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Human Late Memory CD8+ T Cells Have a Distinct Cytokine Signature Characterized by CC Chemokine Production without IL-2 Production

Tae Kon Kim,*‡ Lisa S. St. John, † Eric D. Wieder,* Jahan Khalili, †‡ Qing Ma, †‡ and Krishna V. Komanduri‡‡

Late memory T cell skewing is observed in the setting of immune recovery after cord blood transplantation, and may be associated with inferior control of viral reactivation and cancers. Therefore, we sought to understand how late memory cells differ functionally from earlier stage memory T cells, and whether surface phenotypes associated with differentiation stages were predictably associated with functional signatures. Higher order cytokine flow cytometry allows characterization of human T cells based on complex phenotypic markers and their differential capacity to simultaneously secrete effector proteins, including cytokines and chemokines. We used 8-color, 10-parameter cytokine flow cytometry to characterize the functional activation of human late memory CD8+ T cells defined by CD45RA and CD27 expression (CD27+CD45RA+). We assessed the 15 possible functional signatures of cells defined by production of IL-2, IFN-γ, TNF-α, and MIP-1β alone or in combination, following activation with Ags stimulating bypassing surface proteins (PMA/ionomycin) or through the TCR (e.g., viral Ags). Late memory CD8+ T cells produced abundant amounts of CC chemokines (MIP-1β, MIP-1α, and RANTES) but not IL-2. IL-2/IFN-γ coproduction, characteristic of protective immune responses to viral infections, was absent in late memory CD8+ T cells. These data demonstrate that functional cytokine signatures are predictably associated with CD8+ maturation stages, and that the polarization of late memory CD8+ T cells toward CC chemokine production and away from IL-2 production suggests a unique functional role for this subset.

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In humans, we have had to rely primarily on cross-sectional assessments of Ag-specific immune responses to characterize T cell differentiation. One inherent problem is the need to use surrogate markers of naive and memory T cell differentiation; variations in subsets defined by such surface markers have led to the lack of a consistent language used to define maturation stages. This variation has led to the use of semantic terms (e.g., central, effector, and late memory cells) that are often inconsistent among studies, in part due to varying marker combinations or functional measures. Despite these limitations, we are beginning to better understand the consequences of T cell differentiation, especially as more advanced technological approaches to assess phenotypic and functional characteristics of fine T cell subsets have been developed.

The rapid advancement of flow cytometry has facilitated a more sophisticated understanding of cell surface markers associated with T cell maturation. Initially, CD45R isotypes were found to demarcate naive (RA+/RO+) and memory (RA-/RO+) cells (2, 3). However, with the demonstration by studies that Ag-primed CD45RO+ T cells could revert to CD45RA+ T cells (4–6), additional markers including CCR7 were suggested as a way to divide RA-/RO+ memory cells into earlier (“central” cells, capable of lymph node homing) and later (“effector”) memory cells (7). Additional studies demonstrated that down-regulation of CD27 and CD28 could also be used to define CD4+ and CD8+ T cell maturation stages (8–12). In addition, other markers (e.g., CD57) may define subsets of late memory CD8+ T cells characterized by replicative senescence (13). These studies support the view that the use of at least two surface phenotypic markers (e.g., CD45RA and CD27) is required to divide cells into earlier and later stages of the T cell maturation spectrum.

Fewer studies have focused on the role of late memory T cells, despite their known association with immunodeficiency states in humans. The frequency of late stage memory T cells has been
reported to increase with aging, possibly leading to increased risk for infection in the elderly (14–16). Our prior clinical studies have found that delayed immune reconstitution after cord blood transplantation is associated with skewing of the circulating T cell pool toward late memory cells, a likely result of a failure of thymopoiesis to renew the naive and early memory T cell pool in this setting (17). Other studies have suggested that such late memory skewing may result in T cells with impaired proliferative capacity. Hamann et al. (9) showed that CD45RA+/CD27−CD8+ T cells did not proliferate in response to anti-CD2 and IL-2. Champagne et al. (18) and Geginat et al. (19) demonstrated that CD45RA−CCR7−CD8+ T cells divide less when stimulated with anti-CD3 mAb or allogeneic mature dendritic cells (DC).³

Although traditional studies focused on cytolytic activity of CD8+ T cells (20, 21), it is increasingly apparent that cytokine production, specifically production of IL-2, is strongly associated with the ability of CD8+ T cells to mediate sustained protective immunity against viruses and cancer cells through memory maintenance. Harari et al. (22) found that cells producing IFN-γ alone are associated with progressive disease in HIV-1-infected subjects. Duvall et al. (23) showed HIV-2-specific T cells are more polyfunctional than those specific for HIV-1, which is associated with delayed disease progression. In a murine cancer immunotherapy model,Gattinoni et al. (24) demonstrated that fully differentiated melanoma-specific effector cells had impaired antitumor efficacy, despite their ability to secrete abundant amounts of IFN-γ and their ability to release perforin and induce cytotoxicity in traditional assays of lytic function, whereas earlier memory cells with identical specificity effectively controlled tumor growth.

