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Clonotype Selection and Composition of Human CD8 T Cells Specific for Persistent Herpes Viruses Varies with Differentiation but Is Stable Over Time

Emanuela M. Iancu,* Patricia Corthesy,* Petra Baumgaertner,† Estelle Devevre,† Verena Voelter,* Pedro Romero,‡ Daniel E. Speiser,† and Nathalie Rufer2*†

Protection from reactivation of persistent herpes virus infection is mediated by Ag-specific CD8 T cell responses, which are highly regulated by still poorly understood mechanisms. In this study, we analyzed differentiation and clonotypic dynamics of EBV- and CMV-specific T cells from healthy adults. Although these T lymphocytes included all subsets, from early-differentiated (EM/CD28pos) to late-differentiated (EMRA/CD28neg) stages, they varied in the sizes/proportions of these subsets. In-depth clonal composition analyses revealed TCR repertoires, which were highly restricted for CMV- and relatively diverse for EBV-specific cells. Virtually all virus-specific clonotypes identified in the EMRA/CD28neg subset were also found within the pool of less differentiated “memory” cells. However, striking differences in the patterns of dominance were observed among these subsets, because some clonotypes were selected with differentiation while others were not. Late-differentiated CMV-specific clonotypes were mostly characterized by TCR with lower dependency on CD8 coreceptor interaction. Yet all clonotypes displayed similar functional avidities, suggesting a compensatory role of CD8 in the clonotypes of lower TCR avidity. Importantly, clonotype selection and composition of each virus-specific subset upon differentiation was highly preserved over time, with the presence of the same dominant clonotypes at specific differentiation stages within a period of 4 years. Remarkably, clonotypic distribution was stable not only in late-differentiated but also in less-differentiated T cell subsets. Thus, T cell clonotypes segregate with differentiation, but the clonal composition once established is kept constant for at least several years. These findings reveal novel features of the highly sophisticated control of steady state protective T cell activity in healthy adults. The Journal of Immunology, 2009, 183: 319–331.

As a result of antigenic challenge, naive (N) CD8 T cells undergo clonal expansion and differentiation into memory and effector type lymphocytes (1) whose principal role is to eliminate virally infected and malignant cells. Whereas central-memory (CM) T cells lack immediate effector function and express receptors allowing them to repeatedly circulate to lymph nodes, effector-memory (EM) and effector (EMRA) T lymphocytes preferentially home to peripheral tissues and, upon stimulation, rapidly produce effector cytokines (e.g., IFN-γ) and cytotoxic proteases (2, 3). Several technical improvements of T cell studies have demonstrated that primed Ag-experienced CD8pos T lymphocytes are highly heterogeneous (4–10), varying in terms of cell surface phenotype, function, and history of Ag encounter. Recently, we uncovered additional heterogeneity among EM and effector subsets by studying the functional attributes of such T cells distinguished on the basis of expression of the costimulatory receptors CD27 and CD28 (4–10). This is increasing evidence that the immune response depends on the type and nature of infection (acute vs persistent), the amount of Ag present, the cycles of T cell rest and stimulation, and the site of replication of the pathogen (13–16). Although the existence of distinct subpopulations of CD8pos T cells is now widely accepted, the lineage relationship and differentiation pathways that they follow remain a matter of controversy.

EBV and CMV are common persistent viruses in the human population, infecting ~90 and 60–90% of individuals, respectively. In healthy individuals, there is an equilibrium maintained between viral and immune activity, which keeps the viruses in check and allows the vast majority of carriers to remain asymptomatic throughout their lives. However, these viruses are pathogenic in immunocompromised individuals. CMV can cause cytopathic damage in various organs, and EBV can induce malignancies. Previous studies have shown that following the resolution of acute infectious mononucleosis and upon entering the chronic phase of EBV infection, CD8pos T cells specific for EBV lytic Ags remain high in frequency (17–19) and carry an EM phenotype with low levels of CCR7, variable expression of CD45RA (19, 20), and maintenance of the CD27 and CD28 coreceptor expression (7, 21). Somewhat lower in frequency, the CD8 T cells specific for latent EBV Ags show higher CCR7 expression, lower CD45RA re-expression (19), and high CD27 and CD28 expression (19, 21). In contrast, CMV-specific T cells have a more differentiated phenotype with low CCR7, CD27, and CD28 expression.
frequent reversion from CD45RO\textsuperscript{pos} to CD45RA\textsuperscript{pos}, and higher CD57 expression (20–23).

In humans, dominant EBV- and CMV-specific CD8 T cell responses are primarily directed against the HLA-A2-restricted epitopes GLCTLVAML derived from the EBV lytic protein BMLF1 (referred as A2/GLC), and NLVPVMATV derived from the CMV tegument protein pp65 (referred as A2/NLV) (24, 25). The CD8\textsuperscript{pos} T cell responses to virus infections are characterized by large expansions of activated T cell clonotypes bearing highly restricted TCR. TCR repertoire analyses of EBV GLC/A2-specific T cells have reported a preferential usage of BV gene segments BV2, BV4, BV16, BV22, and of V\textalpha\textbeta\textgamma V15 (26–29), although the highly dominant clones during the acute response do not persist into the chronic phase and are often overtaken or replaced by other clonotypes specific for the same epitope (27). The CMV A2/NLV specific repertoire in healthy donors has also been associated with biased usage of a limited but heterogeneous set of BV segments, including BV8, BV13, BV14, and BV20 (28, 30). Structurally, a large fraction of A2/GLC- and A2/NLV-specific T cells derived from distinct individuals showed several recurrent “public” TCR features with highly conserved TCR CDR3 length and junctional motifs (26, 28–30).

