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Functional Heterogeneity of Cytokines and Cytolytic Effector Molecules in Human CD8\(^{+}\) T Lymphocytes

Johan K. Sandberg, Noam M. Fast, and Douglas F. Nixon

CD8\(^{+}\) T cells use a number of effector mechanisms to protect the host against infection. We have studied human CD8\(^{+}\) T cells specific for CMV pp65\(_{550-552}\) epitope, or for staphylococcal enterotoxin B, for the expression patterns of five cytokines and cytolytic effector molecules before and after antigenic stimulation. Ex vivo, the cytolytic molecule granzyme B was detected in a majority of circulating CMV-specific CD8\(^{+}\) T cells, whereas perforin was rarely expressed. Both were highly expressed after Ag-specific activation accompanied by CD45RO up-regulation. TNF-\(\alpha\), IFN-\(\gamma\), and IL-2 were sequentially acquired on recognition of Ag, but surprisingly, only around half of the CMV-specific CD8\(^{+}\) memory CD8\(^{+}\) T cells responded to antigenic stimuli with production of any cytokine measured. A dominant population coexpressed TNF-\(\alpha\) and IFN-\(\gamma\), and cells expressing TNF-\(\alpha\) only, IFN-\(\gamma\) only, or all three cytokines together also occurred at lower but clearly detectable frequencies. Interestingly, perforin expression and production of IFN-\(\gamma\) and TNF-\(\alpha\) in CD8\(^{+}\) T cells responding to staphylococcal enterotoxin B appeared to be largely segregated, and no IL-2 was detected in perforin-positive cells. Together, these data indicate that human CD8\(^{+}\) T cells can be functionally segregated in vivo and have implications for the understanding of human CD8\(^{+}\) T cell differentiation and specialization and regulation of effector mechanisms. The Journal of Immunology, 2001, 167: 181–187.

Naive CD8\(^{+}\) T cells are activated by Ags presented by MHC class I molecules in secondary lymphoid organs, proliferate, and differentiate to become effector and memory CD8\(^{+}\) T cells. These cells have a broad set of effector mechanisms at their disposal to combat infection, including expression of cytokines, chemokines, and lytic effector molecules such as perforin, granzymes, and CD95L. Secretion of cytokines and chemokines have both local and systemic effects, whereas lysis of infected cells by components of CD8\(^{+}\) T cell cytotoxic granules and CD95L-induced apoptosis depend on cell-cell contact (1, 2).

Cytokines play important and diverse roles in controlling many viral infections (1, 3–5). For example, IFN-\(\gamma\) and TNF-\(\alpha\) can abolish replication of hepatitis B virus and lymphocytic choriomeningitis virus (LCMV) in hepatocytes and clear the infection without destruction of infected cells (6, 7). Although important for immune defense, TNF-\(\alpha\) and IFN-\(\gamma\) also are the primary mediators of septic shock caused by bacterial toxins (8). However, during LCMV infection, effector T cells in mice can rapidly turn on cytokine production on recognition of specific Ag, and rapidly turn off production when Ag stimulation ceases (9). This Ag-specific regulation of cytokine release provides a means to localize the effects of cytokines in the vicinity of infection while limiting the dangerous systemic effects (10).

Another major attribute of CD8\(^{+}\) T cells is their ability to kill cells expressing specific peptide epitopes in complex with MHC class I through the release of cytotoxic granules containing perforin and granzymes A and B (2). Work with mice deficient in perforin have shown that this effector molecule is necessary for clearance of acute LCMV infection and for defense against Theiler’s virus and ectromelia virus (11–13), and it is of less importance for defense against other viruses including cowpox, coxsackievirus, and Semliki Forest virus (1, 2, 11, 13, 14). Perforin facilitates the entry of the granzymes A and B into target cells, where they induce apoptosis (15). The relative role of perforin and granzymes in inducing target cell death is uncertain, although recent evidence indicates that the importance of granzymes may be greater than previously thought (16, 17). Most interestingly, mice lacking granzyme A and B are equally incapable of controlling ectromelia infection as the perforin-deficient mice (18).