Because of increasing evidence that late memory skewing is associated with functional impairment of the immune response, we sought to comprehensively analyze the associations between maturation stage and effector cytokine production in CD8+ T cells from healthy donors. Using 8-color cytokine flow cytometry (CFC) assays, we assessed human CD8+ T cell responses induced by stimuli both bypassing and using the TCR and proximal downstream signaling molecules, including superantigens, phorbol esters, and CMV Ags. We then assessed the production of all possible combinations of the CD8+ effector proteins IL-2, IFN-γ, TNF-α, and the CC chemokine MIP-1β. Our results demonstrate that human CD8+ maturation stages are associated with predictable and distinct cytokine signatures, and that late memory CD8+ T cells have distinct functional properties that suggest a compartmentalized role in the human immune response.

Materials and Methods

Cells

PBMC were obtained by sedimentation using Histopaque 1077 (Sigma-Aldrich) from heparinized whole blood from healthy volunteers (ranging in age from 32 to 60 years) and cryopreserved for further analysis. All cell preparations were >95% viable by trypan blue exclusion.

CFC assays

A total of 10⁶ thawed PBMC were incubated with 10 µg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich) and 1 ng/ml PMA with 1 µM ionomycin together (Sigma-Aldrich) in 96-well V-bottom plates in 200 µl of medium (RPMI 1640; Life Technologies) supplemented with 10% FBS (Invitrogen). For CMV-specific T cell stimulation, pools of pentadecapeptides spanning the CMV pp65 capsid Ag (Jerini Peptide Technologies) were used to stimulate T cells in the presence of Abs to CD28 and CD49d, as previously described (25, 26). After 1 h, brefeldin A (Sigma-Aldrich) was added to enable accumulation of intracellular cytokines. Following an additional 5 h of incubation, we fixed and permeabilized cells with Fix/Perm A/B (Caltag Laboratories), and assessed the simultaneous expression of surface markers and intracellular effector proteins.

Assessment of naive and memory T cells segregation by flow cytometry

FACS analyses were performed using mAbs conjugated with the fluorochromes FITC, PerCP-Cy5.5, allophycocyanin, PE-Cy7, PE, PE-Texas red, allophycocyanin-Alexa Fluor 750, or Pacific blue, and specific for human CD4, CD8, IL-2, TNF-α, and MIP-1β (BD Pharmingen), CD45RA (Beckman Coulter), CD27, and IFN-γ (eBioscience). CD57 expression was assessed using a CD57-specific mAb conjugated with biotin (BD Pharmingen) followed by secondary staining with Qdot705-streptavidin (Invitrogen). After staining, cells were washed, resuspended in PBS with 1% paraformaldehyde, and analyzed by 8-color 10-parameter flow cytometry (or 9-color, for analyses including CD27) in a cytometer (DakoCytometry) or an LSR-II cytometer (BD Biosciences) using FlowJo software (Tree Star). For the most analyses, at least 3 × 10⁶ total events (10⁶ for CMV infections) were analyzed, with sequential gating of PBMC in a lymphocyte region (by scatter). Analyses were completed on T cells by assessing CD4+ or CD8+ and CD45RA−/CD27+ to demarcate naive and memory cells staining for intracellular IL-2, IFN-γ, TNF-α, and MIP-1β. Gates defining cytokine-positive populations were defined based on the upper limits of fluorescence of unstimulated cells stained with the same Abs. The data were also confirmed by using dump channels (using mAbs conjugated with FITC specific for CD14, CD19, CD16, and CD56) to exclude monocytes, B cells, and NK cells, with equivalent results.

Multiplex bead immunoassay

CD8+ T cell subsets sorted by FACS, following staining using mAbs specific for CD45RA, CD27, and CD8 (excluding cells staining in a dump channel positive for CD4, CD14, CD16, CD19, or CD56), were stained with PMA and ionomycin for 6 h. Supernatants were stained using human chemokine 5-Plex reagents (Invitrogen), following the manufacturer’s instructions, and analyzed with a Luminex 100 instrument.