EBV and CMV are genetically stable viruses that provide ideal models for the study of protective and long-lasting immune responses in humans. Despite major efforts over the past decade, the mechanisms that shape clonal selection and dominance of these T cell responses conferring long-term protective immunity still remain elusive. Recently, two independent studies have provided evidence that TCR affinity/avidity is one of the primary driving forces underlying TCR repertoire selection (28, 30). Nevertheless, the currently available data yet fail to describe the specific contribution that dominant and subdominant TCR clonotypes directed against a given EBV or CMV epitope play in the overall ensemble of virus-specific T cells. In this study, we explored both EBV- and CMV-specific CD8\textsuperscript{pos} T cells responses in the context of cell differentiation by determining the lineage relationship between different T cell subsets and examining the dynamics of dominant and subdominant clonotypes within these subsets over time. Lessons learned from these viral specific responses contribute to our understanding of correlates of protection, which can ultimately be used for the design of better immunization therapies against viral infections and cancer.

Materials and Methods

Cell preparation and flow cytometry

Peripheral blood samples and leukapheresis were collected from 13 healthy donors, aged 25–45 years, upon informed consent. This study has been reviewed and approved by an appropriate institutional review committee. At the time of leukapheresis, the donors had normal proportions of CD8-null peptide-MHC multimers with the HLA-A2 H chain mutants HLA-A2/\textalpha/\textbeta/\textgamma (5 x 10⁵ cells/well) were incubated with serial dilutions of HLA-A2/\textalpha/\textbeta/\textgamma/TAP\textsuperscript{neg} cells at 4°C with or without C1R target cells that expressed either wild-type or CD8-null HLA-A2 (1000 cells/well; HLA-A2\textsuperscript{neg}/TAP\textsuperscript{neg}) pulsed with serial dilutions of native T cell clones (50,000 cells/well) were subjected to BV-specific multimers were tested, and selected T cell clones bearing defined clonotypes were expanded in RPMI 1640 medium supplemented with 8% human serum, 150 U/ml recombinant human IL-2 (a gift from GlaxoSmithKline), 1 \mu/ml PHA (Sodiag), and 1 x 10⁵/ml irradiated allogeneic PBMC (3000 rad) as feeder cells. A2/multimer\textsuperscript{pos} T cell clones were expanded by periodic (every 15 days) restimulation in 24-well plates with PHA, irradiated feeder cells, and recombinant human IL-2.

\textit{a3 domain mutant multimer timer}ing, functional INF-\gamma secretion, and cytolytic assays

CD8-null peptide-MHC multimers with the HLA-A2 H chain mutants D277K/T228A were produced by PCR mutagenesis as described previously (35). Seven serial dilutions ranging from 144 to 2.25 mg/\muL mutated multimers were tested, and selected T cell clones bearing defined clonotypes were expanded in vitro-grown T cell clones as described previously (35, 36). A minimum of 20 five-cell aliquots and between 44 and 151 in vitro-generated T cell clones were generated and analyzed per T cell subset. When \textit{CDR3} sequences were too similar such as in the case for EBV-specific TCR BV2 clonotypes, highly specific clonotypic primers could not be designed, and thus all PCR products were sequenced.
Results

Differentiation of A2/GLC EBV-specific and A2/NLV CMV-specific CD8<sup>pos</sup> T cells

Multicolor flow cytometry analysis with CD45RA and CCR7 Abs allows to distinguish four major subsets (N, CM, EM, and EMRA) among total CD8<sup>pos</sup> T cells (Fig. 1A). Dot plots show double staining for CCR7/CD45RA (A) and for CD45RA/CD28 (B) of EBV (HLA-A2/BMLF1 defined as GLC/A2)- and CMV (HLA-A2/pp65 defined as NLV/A2)-specific CD8<sup>pos</sup> T lymphocytes gated using the corresponding peptide/A2 multimers. Analysis of CD45RA/CD28 expression (B) was performed after gating on CCR7-negative multimer-specific CD8<sup>pos</sup> T cells. For comparison, stainings on total CD8<sup>pos</sup> T cells (upper panels) and the distribution of the different subsets according to cell surface expressions is depicted. Quadrant percentages are shown. CD45RA is abbreviated to RA, and CD28 is abbreviated to 28.

The proportions of the four defined subsets of EBV- and CMV-specific CD8<sup>pos</sup> T cells among 13 healthy individuals (three donors had both positive EBV- and CMV-specific responses).