The CD45RA, CD27, and CD57 markers have been used to distinguish subsets of human CD8\(^{+}\) T cells (19). According to this classification, naive cells are believed to be CD27\(^{+}\)CD45RA\(^{+}\)CD57\(^{-}\) and effector cells CD27\(^{-}\)CD45RA\(^{-}\)CD57\(^{+}\), whereas memory cells express CD27 and are negative for CD45RA and CD57, based on functional characteristics on polyclonal stimulation. In contrast, the CD45RO isoform replaces CD45RA in T cells after activation and has thus been used as a memory CD8\(^{+}\) T cell marker (20). Because CD45RO\(^{+}\) cells can reacquire expression of the RA isoform, it has been suggested that long-lived memory cells may be found in the CD45RA/RO double-positive population (21). More recently, Ag-experienced circulating T cells were grouped into effector memory and central memory cells, based on expression of the lymph node homing receptors chemokine receptor 7 and CD62L (22). The corresponding populations also have been described and carefully investigated in mice with CD62L and CD44 (23).

Although it is known that human CD8\(^{+}\) T cells do express cytokines and cytolytic effector molecules, less is known about the phenotype of CD8\(^{+}\) T cells responding with cytokine production...
and cytolyis on recognition of specific Ag. Furthermore, whether different cytokines and cytolytic molecules are uniformly coexpressed on activation with Ag, or form discrete subpopulations, is unknown. In this paper, we have studied human CD8 T cells specific for the HLA-A2-restricted CMV pp65495–503 epitope and cells responding to the superantigen staphylococcal enterotoxin B (SEB). CMV is a ubiquitous human pathogen that persists indefinitely under efficient control by the immune system. However, reactivation of the virus can cause severe disease in immunosuppressed individuals, and strong CD8 T cell responses to CMV are commonly detected in HIV-infected subjects (24–26). SEB is produced by the common human pathogen Staphylococcus aureus and activates T cells bearing TCR with specific Vβ regions (27). We find that CD8 T cells responding to these Ags are functionally more diverse than previously thought. We hypothesize that the CD8 T cell compartment may be able to choose preferential effector mechanisms depending on site of infection, type of infectious agent, and severity of infection. The results are further discussed in relation to the identity of effector and memory T cell populations, and the immunoregulatory role of perforin.

Materials and Methods

Cells, Ags, and study subjects

PBMC from healthy donors were isolated from heparinized whole blood by Ficoll-Paque PLUS density gradient centrifugation (Amer sham Pharma c Biotech, Uppsala, Sweden), and washed three times in RPMI 1640 before use in functional assays or cryopreservation. The study was approved by the institutional review board. B cell lines (BCL) were obtained by EBV transformation and were maintained at 37°C and 5% CO2 in complete tissue culture medium; RPMI 1640 tissue culture medium supplemented with 15% FCS, 50 μg/ml streptomycin, 100 μg/ml penicillin, and 2 mM l-glutamine. The peptide corresponding to the HLA-A2-restricted CMV pp65495–503 NPVPMVATV epitope was synthesized using solid phase F-moc chemistry forin and granzyme B content in CD8 T cells, as determined by HLA-A2/pp65 tetramer staining, for 6 days in complete tissue culture medium in the presence of 0.1 μM of pp65495–503 peptide. These T cell lines were subsequently analyzed for expression of surface markers, intracellular perforin, and granzyme B or used as effectors in a cytotoxicity assay. CTL activity was measured in a standard 3Cr-release assay. Briefly, peptide-coated BCL target cells were prepared by incubating cells with 10 μM peptide for 1 h at 37°C. Coated cells were labeled with 10 μl of 10 mCi/ml 31Cr for 1 h at 37°C. Titrated numbers of effector cells were incubated with 3 × 105 31Cr-labeled target cells for 4 h at 37°C, 5% CO2. After incubation, released radioactivity was measured and specific lysis was calculated according to the following formula: % specific release = (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100.