Statistical analyses

Statistical analyses were performed using Prism software (GraphPad). Intergroup comparisons were performed using Wilcoxon matched pair analysis and paired t test. All p values were two-tailed and considered significant if <0.05. Results were presented using Prism software (GraphPad) and Illustrator (Adobe) by Macintosh computers (Apple).

Results

Higher order CFC

CFC was initially developed by Picker and colleagues (27) to characterize both cell surface markers of human T cells and the production of effector cytokines at a single cell level. Following earlier methods published by de Rosa et al (28), Roederer and colleagues (29), and other studies that have established the importance of polyfunctionalitiy (previously defined by coproduction of IL-2, IFN-γ, TNF-α, and MIP-1β/CCL4 (30–34)), we optimized an 8-color, 10-parameter flow cytometric approach. This strategy allowed us to simultaneously assess CD8+ lineage, maturation stages (defined by CD45RA and CD27), and the production of three cytokines (IL-2, IFN-γ and TNF-α) and one chemokine (MIP-1β).

Analyses for subsequent studies were based on sequential gating of lymphocytes by forward/side light scatter and CD8+ staining, and then of naive and memory T cell subsets defined by the four quadrants demarcated by CD45RA and CD27 staining (Fig. 1A). Although additional markers (e.g., CD62L, CCR7) may assist in the discrimination of naive T cells, CD27 and CD45RA coexpression defines naive human CD8+ T cells with up to 93% specificity, whereas the remaining three quadrants defined by these markers demarcate memory cells (28). Individual studies have used inconsistent terms to describe memory subsets defined by CD27, CCR7, and CD45RA expression, including terms like central memory, effector memory, and late memory subsets. However, it is widely accepted that the loss of CD27 expression is associated with maturation, and that CD45RA re-expression characterizes late memory cells.
We stimulated PBMC with PMA and ionomycin to induce T cell activation. PMA and ionomycin directly activate protein kinase C-β, whereas mobilizing intracellular calcium stores (37); this activation does not require TCR engagement or its most immediate downstream signaling events. Consequently, PMA/ionomycin stimulation reflects the intrinsic ability of a cell to become activated irrespective of the expression of a given surface TCR or cell surface proteins (including CD27 and CD45RA) that might themselves modulate the activation threshold of a T cell. We consistently observed a strong association between maturation stage and functional signature, as determined by cytokine production. The cytokine signatures by maturation stage induced by PMA/ionomycin stimulation in a representative subject are shown in Fig. 1B. These results demonstrated significant polarization in naive cells relative to CD27⁺CD45RA⁻ (M3) cells. In contrast to the polarized production of individual cytokines noted in naive and M3 cells, CD27⁺CD45RA⁻ and CD27⁻CD45RA⁺ and CD27⁻CD45RA⁻ (M1 and M2) cells produced heterogeneous combinations of cytokines (e.g., IL-2⁺ IFN-γ⁺ and TNF-α⁺ MIP-1β⁺ cells). In addition, MIP-1β production increases with progressive maturation of memory cells and is most prominent in M3 CD8⁺ T cells.

Superantigen stimulation via the TCR induces characteristic cytokine signatures defined by maturation stage. Superantigens, including SEB Ags, activate significant fractions of the T cell repertoire (~30%) (38, 39) by bridging MHC class II on APC and cognate TCR Vβ subsets unique to each superantigen (40). Similar to the native activation of T cells by their cognate MHC-peptide ligands, superantigens activate the TCR signaling pathway through Lck-Zap70-PLCγ and other downstream intermediates (41, 42). For this reason, SEB stimulation allows us to model the activation of Ag-specific T cells via the TCR, whereas affording the advantage of activating sufficient proportions of rare T cell subsets to yield informative results. Based on this rationale, we stimulated peripheral PBMC derived from healthy subjects with SEB and examined the production of IL-2, IFN-γ, TNF-α, and MIP-1β within naive and memory (M1, M2, and M3) subsets. Although a lower proportion of CD8⁺ T cells are activated by SEB relative to PMA/ionomycin stimulation, we observed a similar polarization in cytokine signature from naive to progressively mature memory CD8⁺ T cells (see supplemental Fig. 1).4

Late memory CD8⁺ T cells have a unique cytokine signature characterized by MIP-1β production without IL-2