Most dominant virus-specific TCR clonotypes are shared between early RA<sup>neg28pos</sup>- and late RA<sup>pos28neg</sup>-differentiated T cell subsets

To determine whether the early (CD8<sup>pos</sup>)- and the late (CD8<sup>neg</sup>)-differentiated T cell compartments were composed of the same TCR clonotypes or conversely if TCR clonotypes were unique and specific to each differentiation stage, the clonal composition of ex vivo-sorted EBV- and CMV-specific T cell subsets was analyzed by spectratyping, assessing TCR-β chain variable segment usage (BV). To directly reveal the presence or absence of particular

FIGURE 1.
Ex vivo analysis of EBV- and CMV-specific CD8<sup>pos</sup> T cell differentiation. A and B, EBV- and CMV-multimer<sup>pos</sup> T cells were characterized ex vivo by flow cytometry for cell surface expression of CCR7, CD45RA, and CD28. Dot plots show double staining for CCR7/CD45RA (A) and for CD45RA/CD28 (B) of EBV (HLA-A2/BMLF1 defined as GLC/A2)- and CMV (HLA-A2/pp65 defined as NLV/A2)-specific CD8<sup>pos</sup> T lymphocytes gated using the corresponding peptide/A2 multimers. Analysis of CD45RA/CD28 expression (B) was performed after gating on CCR7-negative multimer-specific CD8<sup>pos</sup> T cells. For comparison, stainings on total CD8<sup>pos</sup> T cells (upper panels) and the distribution of the different subsets according to cell surface expressions is depicted. Quadrant percentages are shown. CD45RA is abbreviated to RA, and CD28 is abbreviated to 28. C, The proportions of the four defined subsets of EBV- and CMV-specific CD8<sup>pos</sup> T cells among 13 healthy individuals (three donors had both positive EBV- and CMV-specific responses).
clonotypes, TCR spectratype analysis was performed on specific cDNA after global amplification of expressed mRNA from multiple five-cell samples (32), and the TCR CDR3/H9252 sequence for each sample was determined by either direct sequencing or amplification using a set of clonotypic primers designed for each unique CDR3 sequence (36).

As illustrated in Fig. 3A, and in agreement with previous studies (26–29), the TCR clonotypes that dominated the EBV

![FIGURE 2. Ex vivo analysis of expression of mediators involved in T cell survival and effector functions within virus-specific CD8+ T cell subsets. A and B, Flow cytometry analysis of expression of CD27, IL-7Rα, CD57, granzyme B, and perforin among EBV-specific cells (A) and CMV-specific cells (B). Bulk EMRA (open histogram) and naive (black histogram) CD8+ cells were used to appropriately set the gates between low- and high-expressing populations (CD8t, total CD8; top panels). Dotted lines allow direct comparison. Percentages of cells expressing a given marker are indicated. C, Complete set of data shown as mean percentage and SD of CD27, IL-7Rα, CD57, granzyme B, and perforin expression within RA^{neg28pos}/RA^{28pos}/RA^{neg28neg}, and RA^{28pos} subsets for total CD8 T cells (n = 13), EBV-specific (n = 8), and CMV-specific (n = 8) T cells. Statistically significant differences between EBV- and CMV-specific T cells with p values <0.03 (*) and p values <0.01 (**) using the Welch two sample t test. Of note, both EBV and CMV virus-specific RA^{28pos} subsets significantly exhibited lower expression of perforin as compared with total CD8 T cells (p = 0.0002 for EBV; p = 0.014 for CMV), in line with a previous report (52).]
In vivo analysis of the TCR-BV clonotype composition among EBV-specific (A) and CMV-specific CD8<sup>+</sup> T cell subsets (B). Unique primers corresponding to the CDR3 gene sequence of each identified TCR clonotypes were designed and validated. Clonotypic PCR was performed on cDNA obtained from individually ex vivo sorted five-cell samples of virus-specific RA<sup>−28pos</sup> (nos. 1–10), RA<sup>pos28pos</sup> (nos. 11–20), RA<sup>pos28neg</sup> (nos. 21–30), and RA<sup>−28neg</sup> (nos. 31–40) CD8<sup>+</sup> T cell subsets. Data from 10 independent five-cell aliquots are depicted. Because CDR3 gene sequences were too similar in the case of EBV-specific TCR-BV2 clonotypes, distinct clonotypic primers could not be designed, and all PCR products for the TCR-BV2 subfamily were sequenced. Samples yielding detectable clonotypic-specific signals are depicted in color with those TCR clonotypes which increase in frequency along T cell differentiation shown in green, those which decrease in red, and those which remain stable in gray. Of note, BV13-clono2 (BCL6) was predominant within the RA<sup>−28neg</sup> CMV-specific subset (depicted in blue). Data are representative of four healthy individuals (BCL4, BCL6, BCL7, and BCL8), of which two were used for both EBV and CMV specificities.