Results

Mixed phenotype of CMV pp65 Ag-experienced CD8 T cells

We first were interested in the phenotype of human CD8 T cells specific for a well described MHC class I-restricted peptide epitope, in terms of surface markers, cytolytic effector molecules, and cytokines produced after recognition of Ag. We addressed this by investigating circulating CD8 T cells specific for CMV with four-color cytometry. Of 21 HLA-A2-positive healthy donors (H1–H21), PBMC from donors H3, H7, and H18 had detectable numbers of CD8 T cells specific for the HLA-A2-restricted CMV pp65495–503 epitope as determined by tetramer staining. This CD8 T cell population has experienced Ag, is readily detectable with HLA-A2/pp65 tetramers, and could be expected to have either a memory or an effector phenotype. The frequency of pp65-specific cells ranged from 0.34 to 0.92% of CD3+8 cells in these three donors (Table I). The pp65-specific CD8 T cells displayed a mixed phenotype in terms of surface markers, although there was a dominance of CD45RO over CD45RA expression, and CD27 was more commonly expressed than CD57.

Dichotomy between perforin and granzyme B expression

To assess the functional phenotype of circulating CMV pp65-specific CD8 T cells, the HLA-A2/pp65 tetramer was used together with intracellular staining with Abs against perforin and granzyme B directly ex vivo. Surprisingly, most of pp65-specific cells were low or negative in perforin expression, whereas 43–67% of these cells were positive for granzyme B (Table II and Fig. 1). The relative lack of perforin expression correlated with an absence of cytolytic capacity ex vivo against BCL target cells incubated with the CMV pp65495–503 peptide (Fig. 1).

Intracellular cytokine staining after 6 h of stimulation with peptide in vitro showed that the pp65-specific cells produce IFN-γ, TNF-α, and IL-2 in response to Ag as expected (Table II). However, only around half of pp65-specific CD3+8 cells produced IFN-γ or TNF-α. IL-2 expression could be detected in only <15%
of the specific CD8<sup>+</sup> T cell population. Taken together, these data show that expression of perforin and granzyme B is not coordinately regulated in the CMV Ag-experienced CD8<sup>+</sup> T cell population. Furthermore, only around half of all T cells carrying HLA-A2/pp65-specific TCRs respond with production of any of the cytokines measured on recognition of CMV peptide Ag.

**The phenotypic shift associated with Ag-driven differentiation into effector CTL**

The CMV pp65-specific and HLA-A2-restricted CD8<sup>+</sup> T cells detected in circulation have proliferated and differentiated in response to CMV infection in vivo. A clear majority of CMV pp65-specific CD8<sup>+</sup> T cells in all three subjects do not express the lytic effector molecule perforin, and no specific CTL activity was detected ex vivo. Thus, these cells are most probably not lytic effector CTL, but rather some kind of memory T cells. To investigate the response and phenotypic changes in these T cells on recognition of Ag, PBMC from these donors were cultured for 6 days in the presence of CMV pp65 peptide in vitro. As expected, there was a strong expansion of the CMV pp65-specific CD8<sup>+</sup> T cell population. Furthermore, virtually all of these cells acquired expression of both perforin and granzyme B (Fig. 1). Acquisition of perforin correlated with lytic capacity against pp65-peptide-coated BCL target cells (Fig. 1).

We next investigated the expression of surface markers before and after the 6 day in vitro stimulation (Fig. 2). CD45RA/RO isoform expression changed from a mixed pattern to predominantly CD45RO<sup>+</sup> and CD45RA<sup>+</sup>. CD27, CD28, and CD57 expression remained mixed. Thus, short-term in vitro stimulation of CMV pp65-specific CD8<sup>+</sup> T cells leads to uniform expression of lytic effector molecules and acquisition of lytic capacity. This functional shift is accompanied with up-regulation of CD45RO and down-regulation of CD45RA.

