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Dominant Human CD8 T Cell Clonotypes Persist Simultaneously as Memory and Effector Cells in Memory Phase

Cédric Touvrey,* Laurent Derre,† Estelle Devevre,* Patricia Corthesy,‡ Pedro Romero,* Nathalie Rufer,† and Daniel E. Speiser†

The adaptive immune system plays a critical role in protection at the time of secondary infection. It does so through the rapid and robust reactivation of memory T cells which are maintained long-term, in a phenotypically heterogeneous state, following their primary encounter with Ag. Although most HLA-A*0201/influenza matrix protein-specific CD8 T cells from healthy donors display characteristics typical of memory T cells, through our extensive phenotypic analysis we have further shown that up to 20% of these cells express neither the IL-7 receptor CD127 nor the costimulatory molecule CD28. In contrast to the majority of CD28pos cells, granzyme B and perforin were frequently expressed by the CD28neg cells, suggesting that they are effector cells. Indeed, these cells were able to kill target cells, in an Ag-specific manner, directly ex vivo. Thus, our findings demonstrate the remarkable long-term persistence in healthy humans of not only influenza-specific memory cells, but also of effector T cells. We further observed that granzyme B expression in influenza-specific CD8 T cells paralleled levels in the total CD8 T cell population, suggestive of Ag-nonspecific bystander activation. Sequencing of TCR α- and β-chains showed that the TCR repertoire specific for this epitope was dominated by one, or a few, T cell clonotype per healthy donor. Moreover, our sequencing analysis revealed, for the first time in humans, that identical T cell clonotypes can coexist as both memory and effector T cells, thereby supporting the principle of multipotent clonotypic differentiation. The Journal of Immunology, 2009, 182: 6718–6726.

The activation of Ag-specific CD8 T cells upon acute infection results in cell proliferation and the acquisition of effector functions. During the subsequent contraction phase, most of these cells die by apoptosis, but a relatively small fraction survives and establishes a pool of so-called memory T cells. Memory T cells are heterogeneous but can be divided into two main subsets, central and effector memory, based on their cell surface markers, effector functions, and anatomical location. Memory T cells have enhanced trafficking and proliferative capacities, and specific clones are present in greater frequency compared with the naive cell population. Thus, Ag-specific T cell responses to secondary infection are more rapid and more robust than the primary response (1, 2).

The maintenance of memory T cells is controlled through cytokine-driven proliferation and cell death, in a process called memory turnover (3–6). Properties of memory T cells can change over time and may in part be regulated by the tissue of residence (7–12). In addition to having enhanced trafficking and proliferative abilities, memory cells are also characterized by long-term persistence, self-renewal, and their ability to differentiate into effector cells. Effector cells, whereas, are highly active and are capable of immediate effector functions.

Several studies have shown that steady levels of memory T cells are maintained in the absence of Ag, in contrast to effector T cells which disappear in parallel with the elimination of Ag (13–17). A recent study in mice, however, demonstrated that following recovery from influenza and viral clearance, the T cell population comprised both memory and effector cells, with the latter preferentially found in the lung (17). This observation raises the possibility that CD8 T cell memory for pathogens infecting peripheral organs depends on the continued presence of both memory and effector T cells.

It has been proposed that the heterogeneity that is observed among T cell subsets may be influenced by clonotype because different TCRs, which vary in mechanism and strength of peptide MHC (pMHC)binding, may lead to distinct activation and differentiation. However, it is evident that cellular heterogeneity exists even within the same T cell clonotype, as demonstrated in TCR-transgenic mice (18), and in the elegant study by Stemberger et al. (19), in which it was shown that a single naive CD8 T precursor can differentiate into different effector and memory subsets. Studies of the human TCR repertoire, whereas, are conflicting, with some reporting that effector and memory cells are derived from identical, naive precursor cells (17, 20, 21) and others suggesting that they are recruited from distinct precursor cells (22). We have recently demonstrated that identical T cell clonotypes can differentiate to both CD28+ and CD28− effector memory cells (23).