In vivo selection of dominant EBV-specific T cell clonotypes with differentiation

Although the strategy of combining ex vivo cell sorting with molecular analysis of five-cell aliquots provided the identification of specific T cell clonotypes within the four defined CD8<sup>+</sup> subsets, the current limitation of this technique impeded the determination of precise frequencies of individual clonotypes. We therefore generated a total of 1180 T cell clones by in vitro limiting dilution cultures from both EBV- and CMV-specific T cell subsets. The TCR of each T cell clone was analyzed by sequencing and/or specific clonotypic PCR analysis. More than 40% of the EBV-specific cells from donor BCL4 were BV2 and BV4 clonotypes. This proportion increased even further in the RA<sup>pos28neg</sup> late-differentiated RA<sup>pos28neg</sup> compartment (clonotypes depicted in green), whereas others decreased along the same pathway (clonotypes depicted in red) or remained relatively stable (clonotypes depicted in gray). The observation that the same clonotypes were found in several if not all of the four subsets provides strong evidence that most clonotypes differentiated to multiple CD8<sup>+</sup> T cell compartments.

In contrast to the relatively diverse yet highly BV2/BV4-biased EBV-specific T cell repertoire, the CMV repertoire was restricted to two or three clonotypes that dominated all four subsets. Interestingly, some of the dominant clonotypes were found to increase in frequency from the early-differentiated RA<sup>−28pos</sup> to the late-differentiated RA<sup>pos28neg</sup> compartment (clonotypes depicted in green), whereas others decreased along the same pathway (clonotypes depicted in red) or remained relatively stable (clonotypes depicted in gray). The observation that the same clonotypes were found in several if not all of the four subsets provides strong evidence that most clonotypes differentiated to multiple CD8<sup>+</sup> T cell compartments.

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differentiation was even more apparent (Fig. 4C, Table I). Specifically, BV2-clono1, BV2-clono2, and BV4-clono1 underwent a major increase in frequency with differentiation, whereas BV2-clono4, BV2-clono5, and BV2-clono7 were no longer identified in any clones of the RApos28neg T cell subset, even though they represented 25% of clonotypes in the RAneg28pos compartment (Table I).

In vivo selection of dominant CMV-specific T cell clonotypes with differentiation

In agreement with the ex vivo five-cell sample data (Fig. 3B), the CMV-specific TCR repertoire in all three donors analyzed was much more restricted (Fig. 5) compared with the EBV-specific response (Fig. 4). Although, for EBV-specific responses, we identified between 8 and 11 dominant T cell clonotypes per donor (Table I); for CMV responses, this was restricted to only 2–4 clonotypes (Table II). Despite the decline in frequency of the dominant BV14-clono1 with T cell differentiation in donor BCL4, other CMV-specific clonotypes (BV9-clono1, BV8-clono1, and BV8-clono2) “filled up this space” (Fig. 5A, Table II). Similar results were obtained with donor BCL6 (Fig. 5B) and donor BCL8 (Fig. 5C) in whom we observed the progressive decline in frequencies of BV8-clono1 and BV13-clono1, respectively, along

FIGURE 4. Quantification of dominant EBV-specific T cell clones among distinct CD8pos T cell subsets. Clonotypic PCR and/or sequencing of amplified BV-CDR3-BC products were performed on T cell clones generated in vitro from EBV-specific early (RApos28neg) and late (RAneg28pos)-differentiated T cell subsets from BCL4 (A), BCL7 (B), and BCL8 (C) healthy donors. Results are presented as percentages of BV2 (blue), BV4 (red), BV16 (green), BV18 (orange), and BV22 (yellow) T cell clonotypes within EBV-specific T cell subsets, respectively (see Table I). EBV-specific T cell clonotypes, which increase with differentiation, are shown in dark colors. Nondominant (<3%) EBV-specific T cells bearing miscellaneous TCR BV gene segments (BV2 and BV4 or other BV subfamilies) are defined as others and depicted in gray.
Table I. Proportions of dominant EBV-specific T cell clonotypes among RA<sup>neg</sup>28<sup>pos</sup> and RA<sup>pos</sup>28<sup>neg</sup> CD8<sup>+</sup> T cell subsets