**Analysis of IL-2, TNF-α, and IFN-γ coexpression reveals functional heterogeneity in CD8<sup>+</sup> T lymphocytes**

Although it is well-established that CD8<sup>+</sup> T cells produce diverse cytokines on antigenic stimulation, it is not known whether these cells are homogeneous with regard to coexpression of cytokines. Therefore, we investigated the expression of IL-2, IFN-γ, and TNF-α in CD8<sup>+</sup> cells on direct ex vivo 6-h stimulation of PBMC from donors H3, H7, and H18 with CMV pp65 peptide with four-color flow cytometry and simultaneous triple cytokine staining. Patterns of coexpression of the three cytokines were analyzed by gating and calculating the number of cells staining for one, two, or three cytokines. Initial analysis showed that IFN-γ and TNF-α double-expressing cells were the most prevalent in the CMV pp65-specific CD8<sup>+</sup> T cell population (Fig. 3). CMV-specific cells producing either IFN-γ only or TNF-α only also could be detected as well as a small population, 5–10% of responding cells, expressing all three cytokines.

**Table I. Diverse surface phenotype of circulating CMV pp65-specific CD8<sup>+</sup> T cells in healthy donors**

<table>
<thead>
<tr>
<th>Donor</th>
<th>CMV pp65-Specific T Cells (%)</th>
<th>CD45RO</th>
<th>CD45RA</th>
<th>CD27</th>
<th>CD28</th>
<th>CD57</th>
<th>CD62L</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49</td>
<td>41</td>
<td>81</td>
<td>56</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>H7</td>
<td>0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59</td>
<td>35</td>
<td>64</td>
<td>50</td>
<td>38</td>
<td>27</td>
</tr>
<tr>
<td>H18</td>
<td>0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73</td>
<td>34</td>
<td>44</td>
<td>34</td>
<td>32</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data shows percent CD3<sup>+</sup> HLA-A2/pp65 tetramer<sup>+</sup> cells of all CD3<sup>+</sup> cells.

<sup>b</sup> Data expressed as percent positive of all CD3<sup>+</sup> HLA-A2/pp65 tetramer<sup>+</sup> cells.

**Table II. Functional phenotype of CMV pp65-specific CD8<sup>+</sup> T cells in healthy donors**

<table>
<thead>
<tr>
<th>Donor</th>
<th>CMV pp65-Specific T Cells (%)</th>
<th>Perforin</th>
<th>Granzyme B</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>43</td>
<td>18&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>47</td>
<td>11</td>
</tr>
<tr>
<td>H7</td>
<td>0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>52</td>
<td>44</td>
<td>48</td>
<td>14</td>
</tr>
<tr>
<td>H18</td>
<td>0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18</td>
<td>67</td>
<td>55</td>
<td>47</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data expressed as percent positive of all CD3<sup>+</sup> HLA-A2/pp65<sup>+</sup> tetramer<sup>+</sup> cells.

<sup>b</sup> Cytokine production as measured by intracellular staining after a 6-h stimulation with specific peptide as described in Materials and Methods.

**FIGURE 1.** Differential expression of perforin and granzyme B in circulating vs activated CD8<sup>+</sup> T cells. Dot plots show the expression of perforin and granzyme B in CMV pp65-specific cells within the CD3<sup>+</sup> lymphocyte population in fresh PBMC (left) and after a 6-day in vitro stimulation with pp65 peptide (right). Diagrams show CTL activity against a HLA-A2<sup>+</sup> BCL pulsed with specific peptide (●) or unpulsed (○) in fresh PBMC (left) and after a 6-day in vitro stimulation with pp65 peptide (right).
Although these results showed that there exists heterogeneity in terms of cytokine expression among CD8\(^+\) T cells with specificity for the same epitope, the low frequency of CMV pp65-specific cells and sample availability hampered a more detailed analysis of the CMV-specific population. Instead, to further investigate the kinetics and coexpression patterns of IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) we used the superantigen SEB, which has the advantage of stimulating a population of larger size while still activating T cells via the TCR.