In the present study, we characterized influenza-specific T cells in healthy adults (i.e., the cells are in memory phase) to address the
question of whether or not individual clonotypes can coexist as central memory and effector memory cells. For this work, we focused on the widely studied CD8 T cells specific for the influenza matrix protein 58–66 peptide (Flu-MA) presented by HLA-A*0201. This peptide is shared by all strains of influenza A and it is not free of infection.

To date, studies have shown that human CD8 T cells specific for influenza matrix protein 58–66 peptide are predominantly classical memory T cells, but a detailed phenotypic characterization of the cells is lacking. Previous work has also shown that the human TCR repertoire specific for Flu-MA/HLA-A*0201 is highly restricted to BV17 and ADV10 segment usage (24, 25) and identical TCR sequences have been found in multiple individuals. These “public” TCRs have high avidity for pMHC and efficiently recognize infected cells as well as APCs, indicating that T cells bearing these particular TCRs are highly selected by Ag.

Materials and Methods

Blood donors

Peripheral blood cells were collected from nine healthy donors. In addition, four melanoma patients donated serial blood samples in the context of clinical vaccination studies, whereby influenza-specific T cells were studied as steady reference parameters and were found to be largely unaffected by disease and cancer treatment (26). To assure that the donors had not experienced recent influenza infection, blood was withdrawn outside of the winter season and thus in periods with very low disease prevalence (www.influenza.ch). Note that outside of the fortunately rare influenza pandemics, the incidence of acute influenza infection is low, even during the winter season, much lower than infection with other respiratory "common" viruses. Finally, longitudinal analysis of HLA-A*0201 influenza matrix protein 58–66 multimer + CD8 T cells revealed stable frequencies (data not shown and Ref. 26), further supporting the notion that our donors were free of infection.

Flow cytometry

PBMC were isolated by density centrifugation using Ficoll-Hypaque (Pharmacia) and were cryopreserved. CD8 + T lymphocytes were positively enriched from thawed PBMC using anti-CD8-coated magnetic microbeads (Miltenyi Biotec). Influenza-specific CD8 + T cells were then identified using allophycocyanin- or PE-labeled HLA-A*0201/peptide multimers (also called tetramers) with influenza matrix protein 58–66 peptide GILGFVTL (Flu-MA). Briefly, CD8 T cells were first incubated on ice for 20 min with BV17-FITC Ab where indicated, followed by incubation with multimers for 40 min on ice. Additional surface molecules were added subsequently using appropriate mAbs. Intracellular content of granzyme B and perforin was analyzed without prior stimulation. Briefly, cells were fixed for 20 min at room temperature (PBS with 1% formaldehyde, 2% glucose, and 5 mM sodium azide), followed by staining in permeabilizing buffer (PBS and 0.1% saponin (Fluka), 0.2% BSA, and 50 mM EDTA) with anti-granzyme B-FITC (Southern Biotechnology Associates) or anti-perforin-FITC (mAb 6G9; Ancell) Abs. Cells were directly analyzed with an LSR-II machine using FACSDiva software (BD Biosciences) or sorted into defined populations with a FACSVerse SE using CellQuest software (BD Biosciences). Abs used were: CD8-allophycocyanin-Cy7, CD8-Pacific Blue, CD28-FITC, CD28-PE, CD107a-PE-Cy5, CCR7-PE-Cy7, CCR7 purified, and GAR-allophycocyanin were purchased from BD Biosciences. CD45RA-ECD, CD127-FITC and BV17-FITC were purchased from Beckman Coulter.

Results

Influenza-specific CD8 T cells from healthy donors include subsets with phenotypic features typical of memory and effector cells

Although the discrimination of memory vs effector T cells should be based on functional properties in vivo or ex vivo, this is not always technically or practically feasible. Thus, cells are routinely classified based on their expression of various cell surface receptors which, unfortunately, does not always correlate directly with functional properties (11). It is therefore prudent to assess the expression of functionally relevant molecules, such as cytokines and mediators of cytotoxicity, when defining cell populations. Nevertheless, cell surface phenotypes provide an arbitrary grid that has been very useful for an approximate initial characterization of T cell subsets.