<table>
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<tr>
<th>Donor</th>
<th>Clonotype BV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CDR3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BJ</th>
<th>RA&lt;sup&gt;neg&lt;/sup&gt;28&lt;sup&gt;pos&lt;/sup&gt; Percentage&lt;sup&gt;b&lt;/sup&gt;</th>
<th>RA&lt;sup&gt;neg&lt;/sup&gt;28&lt;sup&gt;pos&lt;/sup&gt; Total&lt;sup&gt;b&lt;/sup&gt;</th>
<th>RA&lt;sup&gt;pos&lt;/sup&gt;28&lt;sup&gt;neg&lt;/sup&gt; Percentage&lt;sup&gt;b&lt;/sup&gt;</th>
<th>RA&lt;sup&gt;pos&lt;/sup&gt;28&lt;sup&gt;neg&lt;/sup&gt; Total&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>BCL4</td>
<td>BV2 clono1*</td>
<td>RDRIGNGY</td>
<td>1.2</td>
<td>1%</td>
<td>151</td>
<td>13%</td>
<td>79</td>
</tr>
<tr>
<td>EBV</td>
<td>BV2 clono2*</td>
<td>RDRTNGGY**</td>
<td>1.2</td>
<td>13%</td>
<td>151</td>
<td>11%</td>
<td>79 --&gt;</td>
</tr>
<tr>
<td>BCL7</td>
<td>BV2 clono1</td>
<td>RDTNGGY</td>
<td>1.2</td>
<td>2%</td>
<td>87</td>
<td>7%</td>
<td>87</td>
</tr>
<tr>
<td>EBV</td>
<td>BV2 clono2</td>
<td>RVGVGNTI**</td>
<td>1.3</td>
<td>13%</td>
<td>87</td>
<td>1%</td>
<td>87 --&gt;</td>
</tr>
<tr>
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<td>MREVNTEA</td>
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<td>2%</td>
<td>87</td>
<td>1%</td>
<td>87</td>
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**T cell repertoire selection and clonotype composition of CMV-specific CD8<sup>+</sup> T cells between early (2002) and late (2006) time points of persistent infection (Fig. 6). The phenotype of Ag-specific cells revealed a high stability of phenotype (Fig. 6A) with again the predominance of both differentiated RA<sup>neg</sup>28<sup>pos</sup> or RA<sup>pos</sup>28<sup>neg</sup> subsets. All dominant clonotypes identified in the peripheral blood of healthy donors BCL8 and BCL6 in the early time point (Fig. 3C and Table II) were also found 1 and 4 years later, respectively (Fig. 6, B and C), indicating long-term persistence of clonally restricted T cell expansions. Most importantly, the proportion of each clonotype within each of the four T cell subsets was remarkably stable over time (Fig. 6C). We observed that BV8-clono1 and BV13-clono1 isolated from donors BCL6 and BCL8, respectively, were again those clonotypes that decreased in frequency from the early-differentiated to the late-differentiated compartment and were not selected with differentiation (for direct comparison, see Fig. 3B). Conversely, those clonotypes initially found at late-differentiated stages were again found within the late-differentiated compartment 4 years later. Interestingly, BV13-clono2 (donor BCL6) was again found to dominate the RA<sup>neg</sup>28<sup>pos</sup> T cell compartment. The two dominant clonotypes from donor BCL4 (BV14-clono1 and BV9-clono1) were also identified 4 years later; however, accurate frequencies could not be determined (data not shown). Finally, EBV-specific T cells also showed a remarkable stability of phenotype of TCR repertoire and of clonotype selection with differentiation over time (data not shown).

In summary, the data described here indicate that throughout the course of persistent EBV and CMV infection in healthy donors,
which represents a steady-state situation, the TCR repertoire diversity and clonotype composition of each virus-specific T cell subset varies with differentiation but remains remarkably stable. At present, we are comparing these findings with situations where potential viral reactivation may occur such as in patients undergoing transient lymphodepletion (E. M. Iancu et al., manuscript in preparation).

Clonotypes enriched in late-differentiated CMV-specific cells expressed TCR of lower CD8 dependency than early-differentiated ones

Two recent studies provided evidence that TCR affinity/avidity for Ag may be one of the driving forces underlying clonal selection along chronic antigenic stimulation (28, 30). We undertook to determine whether such differences in TCR affinities/avidities could explain the changing patterns of dominance along differentiation of CMV-specific T cell clonotypes, hypothesizing that those which are preferentially selected toward differentiation will have less dependency on CD8 coreceptor binding compared with clonotypes, which decline with differentiation. To do so, CMV-specific T cell clonotypes that were selected or that declined with differentiation were tested for their efficacy to stain serial dilutions of multimers containing /H3 domain mutants of the HLA-A2 H-chain precluding CD8 binding. As shown in Fig. 7A, for donors BCL6 and BCL8, the highly dominant clonotypes (BV13-clono1 and BV15-clono1, respectively), which were the
ones that persisted with differentiation, showed higher mean fluorescence intensity with the mutated multimers than the subdominant clonotypes (BV8-clono1 and BV13-clono1, respectively) whose frequencies fell with differentiation. Selection of clonotypes of higher TCR avidity (reduced CD8 dependency) was also observed for donor BCL4, where the highest CD8-null multimer staining was observed for clonotype BV9-clono1, which became more frequent in the late-differentiated RApos28neg subset. However, a strict correlation between high multimer staining and clonotype selection with differentiation was not always found. The CMV-specific BV14-clono1 (BCL4) showed strong staining with the CD8-null multimer (Fig. 7A), despite its reduced frequency in the late-differentiated RApos28neg subset.

We next tested CMV-specific T cell clonotypes for their ability to recognize T2 cells labeled with titrated peptide (NLVPMATV) in chromium release assays (Fig. 7B). Remarkably, all clonotypes showed similar functional avidity because 50% maximal lysis of wild-type T2 cells was found at similar peptide doses (IC50 ranging between 1.6 × 10−11 and 5.2 × 10−11 M; no statistically significant difference). To analyze CD8 dependency of target cell recognition by CMV-specific dominant and subdominant clonotypes, C1R cells transfected with mutant HLA-A2 molecules that abrogate CD8 binding (37) were used as target cells (Fig. 7C). Subdominant clones (BV8-clono1) of donor BCL6 exhibited a slightly inferior but not statistically significant different functional avidity of Ag recognition compared with the dominant clonotype (BV13-clono1), with average IC50 ranging between 2.4 × 10−10 and 2.5 × 10−11 M, respectively. A trend was observed for the maximum average lysis, with significantly (p < 0.03) reduced levels found for BV8-clono1 (53 ± 19%) when compared with those obtained for BV13-clono1 (74 ± 18%). However, these differences remained subtle (Fig. 7C), because we did not find statistically significant differences in functional avidities for the clonotypes tested from donors BCL4 and BCL8 (data not shown).