Initial analysis indicated CD8\(^+\) T cell heterogeneity in cytokine expression 6 h after recognition of SEB (Fig. 4A). Production of IFN-\(\gamma\) and TNF-\(\alpha\) was readily detected after 1 h, and significant IL-2 expression was detected first after 3 h (Fig. 5A). The number of CD8\(^+\) T cells expressing at least one of the three cytokines was close to maximal at 6 h, with only a marginal increase at 15 h. The patterns of coexpression of the three cytokines over time were analyzed by gating and calculating the number of cells staining for one, two, or three cytokines. The dominant population throughout the stimulation period coexpressed IFN-\(\gamma\) and TNF-\(\alpha\) only (Fig. 5B). However, a population that expressed all three cytokines emerged with delayed kinetics to reach a similar frequency at later time points. Minor populations, which expressed TNF-\(\alpha\) only, IFN-\(\gamma\) only, and IL-2 and TNF-\(\alpha\) together, also were detectable. However, cells expressing IL-2 only or IL-2 together with IFN-\(\gamma\) could not be detected in the response to SEB. Thus, we conclude that CD8\(^+\) T cells are clearly heterogeneous with regard to IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) expression after antigenic stimulation.

To determine the dominant cytokine profiles at early and late time points, data was plotted as a percentage of total cytokine-producing CD8\(^+\) T cells at each time point (Fig. 5C). This analysis revealed that expression of TNF-\(\alpha\) alone was the dominant phenotype at 1 h of stimulation. However, coexpression of IFN-\(\gamma\) and TNF-\(\alpha\) rapidly became the most prevalent phenotype representing up to 50% of cytokine-producing cells, followed by IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) triple expression at later time points. Approximately 30% of the cytokine-producing CD8\(^+\) T cells coexpressed IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) at later time points. Taken together, these results demonstrate that single CD8\(^+\) T cells may express one of several different cytokine profiles in response to Ag. Furthermore, the data indicate the sequential acquisition of TNF-\(\alpha\), IFN-\(\gamma\), and IL-2 expression in the subpopulation that coexpresses these three cytokines.

**FIGURE 3.** Heterogeneous cytokine expression pattern in CMV pp65-specific CD8\(^+\) T cells. Relative frequency of cells expressing IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) in PBMC from healthy donors H3, H7, and H18 after 6-h stimulation of fresh PBMC with CMV pp65 peptide epitope as described in Materials and Methods. Data expressed as percentage of the total number of cells expressing at least one cytokine.

**FIGURE 4.** Segregated expression of cytokines and perforin on recognition of superantigen. A, Simultaneous intracellular staining for two cytokines in different combinations in the CD3\(^+\)CD8\(^+\) lymphocyte population. B, Dot plots showing staining for intracellular perforin and single cytokines in the CD3\(^+\)CD8\(^+\) lymphocyte population. PBMC were stimulated for 6 h and analyzed by FACS as described in Materials and Methods. Figure shows one representative of five independent experiments.
Cytokines and perforin segregates into subpopulations of CD8\(^+\) T cells that can be distinguished by surface markers

Next we examined whether circulating perforin-expressing CD8\(^+\) T cells in healthy individuals were capable of cytokine production on recognition of SEB. Virtually no IL-2 production was observed in perforin-expressing cells (Fig. 4A). Both IFN-\(\gamma\) and TNF-\(\alpha\) production could be detected in perforin-positive cells, but at a significantly lower frequency. In light of these data, we were next interested in defining the surface marker phenotype associated with IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) production in CD8\(^+\) T cells (Fig. 6). IL-2-secreting cells were almost exclusively CD27\(^+\), CD28\(^+\), and CD57\(^+\). The pattern of CD45 expression was less distinct, although the majority were CD45RO\(^-\) and CD45RA\(^+\). IFN-\(\gamma\)-expressing cells showed a pattern of cell surface marker expression similar to IL-2-expressing cells. However, although the majority (~80%) of IFN-\(\gamma\)-positive cells were CD27\(^+\), CD28\(^+\), and CD57\(^+\), there also were some IFN-\(\gamma\)-expressing cells within the reciprocal subsets. TNF-\(\alpha\)-expressing CD3\(^+\)8\(^+\) cells showed a similar distribution of markers as the IFN-\(\gamma\)-expressing cells. In contrast, perforin-positive cells were almost exclusively CD57\(^+\) and CD45RA high, while being largely negative for the CD27 and CD28 markers, as well as for CD45RO (Fig. 6). These cells also were CD56\(^-\) and CD161\(^-\) (data not shown). Thus, perforin expression in peripheral blood of healthy individuals is found in CD8\(^+\) T cells with a surface phenotype largely opposite that of IL-2-, IFN-\(\gamma\)-, and TNF-\(\alpha\)-producing cells. Taken together these data indicate that expression of cytokines and perforin segregates into subpopulations of CD8\(^+\) T cells that can be distinguished by surface markers.