Although our approach allowed us to individually analyze all 15 possible combinations of the four cytokines/chemokines, we first examined intracellular production of individual T cell effector proteins. Aggregate results from 10 subjects, consistent across the group, are shown in Fig. 2. For this analysis, the total production of each cytokine/chemokine was summed, irrespective of whether production originated in a cell producing only one or multiple cytokines. As shown in Fig. 2A, the bulk of IL-2 production in PMA/ionomycin-stimulated CD8⁺ T cells emanated from stimulated M1 cells. Although M2 cells did produce substantial amounts of IL-2, M3 cells produced little IL-2. Similar results were seen with SEB stimulation (data not shown), suggesting that cytokine signature is an intrinsic property of cells at a given differentiation stage and independent of whether T cell activation occurs via the TCR (as for SEB) or postactivation by TCR by (for PMA/ionomycin). Production of IFN-γ and TNF-α was relatively consistent across M1, M2, and M3 subsets, and was increased significantly relative (p < 0.01) to the naive subset (Fig. 2A). In contrast, MIP-1β production progressively increased with maturation.

We further determined whether CD57 within the M3 population could define distinct subsets of these cells. Using a 9-color flow cytometric approach similar to that used in previous experiments, but also incorporating staining for CD57, we assessed an additional seven donors to determine whether CD57-positive and -negative M3 cells exhibited unique patterns of cytokine/chemokine production (see supplemental Fig. 2).4 Consistent with our overall conclusion that memory maturation is associated with skewing in

4 The online version of this article contains supplemental material.
MIP-1β/IL-2 production, we found relatively subtle, yet statistically significant, increases in MIP-1β production and decreases in IL-2 production in CD57-expressing M3 cells, consistent with their more differentiated phenotype.

To better define how individual combinations contribute to the overall production of effector cytokines/chemokines within CD8+ T cell subsets, we assessed all of the 15 possible cytokine/chemokine combinations based on IL-2, IFN-γ, TNF-α, and MIP-1β production. The production of each individual cytokine (or combination) was then represented as a percentage of the overall cytokine/chemokine production. In 10 healthy donors (Fig. 2B). These analyses allow us to clearly see which combinations of cytokines/chemokines are dominant within the overall CD8+ population.

The overall composition of the CD8+ repertoire by maturation stage in these subjects is also shown (mean ± SD: N: 33.5 ± 12.4%; M1: 18.6 ± 7.0%; M2: 24.6 ± 8.1%; M3: 23.3 ± 7.2%) (Fig. 2B, inset). This analysis demonstrates that the dominant cytokine-producing combination is one characterized by MIP-1β/TNF-α/IFN-γ coproduction without IL-2; this functional subset primarily consists of M2 and M3 cells. The next most common signatures consist of MIP-1β/IFN-γ coproducing cells (also mostly M2 and M3 cells) and the most polyfunctional (MIP-1β/TNF-α/IFN-γ/IL-2) subset. Notably, all of the four most dominant signatures of cytokine/chemokine production include MIP-1β production, whereas only one signature contains IL-2-producing cells.

The CC chemokines MIP-1α, MIP-1β, and RANTES are abundantly produced by late memory CD8+ T cells

To confirm that late memory maturation is characterized by increasing CC chemokine production and to determine whether the increasing MIP-1β production observed in late memory cells by CFC reflects its actual secretion into supernatants, we performed a Multiplex Bead Immun assay to measure the production of five chemokines (MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, MCP-1/CCL2, and eotaxin/CCL11) simultaneously in supernatants produced by CD8+ T cell subsets (e.g., N, M1, M2, and M3) from five healthy donors, following stimulation with PMA/ionomycin for 6 h. Consistent with the observation by intracellular staining that M3 CD8+ T cells produce abundant MIP-1β (Fig. 2A), the concentration of secreted MIP-1β was highest in M3 CD8+ T cells than in other CD8+ T cell subsets (N, M1, M2, and M3; p < 0.01) (Fig. 3A). Furthermore, we also observed the per cell MIP-1β production, as assessed by measurement of the mean fluorescence intensity of MIP-1β within chemokine-positive cells, was increased significantly in a stepwise fashion from the naive to M3 phenotypic stages (Fig. 3B). We also found that the CCR5-binding CC chemokines MIP-1α and RANTES were also primarily produced in M3 cells (p < 0.05) (Fig. 3A). MCP-1 and eotaxin levels were too low to be determined (data not shown). Taken together, late memory M3 CD8+ T cells are dominant producers of MIP-1β, MIP-1α, and RANTES, supporting our intracellular CFC data and confirming that CC chemokine production is strongly associated with late memory CD8+ T cell maturation.