On the basis of CD45RA and CCR7 expression, four populations of CD8 T cells can be classified: naive cells (CD45RA+CCR7-), central memory (CM) cells (CD45RA-CCR7+), effector memory (EM) cells (CD45RA-CCR7-), and effector (EMRA) cells (CD45RA-CCR7+). Each of these subpopulations is heterogeneous. EM cells, for example, contain at least two subsets distinguished by CD28 expression: EM CD28 + and EM CD28 -.
mainly effector-like cells and EM CD28+/EMRA (CCR7+) cells share many properties with memory cells (30). We analyzed by flow cytometry the expression of CCR7 and CD45RA by influenza-specific CD8 T cells. Blood samples were collected from seven HLA-A2-positive donors. The donors were all healthy, between the ages of 23 and 53, and had previously experienced influenza A infection.

FACS analysis with HLA-A*0201/influenza matrix protein58–66 peptide multimers (Flu-MA multimers) revealed frequencies of epitope-specific cells ranging from 0.03 to 0.84% of CD8 T cells (Table I). The majority of influenza-specific CD8 T cells were naive cells (CCR7+/CD45RA−), consistent with the notion that they had previously encountered Ag and were not naive cells. Surprisingly, the human influenza-specific cells poorly expressed the lymphoid tissue homing receptor CCR7, a marker associated with CM cells. Also, the majority of them expressed CD28, indicating that they had not fully differentiated to effector cells (Fig. 1A).

Subsequently, we compared the profiles of influenza-specific CD8 T cells with total CD8 T cells. Phenotypic analysis with Flu-MA multimers, along with Abs against CD8 and CD45RA revealed extensive heterogeneity among the influenza-specific CD8 T cells (Fig. 1B). CD45RA+/CCR7− cells (naive phenotype) were rarely observed among the influenza-specific cells, but made up ~25% (mean value) of total CD8 T cells (Fig. 1C). Although CM cells (CD45RA−CCR7−) showed relatively consistent frequencies of 4–15%, EM28+ cells (CD45RA−CCR7+) were significantly more frequent within the population of influenza-specific cells than the total CD8 compartment. Among the influenza-specific cells, EM28+ cells were surprisingly well represented, albeit at relatively low frequencies, and were at similar frequencies as observed for the total CD8 T cell population. Influenza-specific EMRA cells were infrequent and expressed lower levels of CD45RA than naive cells. Longitudinal studies revealed that this cellular heterogeneity was relatively stably maintained over many years; in the instance of blood samples from patient LAU205, the heterogeneity was maintained over a period of 10.5 years (Fig. 1D). The majority of influenza-specific cells expressed classical features of memory cells, such as high levels of the IL-7Rα chain CD127 (Fig. 2A).

The naive, CM, and EM cells of both the influenza-specific and the total CD8 T cell compartments expressed similar levels of CD127. Although the proportion of EMRA cells expressing CD127 was more variable between donors, the overall expression levels were higher for the influenza-specific cells than for total CD8 T cells (Fig. 2B). Correspondingly, IL-7Rα chain mRNA (CD127) was easily detectable in all five-cell samples from influenza EM28+ cells and was also frequently expressed by influenza EM28− cells (Fig. 2C). In contrast, expression of IL-15Rα chain and Bcl-2 was more heterogeneous in EM28+ cells and Bcl-2 was less frequently expressed by influenza-specific EM28− cells (Fig. 2C).

Killer cell lectin-like receptor G1 (KLRG1) and programmed death 1 (PD-1) negatively modulate T cell function and are highly expressed by terminally differentiated cells (31–33). As previously shown for influenza-specific T cells, we observed that KLRG1 was infrequently expressed by both EM28+ and EM28− cells (34). Surprisingly, however, PD-1 transcripts were detected in 50% of the five-cell samples (Fig. 2C). This finding was substantiated by FACS staining in which it was shown that ~30% of influenza-specific cells expressed PD-1 on their surface (data not shown). Although the majority of influenza-specific cells exhibited an early