Table II. Proportions of dominant CMV-specific T cell clonotypes among RAneg28pos, RAneg28neg, and RApos28neg CD8+ T cell subsets

<table>
<thead>
<tr>
<th>Donor</th>
<th>Clonotype</th>
<th>BVα</th>
<th>CDR3β</th>
<th>BJ</th>
<th>RAne28pos</th>
<th>RAne28neg</th>
<th>RApo28neg</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL4</td>
<td>BV8 clono1</td>
<td>8</td>
<td>SSVNEA</td>
<td>1.1</td>
<td>0%</td>
<td>49</td>
<td>9%</td>
</tr>
<tr>
<td>CMV</td>
<td>BV8 clono2</td>
<td>8</td>
<td>SSAGGAVGY</td>
<td>1.2</td>
<td>0%</td>
<td>49</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td>BV9 clono1</td>
<td>9</td>
<td>SLLLTGAAEA</td>
<td>1.1</td>
<td>4%</td>
<td>49</td>
<td>39%</td>
</tr>
<tr>
<td>BV14 clono1</td>
<td>14</td>
<td>RLLAGGRSAQ</td>
<td>2.5</td>
<td>82%</td>
<td>49</td>
<td>88%</td>
<td>68</td>
</tr>
<tr>
<td>BCL6</td>
<td>BV8 clono1*</td>
<td>8</td>
<td>SSANYGY</td>
<td>1.2</td>
<td>14%</td>
<td>99</td>
<td>5%</td>
</tr>
<tr>
<td>CMV</td>
<td>BV13 clono1</td>
<td>13</td>
<td>SRQTGAAYGY</td>
<td>1.2</td>
<td>57%</td>
<td>100</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>BV13 clono2</td>
<td>13</td>
<td>SYATGTAYGY</td>
<td>1.2</td>
<td>31%</td>
<td>99</td>
<td>5%</td>
</tr>
<tr>
<td>BCL8</td>
<td>BV13 clono1</td>
<td>13</td>
<td>SSVSGGASNEQ</td>
<td>2.1</td>
<td>15%</td>
<td>80</td>
<td>3%</td>
</tr>
<tr>
<td>CMV</td>
<td>BV15 clono1</td>
<td>15</td>
<td>SDPLTASYEQ</td>
<td>2.7</td>
<td>83%</td>
<td>80</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>BV13 clono1*</td>
<td>13</td>
<td>SSVSGGASNEQ</td>
<td>2.1</td>
<td>15%</td>
<td>80</td>
<td>3%</td>
</tr>
</tbody>
</table>

* TCR-BV nomenclature according to Arden et al. (33).

** Estimated frequency of individual clonotypes is indicated as proportions of positive clonotypes per total number of characterized T cell clones.

*** A public T cell clonotype recently identified (28, 30).
not shown). In line with these results, we neither observed functional differences in IFN-γ secretion when dominant and subdominant T cell clones were stimulated following titrated wild-type or CD8-null CMV-multimers (data not shown).

Collectively, CD8-null multimer staining data indicate that in most instances the T cell clonotypes selected to differentiate are those exhibiting a relative lower dependency for pMHC-CD8 interaction than the ones that declined in frequency. Yet, our observations made by functional assays reveal that most of CMV-specific clonotypes, regardless of being strongly selected for late-differentiation or not, were characterized by TCR of high functional avidity for cognate Ag, suggesting that target cell recognition is constantly kept at high avidity by TCR-dependent and TCR-independent mechanisms.

Discussion

Human persistent viral infections such as EBV and CMV are useful models to study how Ag-specific memory T cell populations are generated and maintained over the lifetime of a healthy individual to ensure long-lasting protection from viral spread. During chronic infection with EBV and CMV, CD8pos T cell responses to A2/GLC and A2/NLV viral Ags are composed of heterogeneous populations of T cells of various differentiation stages, with EBV-specific responses being less differentiated than the CMV-specific ones (4–8, 10–12, 19–23). We focused our study on four distinct virus-specific T cell subsets, RAneg28pos, RApos28pos, RAneg28neg, and RA pos28neg, and found that each of these subsets could be identified within both EBV- and CMV-specific responses, but that their proportion differed. CMV-specific T cell responses were mostly composed of CD28-negative subpopulations and showed more advanced differentiation than EBV-specific T cells. Our data are in agreement with the model according to which, despite these differences in subset composition, EBV- and CMV-specific T lymphocytes follow the same pathway of differentiation from the RAneg28pos subset toward the RA pos28neg subset. This process is accompanied by the progressive loss of costimulatory receptors.
and up-regulation of cytotoxic molecules. This appears to be a general picture for human T cell differentiation, because it is not restricted to T cells specific for persistent viruses (8) but is also found in bulk CD8\(^{\text{pos}}\) T lymphocytes (11, 12) in other types of T cells (\(\gamma^\delta\),CD4\(^{+}\) (38)), as well as in T cells specific for self-tumor Ags such as Melan-A/MART-1 and NY-ESO-1 in melanoma patients (35, 36).