MIP-1β/TNF-α/IFN-γ coproducing cells rarely exist beyond the M2 stage

Recent studies have strongly suggested that the expression of individual effector cytokines (especially IFN-γ) may be insufficient to identify human T cells most important for protective immunity to persistent viral pathogens (22, 43–46). Pantaleo and colleagues (22) demonstrated that polyfunctional T cells, defined by IL-2 and IFN-γ coproduction, were critical for protective immune responses in studies of chronic and cleared infections. Based on our prior studies, we hypothesized that IL-2/IFN-γ coproducing CD8+ T cells would be concentrated at certain stages of the maturation spectrum.

Confirming this view, we found that the naive and M3 CD8+ T cell compartments rarely contained cells capable of IL-2/IFN-γ coproduction, following either PMA/ionomycin or SEB stimulation (with results from 10 subjects shown in Fig. 4A). For example, after PMA/ionomycin stimulation, the proportion of CD8+ T cells (mean ± SD) with the IL-2/IFN-γ phenotype by maturation stage was as follows: N, 7.2 ± 4.0%; M1, 53.0 ± 11.2%; M2, 35.8 ± 11.1%; M3, 3.8 ± 2.3%. Similar results were seen in CD8+ T cells following SEB stimulation (data not shown). These results demonstrate that differentiation to an M3 CD8+ T cell is...
associated with the loss of the IL-2/IFN-γ coproduction. Furthermore, we examined the production of TNF-α and MIP-1β in IL-2/IFN-γ coproducing cells and found that the vast majority of such cells also produced either TNF-α or MIP-1β (Fig. 4B). Taken together, IL-2/IFN-γ coproducing cells are confined to the M1 and M2 subsets, and rarely exist at the M3 subset. Moreover, these cells are heterogeneous and usually produce other cytokines in addition to IL-2 and IFN-γ.

These findings were also confirmed by an analysis of the maturation subsets producing each individual cytokine/chemokine or possible combinations. In Table I, the location within the CD8⁺ T cell maturation spectrum of the primary and secondary producer of each possible cytokine combination, in data derived from 10 healthy subjects, is shown; the subset producing the greatest amount of each cytokine is highlighted among the location of the second-most prolific producer. Cytokine signatures of CD8⁺ T cells stimulated by PMA/ionomycin (or SEB) are shown in Table I. It is evident from this analysis that the production of individual cytokines/chemokines (e.g., IL-2 without others) occurs primarily at the naive or M1 stages; polyfunctional cells are increasingly produced at M1 and M2 stages, whereas progression to the M3 stage is again associated with monofunctionality, with prominent MIP-1β production. This analysis also reaffirms that the subset defined by Pantaleo and colleagues (22) as polyfunctional on the basis of IL-2 and IFN-γ coproduction is actually quite heterogeneous, with IL-2/IFN-γ coproducing cells rarely producing these two cytokines without additionally producing TNF-α or MIP-1β. Finally, whereas M3 cells are the dominant source of MIP-1β production, they rarely produce IL-2.

The production of MIP-1β, and not IFN-γ, by late stage memory cells is the hallmark of the CMV-specific T cell response

It has recently been suggested that mature CMV-specific CD4⁺ T cells acquire antiviral effector function and that their predominant combination of cytokine production is IFN-γ/TNF-α/MIP-1β (31). We sought to more carefully examine the cytokine signature of CD8⁺ CMV-specific T cells, and the relationship of cytokine/chemokine production to maturation stage. Therefore, we stimulated PBMC from eight healthy CMV-seropositive donors with a CMV pp65 peptide mixture (25, 26), and examined maturation and function.

Consistent with prior observations suggesting the relative maturity of peripheral CMV-specific T cells (10), we found that most functional CMV-specific T cells were in the M2 and M3 stages, as shown for a representative subject (Fig. 5A). We then analyzed the relative contribution of individual cytokines/chemokines (and combinations) to overall cytokine production in eight separate donors (Fig. 5B). The most striking finding was the most abundant functional subsets consisted of cells producing either MIP-1β alone (>60% of the total cytokine/chemokine response) or MIP-1β and other cytokines (especially IFN-γ/TNF-α/MIP-1β). Consistent with our earlier observations that memory differentiation is associated with increased production of MIP-1β, M2 and M3 cells were the primary source of this chemokine in CMV-specific CD8⁺ T cells. Finally, we analyzed the production of IL-2/IFN-γ coproducing cells. These data (Fig. 5C) demonstrate that the distribution of these cells was consistent with that observed