### Table I. Donor age and T cell frequency

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (years)</th>
<th>% Multimer+ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCB52</td>
<td>Unknown</td>
<td>0.18</td>
</tr>
<tr>
<td>BCB74</td>
<td>38</td>
<td>0.42</td>
</tr>
<tr>
<td>BCB100</td>
<td>53</td>
<td>0.44</td>
</tr>
<tr>
<td>BCB117</td>
<td>37</td>
<td>0.49</td>
</tr>
<tr>
<td>BCB122</td>
<td>52</td>
<td>0.07</td>
</tr>
<tr>
<td>BCB123</td>
<td>24</td>
<td>0.26</td>
</tr>
<tr>
<td>BCB129</td>
<td>38</td>
<td>0.18</td>
</tr>
<tr>
<td>BC26</td>
<td>23</td>
<td>0.84</td>
</tr>
<tr>
<td>LAU205</td>
<td>24</td>
<td>0.2</td>
</tr>
<tr>
<td>GEN207</td>
<td>70</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Frequency of HLA-A*0201/influenza M58–66-specific cells is expressed as percentage of total circulating CD8 T cells. Age of donors at the time of first blood withdrawal is given. LAU205 and GEN 207 are melanoma patients.
differentiated phenotype (CD45RA<sup>−</sup> and CD28<sup>+</sup>), and frequently expressed “survival genes” (CD127<sup>+</sup> and Bcl-2<sup>+</sup>), 5–30% of influenza-specific cells had down-regulated CD28 (Fig. 1A) and 10–30% expressed differentiation markers associated with effector T cells (i.e., PD-1). Thus, both our cytometric analysis and PCR studies demonstrated the heterogeneity of influenza-specific T cells in memory phase.

Since previous studies have shown that CD8 T cells up-regulate perforin and granzyme concomitantly with the down-regulation of CCR7 and CD28, we next sought to investigate the expression of these lytic proteins. Because EMRA cells were observed too infrequently for reliable results, we focused our analysis on EM28<sup>+</sup> and EM28<sup>−</sup> cells. Although the majority of influenza-specific T cells were negative for these lytic proteins, small but sizable cell fractions clearly expressed granzyme B and perforin (Fig. 3A). This was the case for all donor samples (n = 6) tested. Similar to our previous observations for total CD8 T cells (30) and tumor Ag-specific CD8 T cells (23), EM28<sup>+</sup> cells were mostly negative and EM28<sup>−</sup> cells were frequently positive for both lytic molecules (Fig. 3A).

These observations, regarding the expression of granzyme B and perforin, raised the question of whether influenza-specific effector cells are present among circulating CD8 T cells in the absence of infection. Thus, we quantified granzyme B expression for PBLs withdrawn over extended periods of time from four individuals (Fig. 3B). Frequencies of influenza-specific T cells remained quite stable over time (data not shown). Although granzyme B was detectable for both CD8 T and influenza-specific cells at all time points tested, the proportions of such cells fluctuated more significantly (Fig. 3B). In some instances, changes in granzyme B expression by influenza-specific cells paralleled those of total CD8 T cells, suggesting that granzyme B expression may have been triggered through bystander activation.
Analysis of granzyme B expression in 40 PBMC samples demonstrated a significant positive correlation between levels of expression in total CD8 T cells and in influenza-specific cells (Fig. 3C, \( r = 0.425, p < 0.01 \)). This finding confirmed our hypothesis that granzyme B expression by influenza-specific T cells is enhanced when total CD8 T cells have increased granzyme B levels. Thus, taken together, our data indicate that granzyme B expression is regularly seen in small but sizable fractions of influenza-specific CD8 T cells and that the frequency of such cells may fluctuate in the absence of influenza infection.

**Immediate cytotoxic function by influenza-specific T cells from healthy adults**

Studies in mice have shown that CD8 memory T cells are not lytic ex vivo and require more than 12 h of in vitro stimulation to acquire cytotoxic function (35). Furthermore, the demonstration of lytic protein expression by effector cells is not proof of functionality. Thus, taking advantage of the LiveCount assay recently developed in our laboratory, we sought to characterize the activity of influenza-specific CD8 memory T cells (27). For the assay, multimer-positive cells were sorted and incubated for 4 h with Flu-MA peptide-pulsed T2-CMTMRlow target cells, as well as with irrelevant peptide pulsed T2-CMTMRhigh target cells. Subsequently, the killing of specific and irrelevant target cells was quantified by flow cytometry.