The comparison of the diversity of the T cell repertoire in different virus-specific CD8\(^{\text{pos}}\) subsets using TCR spectratyping combined with clonotyping is a powerful tool to dissect the process of T cell differentiation. In particular, the question of whether each T cell subset contains its unique set of clonotypes or whether dominant clonotypes are found throughout numerous stages of differentiation could be specifically addressed in this study. We found a striking restriction in the TCR repertoire of CMV-specific cells (2–4 clonotypes) that contrasted with the wider variety of EBV-specific T cells (8–11 clonotypes). Because such contrasting TCR repertoires coexisted in two healthy donors (BCL4 and BCL8), our observed differences in phenotype and TCR repertoire between these two viral specificities are indeed Ag specific rather than donor specific. Our results are in good agreement with previous studies looking at responses to EBV A2/GLC (28, 29) but are somewhat contradictory with data from studies on CMV A2/NLV-specific T cells, which reported a more diverse repertoire in healthy donors (28, 30) but a drastic restriction in situations of chronic inflammation, which resulted in selection of a single clonotype (30). One possible explanation for this discordant finding is that our study, unlike the previous ones, carried the analysis to the differentiation level of Ag-specific T cells, whereby the differentiated cells exhibited the highest restricted TCR-V\(\beta\) usage. Alternatively, we cannot formally exclude some bias due to the in vitro T cell cloning approach, because CMV-specific T cells are mostly composed of cells that are less likely to survive and expand in long-term cultures than EBV-specific cells.

Although the reasons behind the higher degree of differentiation and repertoire restriction of CMV-specific T cells compared with EBV-specific T cells are not fully understood, it has been suggested that these are Ag specific and due in part to differences in the biology of these viruses. Specifically, since latent CMV is harbored in cells of the myeloid lineage and their differentiation into monocytes and dendritic cells results in CMV reactivation, these events may occur more often than those of EBV reactivation from latently infected B cells (21). However, in the absence of more precise knowledge of viral spread in acute and chronic phases, it is difficult to determine whether the CMV-specific cells are more differentiated and more selected as a result of a stronger immune stimulus during the acute phase, or whether it is a result of factors involved in the selection and maintenance of the immune response during the chronic phase of infection. One longitudinal study reported that the TCR repertoire in the response to CMV primary infection was diverse but that this usage rapidly focused on few high avidity TCR clonotypes (39). Future techniques may allow us to localize and quantify viral spread more precisely to address these questions.

A major finding was that virtually all virus-specific clonotypes identified in the late-differentiated subsets (RA\(^{\text{neg28neg}}\), RA\(^{\text{neg28pos}}\)) were as well found within the pool of less differentiated “memory” cells (RA\(^{\text{pos28pos}}\)). In line with these observations, the proportion of other and infrequent TCR was greater in the RA\(^{\text{neg28pos}}\) EBV-specific subset (27–48%) than among the more differentiated RA\(^{\text{pos28neg}}\) subset (9–39%). Altogether, our data provide evidence for a linear model of human T cell differentiation, similar to the one described in a mouse model (13), in which a small number of clonotypes are selected to differentiate from a larger pool of less-differentiated “memory” T cells. This view is supported by previous studies reporting that murine TCR repertoires of CM and EM Ag-specific CD8\(^{\text{pos}}\) T cells are largely overlapping (40, 41). Recently, we also showed that the T cell response specific for the tumor Ag NY-ESO-1,157–165 was restricted to several codominant clonotypes and that both EM28\(^{\text{pos}}\) and EM28\(^{\text{neg}}\) compartments comprised the same T cell clones, revealing a tight interplay of T cells in early- and late-differentiated stages (36). The relatively high abundance of a given subset within the overall Ag-specific response does not correlate with increased TCR repertoire diversity and thereby should not influence our analysis. This was best illustrated with the CMV-specific RA\(^{\text{neg28pos}}\) subset that was the most prevalent one within the CMV-specific response but was not more diverse than the others.

Because our analysis was performed on Ag-specific T cells sorted according to their differentiation stage, it revealed for the first time that some virus-specific T cell clonotypes are preferentially selected with progressing cell differentiation, whereas others declined in frequencies and were eventually lost with differentiation. This observation points to the existence of not one but several orders of clonotype selection during an antiviral immune response against persistent herpes viruses. During the acute phase of infection, the first level is the primary response with the selection and generation of highly efficient effector T cells (17, 19, 39, 42, 43). The second level is the establishment of a stable memory response during which T cell clonotypes from the primary phase may either completely disappear or become subdominant, while new clonotypes may emerge and are chosen to dominate (27, 43). During the latent phases of infection, the third level as described in the present study represents the process of “clonotype bias with differentiation” by which a set of clonotypes is preferentially selected to further differentiate into effector cells. Finally, the remarkable persistence of both dominant and subdominant memory T cell clonotypes over time defines a fourth level of selection that will be discussed later on.