Discussion

In this paper, we have investigated the expression patterns and kinetics of three cytokines and two cytolytic effector molecules that human CD8\(^+\) T cells use to protect the host against infection. By studying circulating CD8\(^+\) T cells specific for the HLA-A2-restricted CMV pp65 epitope as well as the population responding to the superantigen SEB, we find that the expression patterns of cytokines, perforin, and granzyme B in these cells are complex.

The finding that circulating CMV pp65-specific CD8\(^+\) T cells express granzyme B much more frequently than perforin indicates that these effector molecules are differentially regulated (Fig. 1 and Table II). Tight control of perforin expression seems rational given the cytotoxic potential of this agent. Granzymes need to act together with perforin to induce apoptosis in target cells, and regulating perforin may thus obliterate the need for a tight control of the granzymes (18, 32). In the mouse, memory CD8\(^+\) T cells established after LCMV infection express perforin mRNA while not being cytotoxic directly ex vivo (33). Taken together, these data suggest that when T cells are not actively recognizing Ag, they down-regulate perforin expression by a posttranscriptional mechanism but keep expressing the granzymes.

Production of cytokines segregates into discrete subpopulations and occurs with different kinetics. Detailed analysis indicates that superantigen-stimulated CD8\(^+\) T cells first initiate TNF-\(\alpha\) production, tightly followed by IFN-\(\gamma\), and IL-2 production comes late (Fig. 5). Thus, the triple-expressing phenotype is very rare after 1–2 h, although it becomes more prevalent later on. However, this phenotype does not become dominant. Instead, TNF-\(\alpha\) and IFN-\(\gamma\) double-expressing cells are dominant at 6 h of stimulation. Minor populations comprising up to 10% of responding CD8\(^+\) T cells include IL-2 plus TNF-\(\alpha\) double-expressing cells and TNF-\(\alpha\) and IFN-\(\gamma\) single-expressing cells. The combination of IL-2 plus IFN-\(\gamma\) and IL-2 single-expressing cells are virtually absent. We observe similar dominance of TNF-\(\alpha\) and IFN-\(\gamma\) double expression in analysis of CMV pp65-specific cells, although detailed analysis is harder because of lower cell numbers (Fig. 3). Our data suggest that cells expressing TNF-\(\alpha\) only or IFN-\(\gamma\) only are more common and that cells producing all three cytokines are less common in the CMV-specific population compared with the more diverse SEB-reactive population. In support of the segregation we observe in cytokine expression, single-cell analysis at the molecular level of IL-2 gene expression in mouse models have recently indicated that T cells are heterogeneous with regard to IL-2 expression (34) and that IL-2 and IFN-\(\gamma\) are rarely coexpressed after mitogenic stimulation (35).

![Graph A](#)

**FIGURE 5.** Complex cytokine expression patterns in CD8\(^+\) T cells responding to superantigen assessed by four-color flow cytometry. A, Kinetics of IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) expression by CD8\(^+\) T cells stimulated with SEB. B, Frequency of CD8\(^+\) cells displaying cytokine coexpression profiles expressed as percentage of the total number of CD8\(^+\) cells. FACs data was gated to determine the frequency of cells producing single cytokines alone, two cytokines in combination, or all three cytokines. C, Sequential acquisition of TNF-\(\alpha\), IFN-\(\gamma\), and IL-2 in CD8\(^+\) T cells responding to Ag. The relative frequency of CD8\(^+\) cells displaying one of the different cytokine coexpression profiles was calculated and expressed as percentage of the total number of CD8\(^+\) cells expressing at least one cytokine at each time point. Legend in C same as in B. Figure shows one representative of five independent experiments.
The functional heterogeneity in CD8\(^+\) T cells that we have observed may reflect an ability to adjust immune effector mechanisms depending on the task. Experiments with gene knockout mice have shown that perforin is necessary in defense against ectromelia virus, whereas perforin deficiency had little impact on resistance to cowpox virus (13). Also, IFN-γ together with TNF-α can clear hepatitis B virus and LCMV from hepatocytes noncytopathically (6, 7). Our hypothesis is that the CD8\(^+\) T cell compartment may be able to choose preferential effector mechanisms depending on site of infection, type of infectious agent, and severity of infection.