Data obtained from the LiveCount assay demonstrated surprisingly strong cytotoxicity for the influenza-specific T cells from healthy donors, with lytic activity of similar intensity to the CTL clones used as positive controls (Fig. 4A). In addition, \( \sim 20\% \) of the influenza-specific CD8 T cells from healthy donors expressed the degranulation marker CD107a (data not shown), notably comparable to the frequency of cells expressing granzyme B and/or perforin (Fig. 3). As expected, upon antigenic stimulation, the influenza-specific cells rapidly produced inflammatory cytokines (Fig. 4B). These data demonstrate that healthy donors in memory phase maintain CD8 T cells that are immediately cytotoxic ex vivo (i.e., without prior in vitro stimulation). We hypothesize that these cells in memory phase are similarly ready to kill in vivo.

**Dominant T cell clonotypes differentiate to both memory and effector cells**

Our observations regarding influenza-specific CD8 T cells indicate that long-term T cell memory is composed of both memory and effector T cells. We thus sought to characterize their clonality to determine whether individual T cell clonotypes are able to differentiate into both types of cells. Since most A2/Flu-MA-specific TCRs are known to be BV17+ (24, 25, 36), we limited our analysis to this β-chain family.

We sorted CM, EM28+, EM28−, and EMRA cells from three healthy donor samples and generated 179 Flu-MA-specific T cell clones. Subsequently, BV17 transcripts were amplified by PCR, the products were sequenced, and the CDR3 regions of the TCR β-chains were analyzed (Table II). T cell clones were characterized as being a “dominant clonotype” when the identical β-chain sequence was found for \( \geq 5\% \) of clones analyzed per donor. Dominant clonotypes were identified for all three donors but each donor showed variable degrees of clonal diversity. Moreover, dominant clones were found among both the memory and effector subpopulations, thus demonstrating that human clonal CD8 T cell responses can give rise to T cells that differ in phenotype and in functionality (Fig. 5A). Interestingly, the full consensus motif described for BV17 CDR3 (\( S_{96-R-R-S/S-A_{100-X-X-X}} \)) was only found in three of five dominant clonotypes while the minimal \( R_{95-S_{99}} \) motif was present in all clonotypes (Table II) (37). Clones sharing identical TCR β-chains also shared identical α-chains (data not shown), thereby confirming that we identified true T cell clonotypes.

The VB17 repertoires characterized for all three donors were relatively diverse in the CM population as nondominant clonotypes made up a large percentage of those cells (C. Touvrey and D. Speiser, manuscript in preparation). For donors BCB74 and

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Immediate cytotoxic activity detected ex vivo by influenza-specific CD8 T cells from healthy donors. A. Cytotoxicity was assessed using the LiveCount assay (27). Influenza-specific cells from two healthy donors were sorted and coincubated for 4 h with titrated amounts of T2 target cells. Influenza-specific T cell clones were used as positive controls. B. Intracellular staining with TNF-α-specific Ab. PBMC from healthy donors were stimulated for 4 h with influenza matrix peptide (gray filled peak) or with HIV peptide (black solid line).

<table>
<thead>
<tr>
<th>Donor</th>
<th>Dominant Clonotype</th>
<th>BV17 CDR3 Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCB74</td>
<td>1</td>
<td>CASRSESGLFFGEG</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CASRSDTDQYFGPG</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CASRSDTDQYFGPG</td>
</tr>
<tr>
<td>BCB103</td>
<td>1</td>
<td>CASRSDTDQYFGPG</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CASRSDTDQYFGPG</td>
</tr>
<tr>
<td>BCB129</td>
<td>1</td>
<td>CASRSDTDQYFGPG</td>
</tr>
</tbody>
</table>