**FIGURE 3.** Late memory (M3) CD8⁺ T cells are the predominant producer of CC chemokines. A, CD8⁺ T cells were FACS sorted from PBMC from n = 5 healthy donors and stimulated with PMA/ionomycin for 6 h. Secreted chemokines (MIP-1β/MIP-1α/RANTES) were measured using a multiplex bead immunoassay. *, p < 0.05 and **, p < 0.01. B, Increasing MIP-1α production per cell is a characteristic of memory maturation. We calculated the mean fluorescence intensity (MFI) of the MIP-1β population within CD8⁺ T cell maturation subsets naive (N: CD27⁻/CD45RA⁻), early memory (M1: CD27⁺/CD45RA⁻), intermediate memory (M2: CD27⁺/CD45RA⁻), and late memory (M3: CD27⁻/CD45RA⁻). Results depict aggregate results from n = 10 healthy donors. *, p < 0.05.

**FIGURE 4.** IL-2/IFN-γ coproducing cells exist primarily at M1 and M2 stages. A, IL-2/IFN-γ coproducing cells, known to be critical for protective immunity, were assessed for their production across the CD8⁺ T cell naive/memory and memory stages (10, 22). Most IL-2/IFN-γ coproducing cells also produce TNFα and/or MIP-1β. We assessed the polyfunctionality of IL-2/IFN-γ coproducing CD8⁺ T cells stimulated with PMA/ionomycin. IL-2/IFN-γ/TNF-α/MIP-1β⁻ (□), IL-2⁺/IFN-γ⁻/MIP-1β⁺TNF-α⁻ (□), IL-2⁺/IFN-γ⁻/TNF-α⁻/MIP-1β⁻ (■), and IL-2⁻/IFN-γ⁻/TNF-α⁻/MIP-1β⁻ (●) shown.

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following SEB and PMA/ionomycin stimulation, reinforcing the notion that differentiation stage, rather than the nature of the activating stimulus, determines the functional signature. Furthermore, these data suggest that skewing of the memory response to M3 results in functional T cells that produce abundant MIP-1β, but lack the ability to produce IL-2, a characteristic associated with pathogen clearance.

### Discussion

Recent technological advances, including “polychromatic” flow cytometry using mAbs recognizing intracellular proteins, has led to the recognition that there is dramatic phenotypic and functional heterogeneity of the peripheral T cell repertoire. To better characterize the relationship between CD8⁺ T cell maturation stages and their cytokine signatures, we examined peripheral human CD8⁺ T cells in PBMC, stimulated with polyclonal activators and viral Ags, and systematically characterized the production of IL-2, IFN-γ, TNF-α, and MIP-1β. Our results confirm the following: 1) late memory (M3) CD8⁺ T cells produce little IL-2 (including IL-2/IFN-γ coproduction), but are prolific producers of MIP-1β, MIP-1α, and RANTES; 2) MIP-1β is the most abundant cytokine produced by CMV-specific CD8⁺ T cells; 3) nearly all IL-2 production occurs in N, M1, and M2 CD8⁺ T cells, whereas nearly all IL-2/IFN-γ coproduction occurs at the M1 and M2 stage; and 4) functional cytokine signature is strongly associated with T cell maturation stage in CD8⁺ T lineage and is largely independent of the type of stimulus initiating activation.

Using both TCR-dependent stimulation (e.g., CMV Ags) and stimuli that bypass the TCR, requirements for costimulation and proximal signaling events (PMA/ionomycin) we found that maturation stage was closely tied to cytokine signature. Although we observed complexity in the various combinations of cytokines/chemokines produced by early and late memory CD8⁺ T cells, relatively simple rules could be used to model the effects of maturation on cytokine signature: 1) early stage CD8⁺ T cells, including naive cells, were more likely to be monofunctional with respect to cytokine/chemokine production, with IL-2 and TNF-α production defining their cytokine signature; 2) differentiation from naive to the M1/M2 stages of CD8⁺ T cells is associated with increasing polyfunctionality, including coproduction of IL-2 and IFN-γ, whereas progression to the M3 stage is again associated with monofunctionality; and 3) M3 CD8⁺ T cells are characterized by the production of abundant amounts of MIP-1β (often in concert with other cytokines) and other CC chemokines (MIP-1α and RANTES) and little, if any, IL-2. These results are consistent with the results of Hamann et al. (9) and Sandberg et al. (47), who also found that CD27⁺CD8⁺ T cells rarely produce IL-2, although these cells produce perforin following polyclonal stimulation. They also confirm the findings of Duvall et al. (23), who demonstrated that subsets of HIV-2-specific CD8⁺ T cells making MIP-1β were more likely to express markers associated with more differentiated maturation states.

