models, this occurs through antigen transfer to CD8+ dendritic cells (Allan et al., 2003). Therefore, it is probable that HSV is not entirely hidden from the immune system of the human host and, like CMV, can stimulate T-cells repeatedly upon reactivation. Nonetheless, its effect on T-cell subset distribution in infected individuals is very different from that of CMV, at least in the cohort of 93 individuals studied here. Of course, it cannot be excluded that this difference is due to higher reactivation rates of CMV than HSV, although to the best of our knowledge there are no data supporting this interpretation. It also remains possible that analysis of HSV-1 or HSV-2 separately might show different results.

The apparently unique impact of CMV on the immune system was confirmed further when the distribution of different T-cell subsets was correlated with the level of antibodies in seropositive individuals. In this analysis, we were also able to explore the impact of EBV, which, similarly to CMV, also resides and proliferates in certain types of antigen-presenting cell. Despite this, unlike CMV,
there was no correlation between the titres of antibody against this virus and the accumulation of late-differentiated CD4+ T-cells. The same was true for HSV, although the number of individuals analysed was rather small in some groups. Higher levels of anti-CMV antibody might reflect a more frequent reactivation history of the virus, thus leading to accumulation of late-differentiated T-cells. It is surprising that this effect was limited to the CD4 T-cells and was not observed in the CD8 compartment. This might be explained by the helper role of the former in generating humoral responses. However, this does not seem to be a general rule, as this effect was only seen in the case of CMV and not two other persistent viruses analysed.

Although no significant impact of HSV infection on the distribution of T-cell subsets was observed in our study, there was a higher inter-individual variability, especially for CD4+ subsets in individuals co-infected with CMV and HSV. As almost all of the individuals were seropositive for EBV and VZV, we can exclude a simple co-infection with these persistent herpesviruses as the reason for this large variability. One explanation for this could be different rates
of reactivation of HSV and CMV, or superinfection with the latter, in different individuals, and thus different degrees of encounter of the immune system with them. Because reactivations can occur without any clinical symptoms (Crough & Khanna, 2009; Mark et al., 2008), this is very difficult to establish. CMV is reported to impair the immune response against EBV (Khan et al., 2004). The same could be true for HSV; thus, in individuals with a latent infection with CMV, reduced immunosurveillance against HSV might lead to more frequent reactivation of the virus, which may then

**Fig. 6.** Correlation analysis between IgG titres against HSV and EBV and the distribution and absolute numbers of late-differentiated CD4+ T-cell subsets. Individuals were grouped according to presence of absence of a latent CMV infection and the level of anti-IgG antibodies against HSV (a) and EBV (b). The frequency (upper panel) and absolute number (lower panel) of late-differentiated CD4+ effector memory 3 (CD45RA-CCR7-CD27-CD28-, left-hand panels), effector (CD45RA+CCR7-CD27-CD28-, middle panels) and CD57-expressing cells (right-hand panels) were compared.
induce the differentiation of the CD4+ T-cell compartment. So far, no data addressing this point are available. The large inter-individual differences observed could also be due to the duration of a latent infection with these viruses. This is a very important parameter to take into account; however, to our knowledge, this cannot be determined using current techniques, due to the asymptomatic course of most infections.

We have previously documented a large impact of CMV on the distribution of different CD8+ T-cell subsets in the cohort analysed here (Derhovanessian et al., 2010), in line with several published reports (Almanzar et al., 2005; Chidrawar et al., 2009; Khan et al., 2002; Looney et al., 1999). In the current study, we present data showing that CMV also has an impact on the CD4 compartment in a cohort of middle-aged individuals. The existence of large pools of memory CD4+ T-cells (albeit in lower frequencies than CD8+ T-cells in middle-aged individuals (Sester et al., 2002; Sylwester et al., 2005)) suggested changes to the CD4+ pool as well as the CD8+ pool in individuals harbouring CMV. We observed significantly higher proportions and numbers of late-differentiated CD4+ memory subsets lacking the expression of CD27 and CD28, as well as T-cells carrying the putative senescence marker CD57 in CMV-seropositive people. Similar findings have been reported in at least two other studies (Fletcher et al., 2005; Pourghesari et al., 2007). Fletcher et al. (2005) demonstrated that a mean of 50–75% of CMV-specific CD4+ T-cells in young and elderly individuals, respectively, lack the expression of CD27 and CD28. The fraction of double-negative cells among CD4+ T-cells specific for other pathogens was also increased in association with a latent CMV infection, suggesting the fascinating possibility of a bystander effect, whereby CMV-specific T-cells drive non-CMV-specific CD4+ T-cells towards a more late-differentiated phenotype. Interestingly, in contrast to CD8+ T-cells, such increased proportions of late-differentiated T-cell subsets were not accompanied by reduction in the percentage of naïve, central memory and effecter memory 1 subsets (carrying both CD27 and CD28), suggesting different differentiation pathways for CD4+ and CD8+ T-cells. As mentioned previously, unlike CMV, the presence or absence of HSV did not have any significant impact on the distribution of the different CD4+ T-cell subsets analysed here.