\( ^a \) BV17 CDR3 sequence is given of each dominant clonotype. Clonotypes were considered dominant when \( \geq 5\% \) of clones expressed a given TCR α− and β− chain (α-chain, data not shown). Clonotype numbers were attributed by order of frequency, the same numbering is used in Fig. 4A.
BCB103, the TCR diversity appears to have decreased with progressive states of differentiation as the repertoire of the EM28$^+$ cells was dominated by a single clonotype (Fig. 5A). For both of these donors, the dominant clonotypes were found in each of the analyzed stages of differentiation. However, although dominant clonotypes were identified for donor BCB129, they made up only a minor fraction of the whole A2/Flu-MA-specific population. Furthermore, the dominant clonotypes for this donor were less broadly distributed among cells at varying stages of differentiation. Nevertheless, the dominant clonotype of this donor was expressed by 50% of the EM28$^+$ cells. Thus, for all three donor samples that were analyzed, we identified dominant clonotypes that were found among multiple memory and effector cell subsets.

**Dominant T cell clonotypes express high-avidity TCRs**

T cell clones were functionally tested in $^{51}$Cr release assays with titrated peptide. These assays revealed that the TCRs recognized A2/Flu-MA with strong avidity, as 50% maximal lysis (IC$_{50}$) was at $\sim 10^{-10}$ M peptide (Fig. 5B). Interestingly, similar functional avidity was observed for sister clones derived from CM, EM28$^+$, EM28$^-$, and EMRA cells (Fig. 5C). This finding suggests that it is the TCR and not the state of differentiation that controls the functionality of the cells. However, because the assays were conducted with cells that had undergone several weeks of in vitro culture, it is possible that culture conditions somehow influenced their capacity to interact with Ag-bearing cells. Therefore, we cannot rule out the possibility that in vivo a clonal response may give rise to early and late differentiated T cells which differ in their Ag recognition efficacy. Nevertheless, our results indicate that identical clonotypes are found as memory and effector cells, demonstrating for the first time that human T cell memory is maintained by clonotypes that persist simultaneously in multiple differentiation stages in the absence of Ag.

**Discussion**

The phenotypes for both human and murine, influenza-specific CD8 T cells in memory phase have been widely characterized by single parametric analysis (38, 39). For this work, we took advantage of multiparameter flow cytometry, coupled with ex vivo analysis, to further elucidate human influenza-specific CD8 T cells. We found that influenza-specific T cells comprise a large array of subsets, including CM, EM28$^+$, EM28$^-$, and EMRA cells. Therefore, influenza-specific CD8 T cells in memory phase have phenotypic characteristics of both memory and effector cells. We also found that these cells persist stably for many years. Our findings thus confirm and extend the notion that memory T cells are heterogeneous and are maintained long term (29, 40–43).

Among the identified subsets, EM cells were predominant, and mostly displayed an early differentiated phenotype (CD127$^+$ CD28$^-$). This observation is in accordance with previous work in which it has been shown that, after Ag clearance, the majority of CD8 T cells continue to express CD28 and CD127 or they progressively re-express CD127 (7, 8). In the mouse model of influenza infection, the resolution of infection is followed by a rapid increase of percentages of CM cells which become the dominant population (17). In contrast, we found that only a small proportion of influenza-specific CM cells (~10% of total specific CD8 T cells) remained stable over time and EM28$^+$ cells appeared as the dominant population.

More surprising was the consistent detection of both EM28$^-$ cells (~10%) and EMRA cells (~5%). Down-regulation of CD28 has been described in T cells specific for persisting viruses such as CMV (40, 42), as well as for tumors (9). Thus, it was rather unexpected to find such cells among a population of influenza-specific T cells. In addition, for donors with relatively low frequencies of influenza A2/Flu-MA multimer$^+$ cells (Table I), a large proportion of the EM28$^-$ cells expressed granzyme B and perforin. The one exception to this observation was for donor BCB115. Cells from this donor comprised a large percentage of influenza-specific cells (2.82% of CD8 T cells) with high levels of granzyme B, perforin, and PD-1 expression. Due to the possibility that this individual may have recently recovered from influenza infection, the sample was excluded from subsequent phenotypic analysis.
A recent study suggests that the permanent expression of perforin and granzymes is not necessary because they can be rapidly up-regulated upon cell activation (44). The study also showed that activation induces conformational changes in perforin, resulting in its lack of detection by the commonly used anti-perforin mAb 8G9. Therefore, activation- induced perforin up-regulation may in fact be underestimated. Nevertheless, our detection of perforin and granzyme B indicates that these molecules are regularly expressed by a small fraction of circulating T cells, despite the absence of Ag.