Our analysis of CMV-specific T cells confirmed the results of other studies demonstrating the abundance of late memory cells (i.e., M2 and M3 cells) within this Ag-specific population. In contrast to our expectation that the most abundant product of CMV-specific T cells would be either IFN-γ or TNF-α, which have been...
most commonly used as markers of Ag-specific T cells (22, 25–27, 31, 48, 49), we found that MIP-1β was by far the most abundant cytokine produced by CD8⁺ CMV-specific T cells stimulated ex vivo. Besides cells producing MIP-1β alone, we found a fairly robust number of cells producing MIP-1β in conjunction with effector cytokines, including IFN-γ/TNF-α/MIP-1β, IL-2/IFN-γ/TNF-α/MIP-1β, and IFN-γ/MIP-1β were observed. These data suggest that MIP-1β may actually be a more sensitive marker than IFN-γ for the detection of CMV-specific T cells, and that conventional approaches (e.g., CFC or ELISPOT assays based on IFN-γ detection alone) may significantly underestimate the frequency of CMV-specific T cells. In addition, late memory (M3) CMV-specific T cells are also characterized by the lack of IL-2 production.

Our own studies of late memory CMV-specific T cells (e.g., those expressing CD57, which defines cells that would most likely have been defined as M3 in this study) suggests that a greater frequency of cells residing within this population are associated with poor control of early CMV reactivation after stem cell transplantation, supporting the view that polyfunctional cells producing IL-2 may be better at controlling pathogenic viral infections. Studies by Restifo and colleagues (24) in a cancer immunotherapy model also suggest that late effector T cells, characterized by strong cytotoxic function and IFN-γ production, may be paradoxically inferior in their ability to control cancer growth. Although high-order cytometric analyses were not reported, this study showed that late effector cells, whereas producing IFN-γ and having greater lytic capacity, were deficient in IL-2 production. Taken together with prior translational studies in the setting of human infections, our present results suggest that human clinical studies in the setting of cancer and infectious disease should consider not only the overall precursor frequency of responsive T cells, but also differentiation stage, polyfunctional capacity (especially that of IFN-γ/IL-2 coproduction) or both. Additionally, our results confirm that assays that measure IFN-γ alone (including ELISPOT assays) will measure cells across the memory spectrum and may significantly underestimate the precursor frequency of Ag-specific CD8⁺ T cells, especially those at later stages of the maturation spectrum.

Our conclusions should be considered in light of some limitations of our experimental approach. First, our quadrant-based gating strategy, using two well-validated (but not exhaustive) markers, is inherently imperfect. Such gating strategies may not always classify cells in transitional phenotypic compartments. We also acknowledge that the use of additional markers to define naive and memory subsets, whereas adding complexity to our experimental approach, would likely have defined both naive and memory subsets with greater specificity. For example, de Rosa et al. (28), found that the addition of a third memory/naive marker increased the specificity of definition of naive CD8⁺ T cells from up to 87–93% to ~96%. Additionally, our studies of CMV-specific CD8⁺ T cells should be cautiously interpreted with respect to their relevance to T cell responses to other pathogens. It is very likely that individual viruses differ in their susceptibility to individual mechanisms of CD8⁺ T cell control; the caveat that “individual results may vary” should be considered when considering the unique functional profiles that constitute protective immune responses to specific pathogens.

Although studies like this one cannot directly confirm mechanisms operative in vivo, our results suggest that late memory CD8⁺ T cells that are abundant producers of MIP-1β, MIP-1α, and RANTES may have a unique functional role in human immune responses. It is known that CCR5 up-regulation occurs with T cell activation (50), raising the possibility that late memory (M2 and M3) cells may have an autocrine or paracrine role in regulating immune responses. Indeed, Ahmed and colleagues (51) demonstrated up-regulation of MIP-1β and MIP-1α mRNAs in murine model, and suggested that chemotactic properties of murine CD8⁺ exhausted cells might “sound the alarm”. Consistent with this hypothesis, MIP-1β, MIP-1α and RANTES are potent chemotactic factors, inducing migration of monocytes and macrophages to the site of local elaboration. This raises the possibility that the activation of late memory CD8⁺ T cells (especially those specific for CMV and other herpes viruses that reside mostly in late memory stages) may serve as important attractors of cells capable of clearing debris and magnifying Ag presentation locally. The balance between these functions and those of earlier memory cells that appear to be more important for viral clearance (e.g., polyfunctional, M1, M2 cells) deserves further investigation. It will also be important to elucidate the mechanisms that govern maturation and migration through activated functional stages and to determine how the production of CC chemokines is associated with CCR5 expression on T cells and myeloid subpopulations in vivo. Finally, to understand how functional differentiation is programmed and might be modulated in vivo for therapeutic benefit, it will be critical to better define the role of genetic alterations (52, 53) and epigenetic changes (54, 55) associated with phenotypic and functional differentiation.