Immune responses to viruses (influenza, EBV, CMV, and HIV) are characterized by massive expansions of CD8\(^{\text{pos}}\) T lymphocytes (17, 42, 44) and by the preferential selection of a particular TCR-BV gene, as well as for the expansion of public clonotypes bearing common motifs in the amino acid sequence of the CDR3\(\beta\) region (26–29). Although it is generally believed that this process serves to select high-avidity Ag-specific CD8\(^{\text{pos}}\) T cells that will most efficiently recognize target cells (45), recent studies suggest that chronic viral infections such as EBV and CMV lead to the expansion of T cells of variable TCR affinities/avidities for pMHC complexes (28, 30, 46). Indeed, TCR affinity/avidity, defined as the strength of binding of one TCR molecule to the pMHC complex, may be one of the driving forces that determines T cell clonotype selection with differentiation. Moreover, the variable contribution of CD8 coreceptor may compensate for those differences in TCR affinities/avidities by keeping heterogeneous T cell clonotypes within a narrow range of functional avidities (28, 30, 46).

The work reported here extends these previous findings by showing in the context of T cell differentiation that those Ag-specific clones that are preferentially selected to differentiate are also those that typically express TCR of higher affinities/avidities, compared with those that decline in frequency with differentiation. Yet, we did not observe striking differences between clonotypes when IFN-\(\gamma\) secretion was assessed as a functional readout (data not shown). Intriguingly, these results do not exactly support those obtained by Price et al. (28) in which subdominant virus-specific T cell clonotypes were also those characterized by lower functional IFN-\(\gamma\) sensitivity. One potential explanation for these discordant results could be due to changes in functional avidities that may...
have developed within T cell clones generated in vitro, when compared with T cells that were analyzed directly ex vivo (28). Altogether, our data based on in vitro-generated T cell clones favor the view of a compensatory role of CD8 coreceptor in the clonotypes of lower TCR affinity (i.e., clonotypes not preferentially selected with differentiation), thus assuring a certain degree of clonotype diversity during T cell differentiation. Because we found that most clonotypes, regardless of whether they were preferentially selected with differentiation or not, recognized and killed within a tight window of functional avidity, target cells in the absence of CD8 coreceptor interactions, these data further suggest that not only the CD8 coreceptor but other accessory molecules as well (presumably costimulation and adhesion molecules) could modulate TCR-pMHC interactions. Finally, assessment of the functional avidity in ex vivo individual clonotypes would be informative and would allow validating the corresponding data obtained with clones. However, this would require the generation of clonotype-specific mAbs to identify the individual clonotypes in the flow cytometry-based ex vivo cytokine release assays, which remains a very technically challenging task.

Long-term persistence of clonally restricted CD8 T cell expansions has been observed in response to chronic Ag exposure, e.g., in viral infection (47–51) or in the tumor-bearing melanoma patients (35, 36). Yet, most of these studies provide limited information in regards to the evolution of distinct dominant and subdominant T cell clonotypes over time. For instance, one might speculate that subdominant clonotypes or those, which are not preferentially selected to enter the late-differentiated compartment, e.g., due to lower TCR affinity/avidity, might eventually disappear with time. Our data clearly indicate that this is not the case, and that such T cell clones were in fact maintained stably over time, since similar proportions of these clones were found within the early-differentiated subsets when we compared the two distinct time points. This is a remarkable observation as one might expect that during persistent viral infection characterized by low Ag levels, there may be a progressive constriction toward T cells expressing TCR of high affinity/avidity. Moreover, this study shows for the first time that the differentiation-dependent selection of clonotypes is preserved over time, with those clonotypes initially found at late-differentiated stages that were again present within the late-differentiated compartment 4 years later. Altogether, our data show that the clonal composition, composed of heterogeneous T cell clonotypes bearing TCR with various affinities/avidities, once established is kept constant for at least several years. In this regard, we propose that all clonotypes, whether they differentiate into effector cells or whether they retain a memory phenotype, serve important but likely distinct purposes to the overall EBV- and CMV-specific immune response. Finally, these observations point not only to a remarkable stability over time of the clonal composition of CMV-specific lymphocytes, but also of the factors that drive the selection of T cell clonotypes with differentiation. Although these may hold true for healthy donors where the infection is efficiently kept under immunological control, future studies are needed to investigate the level of persistence of TCR repertoire in situations of immunosuppression and disease associated with large scale and unrestrained viral reactivation and with potential alterations in the TCR repertoire.

In summary, we found that a highly proficient antiviral immune response is the result of several levels of T cell clonotype selection, with subsequent mechanisms involved in ensuring its stability over time. Understanding the dynamics involved in the formation and persistence of T cell-mediated protection should help us to identify specific deficiencies in antiviral and antitumor T cell responses and to optimize therapeutic strategies against infection and cancer.

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Disclosures
The authors have no financial conflict of interest.

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