In memory phase, T cells are thought to be devoid of immediate cytotoxic function (15, 35). The discovery of basal to intermediate levels of cytotoxic function in immune mice in memory phase has challenged this view (10, 45). In this study, we report that human influenza-specific T cells exert Ag-specific cytotoxicity directly ex vivo, indicating that steady-state CD8 T cell memory includes a population of functional effector cells. The cytotoxic function observed was of considerable intensity, comparable to fully functional T cell clones, and thus greater than previously described in mice (10, 45). Thus, taken together our results demonstrate that perforin- and granzyme-expressing effector cells against influenza virus, with immediate cytotoxic function, persist in humans after clearance of Ag.

Memory T cells lack the high-avidity IL-2R, but depend on other common γ-chain receptor cytokines such as IL-7 and IL-15. Although IL-7 supports survival of memory cells more than effector cells (7, 8, 46, 47), IL-15 promotes proliferation of both memory and effector cells. We found corresponding expression of these two cytokine receptors, with frequent expression of IL-7Rα by influenza-specific memory T cells (CM and EM28+ cells). EM28+ cells also expressed IL-15Rα, albeit at a lower frequency, and the antiapoptotic factor Bcl-2 that is regulated via IL-7 and IL-15 (48). Bcl-2 expression, however, was heterogeneous and did not strictly correlate with IL-7Rα or IL-15Rα expression, possibly due to the involvement of additional factors in Bcl-2 regulation.

In contrast to the influenza-specific memory populations (CM and EM28+ cells), influenza effector (EM28+ cells) expressed less IL-7Rα and Bcl-2, suggesting that they are more prone to apoptosis than the memory cells. EM28+ cells also showed enhanced expression of PD-1. PD-1 attenuates TCR-mediated activation of IL-2 production through the modulation of ZAP70 phosphorylation. It has also been shown that blockade of the IL-1 pathway, with Abs against PD-L1, restores proliferation of so-called exhausted cells, i.e., effector CD8 T cells with reduced function in chronic infection (31–33). This may seem inconsistent with our findings because we showed that influenza-specific effector cells express PD-1 and at the same time are able to persist in vivo for >10 years and are immediately functional ex vivo. One possible explanation regarding PD-1 expression by the EM28− cells comes from the work of Dong et al. (49); they demonstrated that PD-L1 can stimulate T cell proliferation. The apparent discrepancies regarding the role of PD-1 in cell proliferation could be explained by the fact that PD-L1-specific Abs (31–33) inhibit interactions, not only between PD-1 and PD-L1, but also between B7-1 and PD-L1 (50), an interaction implicated in the control of cell proliferation. Lastly, PD-1 expression is linked to T cell differentiation and is rapidly increased following TCR stimulation (51, 52), suggesting that it may control T cell function before exhaustion.

Along with PD-1, the EM28− cells also expressed KLRG1, an NK receptor that transmits inhibitory signals through its interactions with E-cadherin. Others have shown that KLRG1 is found on effector CD8 T cells, during both acute and chronic viral infections and that KLRG1+ T cell proliferate poorly in vivo and in vitro after TCR challenge (34, 53, 54). KLRG1-expressing T cells can be divided into two populations based on the expression of CD57. The KLRG1+CD57− fraction has characteristics of functional memory cells (CD28+CD27+CD127+), whereas the CD57+ fraction is terminally differentiated (55). Indeed, we observed significant CD57 expression (7.5 ± 5.8%) by influenza-specific T cells, the majority of which were also CD28− (data not shown). We have recently reported that melanoma Ag-specific T cell clones do not express KLRG1 without impairing in vivo proliferative and cytotoxic capacities (23), further supporting the notion that at least some KLRG1+ CD8 T cells are functional in vivo.