Due to the essentially ubiquitous prevalence of EBV and VZV in our cohort, we were not able to determine the impact of a latent infection with these viruses on the distribution of different T-cell subsets. However, at least for the CD4+ compartment, the absence of late-differentiated memory subsets in CMV-seronegative individuals, despite latent infections with EBV and VZV, does exclude these viruses as a factor in inducing such differentiation. Our data thus demonstrate the apparently unique impact of CMV, not shared with another persistent herpesvirus, HSV, in driving both the CD4+ and the CD8+ T-cell compartments towards a more late-differentiated phenotype in middle-aged individuals.

METHODS

Subjects. One hundred subjects representing the general population recruited as controls in the Leiden Longevity Study (Schoenmaker et al., 2006) were analysed. Detailed characteristics of these donors have been published previously (Derhovanessian et al., 2010). Briefly, the cohort, with a median age of 61.2 years (range 42–81 years) consisted of 61% females, median age of 59.5 years (range 42–74 years), and 39% males, with a median age of 65 years (range 49–81 years).

Virological analyses. CMV serostatus was determined by ELISA using the CMV-IgG-ELISA PKS assay (Medac). HSV and VZV serostatues were determined by ELISA using the Enzymost anti-HSV/IgG (detecting both HSV-1 and HSV-2) and Enzygnost anti-VZV/IgG assay, respectively (Siemens Healthcare Diagnostics). Liaison EBNA IgG chemiluminescence immunoassay (CLIA) was used for determination of EBV serostatus (DiaSorin). EBNA IgG-negative sera were additionally tested for viral capsid antigen (VCA) IgG and considered EBV-seropositive if VCA IgG was detectable.

Flow cytometry. After thawing, PBMCs were stained for surface markers as reported previously (Derhovanessian et al., 2010). Briefly, after treatment with human Ig (GAMUNEX; Talecisi Biotherapeutics) and ethidium monoazide (EMA; Invitrogen), cells were first stained indirectly with anti-KLRG-1 primary antibody (kindly provided by Professor Hans-Peter Pircher, University of Freiburg, Germany) and Pacific Orange-conjugated goat anti-mouse IgG (Invitrogen). After blocking with mouse serum (Chemicon, Millipore), cells were labelled with directly conjugated mAbs CD3–PE (Caltag, Invitrogen), CD4–PerCP, CD8–APC–Cy7, CC7–PE–Cy7 (BD Biosciences), CD27–APC, CD45RA–Pacific Blue, CD28–Alexa Fluor 700 (BioLegend), CD57–FITC (Immumotools) and analysed immediately on an LSR II cytometer with FACSDiva software (BD Biosciences). The spectral overlap between all channels was calculated automatically by the BD FACSDiva software, after measuring negative and single-colour controls.

Data analysis. Data were analysed using FlowJo software (Tree Star). The detailed gating strategy has been published previously (Derhovanessian et al., 2010). After exclusion of EMA-positive dead cells, lymphocytes were gated in an forward scatter versus side scatter dot-blot according to their size and granularity. T-cells within the lymphocyte gate were characterized as CD3+ cells. T-cell subsets were characterized according to surface expression of CD45RA, CCR7, CD27 and CD28 as naïve (N, CD45RA+CCR7+CD27+CD28+), central memory (CM, CD45RA–CCR7–CD27+CD28+), effector memory 1 (EM1, CD45RA–CCR7–CD27+CD28+), effector memory 3 (EM3, CD45RA+CCR7–CD27+CD28+), and effector (E, CD45RA+CCR7+CD27+CD28+) according to previously published models (Koch et al., 2008; Romero et al., 2007). The absolute number of cells in each subset per unit of blood was calculated by multiplying the frequency of the subset within the lymphocytes by the absolute number of lymphocytes calculated during cytometric analysis of fresh whole blood. Flow cytometry stainings and data analysis were performed on blinded samples.

Statistical analyses were performed by GraphPad Prism v4. Mann–Whitney testing was used for comparison of two independent groups. Differences between two groups for the categorical variables were assessed with the χ2 test and χ2 test for trend for different age groups. Binary logistic regression analysis was used to adjust for gender.

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