In conclusion, our results establish an unequivocal link between maturation stage and functional cytokine/chemokine signature in CD8⁺ T cells, and demonstrate that late memory maturation results in polarization toward CC chemokine production without IL-2 production. We also found that CMV-specific CD8⁺ T cells in healthy donors were heavily skewed toward late memory subsets, and that MIP-1β production dominates the functional signature of the human CMV-specific T cell response. Further studies in model systems and in the context of clinical trials, will be required to determine how the accumulation of late memory cells that are polarized toward CC chemokine production influences outcomes in the setting of human disease states.

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Disclosures

The authors have no financial conflict of interest.

References

Supplementary Figure 1. CD8+ T cell maturation is associated with stage-specific cytokine signatures, including increased MIP-1β and decreased IL-2 production in late memory cells. As in Fig. 1, but following stimulation of T cells using the superantigen staph enterotoxin B (SEB) instead of P:I, we assessed the relationship between maturation stage and functional signature following activation of healthy donor PBMC, as shown for a representative subject. 2-D dot plots depict IL-2 vs. IFNγ staining and TNFα vs. MIP-1β staining within CD8+ T cell maturation subsets (Naïve, N: CD27+CD45RA+, Early memory, M1: CD27+CD45RA-, Intermediate memory, M2: CD27-CD45RA-, Late memory, M3: CD27-CD45RA+). Little background stimulation is evident, as shown for two untreated biological control samples. Results from this subject were representative of over 10 healthy donors similarly studied.

Supplementary Figure 2. CD57 expression further characterizes cytokine production in late memory M3 CD8+ T cells. A. The relationship between CD57 expression and cytokine production in M3 CD8+ T cells. We assessed the relationship between CD57 expression and functional signature in PMA-stimulated healthy donor PBMC, as shown for a representative subject. 2-D dot plots depict IL-2 vs. IFNγ staining and TNFα vs. MIP-1β staining within CD57+/- M3 CD8+ T cell maturation subsets. B. CD57 expression further subsets the M3 CD8+ T cell population. Cytokine/chemokine production following PMA:Ionomycin (P:I) stimulation was calculated within CD57- M3 and CD57+ M3 stages in the CD8+ T cell population, irrespective of whether such production originated in a cell producing only one or multiple cytokines. This figure depicts aggregate results from 7 healthy donors. * P<0.05
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<th>Untreated control</th>
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<td>IFNγ</td>
<td>0.0</td>
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Supplementary figure 1
Supplementary Figure 2

A

CD8+ M3

\[
\begin{align*}
\text{M3 CD57+} & \quad 19.3 \\
\text{M3 CD57-} & \quad 77.2
\end{align*}
\]

CD57

CD45RA

M3 CD57-

IL-2

TNF\(\alpha\)

IFN\(\gamma\)

MIP1-\(\beta\)

B

**IL-2**

\[
\begin{align*}
\text{% IL-2} & \quad 0.3 & \quad 0.3 \\
\text{CD57- M3} & \quad 52.4 & \quad 47.0 \\
\text{CD57+ M3} & \quad 18.8 & \quad 53.1
\end{align*}
\]

**TNF\(\alpha\)**

\[
\begin{align*}
\text{% TNF\(\alpha\)} & \quad 0.6 & \quad 0.6 \\
\text{CD57- M3} & \quad 14.3 & \quad 48.8 \\
\text{CD57+ M3} & \quad 18.8 & \quad 53.1
\end{align*}
\]

**IFN\(\gamma\)**

\[
\begin{align*}
\text{% IFN\(\gamma\)} & \quad 0.3 & \quad 0.3 \\
\text{CD57- M3} & \quad 31.2 & \quad 68.2 \\
\text{CD57+ M3} & \quad 68.2 & \quad 31.2
\end{align*}
\]

**MIP1-\(\beta\)**

\[
\begin{align*}
\text{% MIP1-\(\beta\)} & \quad 1.6 & \quad 26.5 \\
\text{CD57- M3} & \quad 36.3 & \quad 48.8 \\
\text{CD57+ M3} & \quad 53.1 & \quad 18.8
\end{align*}
\]

\* \(P<0.05\)