Recent studies have demonstrated that CM CD8 T cells (CD27+CD127+CD62L+) became CD27− and CD127− after adoptive transfer to the lungs of recipient mice (56). Interestingly, this effector phenotype was adopted by all CD8 T cells and was maintained for extended periods of time (56, 57). These findings suggest that pulmonary infection somehow causes the differentiation of effector cells in an Ag-independent manner. Two possible mechanisms can explain the activation of “unspecific” T cells: 1) TCR cross-reactivity to other Ags/pathogens and 2) Ag- nonspecific bystander activation. The former mechanism was proposed by Ostler et al. (58) after their observation that the “bystander recruitment” of lymphocytic choriomeningitis virus-specific memory CD8 T cells to the lungs of respiratory syncytial virus-infected, lymphocytic choriomeningitis virus-immune mice, not only failed to enhance immunity (i.e., bystander activation did not occur), but also delayed the recruitment of respiratory syncytial virus-specific T cells (58). However, given the specificity of TCR-pMHC interactions (even single amino acid changes in the TCR-pMHC interface can abolish binding), the probability of bona fide cross-reactivity resulting in TCRs signals that are strong enough for T cell activation is likely very low.

Evidence for the mechanism of nonspecific bystander activation is quite extensive. It has been shown, for example, that in microenvironments where innate and specific immune responses develop in parallel, neighboring T cells with largely unrelated TCRs can be coactivated (59–62). The recruitment of nonspecific T cells to inflammatory sites has also been demonstrated (63). Even in Ag-nonspecific bystander activation, however, it appears that TCR signaling plays a role in activation because the mechanism has been shown to be MHC class I dependent (64). A recent study of mice infected with mycobacteria (65) demonstrated that bystander activation can trigger large numbers of T cells with unrelated TCRs via mechanisms similar to those responsible for homeostatic CD8 T cell proliferation (i.e., through low-affinity TCR interactions, presumably with self-peptide-MHC, along with stimulation by IL-7, IL-12, IL-15, and IFN-αβ) (6, 66, 67). In our study, we found that levels of granzyme B expression in influenza-specific CD8 T cells paralleled, with statistical significance, levels in the total CD8 T cell population. This suggests that immune responses to unrelated pathogens may indeed boost influenza-specific T cells. Therefore, the maintenance of activated effector, influenza-specific human CD8 T cells in memory phase may be, at least in part, mediated by bystander activation.

Our data provide evidence that human influenza-specific CD8 T cell memory is composed of at least two functionally distinct populations of cells that are stably maintained long term. Because identical clonotypes were found among both populations, they are probably derived from shared naive T cell precursors and are thus related. This finding is in contrast to an early study by Baron et al. (22), in which it was suggested that the repertoires of circulating human CD8 CM and EM T cells are largely distinct (22). The characterization of TCR repertoires is challenging and technical obstacles preclude the sequencing of all TCR sequences in an immune response. However, several other recent studies have also
shown that many identical TCR sequences are shared among memory and effector T cell populations (17, 20, 21), further supporting the notion that the cells are related. Moreover, a recent adaptive transfer study in which it was shown that a single T cell can give rise to the full spectrum memory and effector T cells (19) provides proof of principle for multipotent clonotypic differentiation. Therefore, we propose that the clonotypes shared among the various populations of anti-influenza CD8 T cells characterized in this study are in fact related.

The human influenza-specific effector cells identified in this study have immediate cytotoxic function, and they are commonly and stably maintained long-term postinfection. It is therefore likely that they play a significant role in protection at the time of viral re-exposure. Although memory cells give rise to accelerated immune responses, compared with the primary immune response, they may fail to provide effective defenses immediately following infection during the time needed for them to proliferate and differentiate. The effector cells that we characterized in this study, with their immediate cytotoxic capabilities, may therefore play a critical role in the killing of infected cells during the early phase of pathogen invasion and multiplication. Through their immediate cytotoxic activity they may help to limit viral titers while a fully protective secondary immune response is mounted. Whether or not effector T cells in memory phase are a feature common to protection against other pathogens or malignant diseases should be explored. Furthermore, elucidation of the underlying mechanism of protection by effector T cells in memory phase, as well as how they evolve after the primary infection, may enable the development of novel immunotherapeutic treatments.

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