Circulating CMV pp65-specific CD8\(^+\) T cells are heterogeneous with regard to the markers CD45RO, CD45RA, CD27, CD28, CD57, and CD62L (Table I and Ref. 36). Because perforin expression correlates with CMV pp65-specific CTL activity, expression of perforin may be a reliable marker to distinguish a lytic effector T cell population from a nonlytic memory cell population. The granzyme B\(^+\) perforin\(^-\) population may fall within the effector memory population previously suggested by others (22). Interestingly, immunohistochemical analysis of lymphoid tissue from HIV-infected subjects have indicated previously a relative lack of perforin compared with granzyme A (37). CMV pp65-specific T cells in HIV-infected subjects were recently found to express perforin more frequently than observed here in healthy individuals (38). This difference may relate to reactivation of CMV in HIV-infected subjects.

Perforin-expressing cells within the circulating polyclonal CD8\(^+\) T cell population in healthy donors is generally positive for CD45RA and CD57, while being mostly negative for CD27, CD28, and CD45RO. The finding that circulating perforin-positive cells are generally CD27\(^-\) and CD45RA\(^+\) previously have been taken as evidence that these two markers can be used to distinguish specific effector CTL (19). However, we observe that differentiation of circulating CMV pp65-specific T cells into lytic CTL in vitro is associated with a down regulation of CD45RA. Furthermore, CD45RO is up-regulated, and no clear pattern of CD27, CD28, or CD57 expression can be detected (Fig. 2). Thus, by studying an epitope-specific CTL population we get indications that active effector CTL are characterized by high CD45RO expression and absent or low CD45RA expression. This is supported by the findings in patients with infectious mononucleosis by Callan et al. (30), indicating that EBV-specific CD8\(^+\) T cells are largely CD45RO\(^+\) and CD45RA\(^-\) during the acute phase of disease when Ag is abundant.

Our results indicate that neither circulating CMV-specific CD8\(^+\) T cells nor activated CMV pp65-specific effector CTL are contained within the CD45RA\(^+\)CD57\(^-\) perforin\(^+\) triple-positive population of circulating CD8\(^+\) T cells. Perforin generally is found in cells with a surface phenotype opposite that of the cells producing cytokine in response to SEB, and cytokine-producing cells are rarely found in the perforin-containing population (Figs. 4B and 6). Thus, perforin expression and cytokine production in response to SEB appears largely segregated in healthy individuals. Increasing evidence indicates that perforin plays an important role in downregulating immune responses and autoimmunity (39–43). Furthermore, CD57\(^+\) T cells are expanded in HIV infection (44) and can suppress generation of EBV-specific CTL in vitro (45). Oligoclonal expansions of CD57\(^+\) CD8\(^+\) T cells have been shown to occur in CMV infection (46). However, only a minority of these cells were CMV-specific and MHC-restricted, and the major activity displayed by these cells was MHC-unrestricted proliferation (46). Taken together, these results open the possibility that the CD45RA\(^+\)CD57\(^+\) perforin\(^+\) population contains cells with an immune-regulatory role rather than being CTL-engaged in immune defense against pathogens.

In summary, we have shown that the expression patterns of five different molecules that human CD8\(^+\) T cells use to respond against infection are unexpectedly complex. We have further analyzed the phenotype and function of an epitope-specific CD8\(^+\) T cell population and the identity of perforin-expressing cells ex vivo. These aspects of CD8\(^+\) T cell biology are of importance for our understanding of how these cells function to protect the organism against infectious disease.

Acknowledgments
We thank Graham S. Ogg for kindly providing the HLA-A2 tetrameric complexes used in this study.

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co-expressed with granzyme A within cytotoxic granules in CD8+ T lymphocytes

present in lymphoid tissue during chronic HIV infection. AIDS 12:1325.


