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The Functional Profile of Primary Human Antiviral CD8+ T Cell Effector Activity Is Dictated by Cognate Peptide Concentration

Michael R. Betts,* David A. Price,† Jason M. Brenchley,† Karin Loré,* F. Javier Guenaga,† Anna Smed-Sorensen,‡ David R. Ambrozak,* Stephen A. Migueles,§ Mark Connors,§ Mario Roederer,‡ Daniel C. Douek,† and Richard A. Koup*

Antiviral CD8+ T cells can elaborate at least two effector functions, cytokine production and cytotoxicity. Which effector function is elaborated can determine whether the CD8+ T cell response is primarily inflammatory (cytokine producing) or antiviral (cytotoxic). In this study we demonstrate that cytotoxicity can be triggered at peptide concentrations 10- to 100-fold less than those required for cytokine production in primary HIV- and CMV-specific human CD8+ T cells. Cytolytic granule exocytosis occurs at peptide concentrations insufficient to cause substantial TCR down-regulation, providing a mechanism by which a CD8+ T cell could engage and lyse multiple target cells. TCR sequence analysis of virus-specific cells shows that individual T cell clones can degranulate or degranulate and produce cytokine depending on the Ag concentration, indicating that response heterogeneity exists within individual CD8+ T cell clonotypes. Thus, antiviral CD8+ T cell effector function is determined primarily by Ag concentration and is not an inherent characteristic of a virus-specific CD8+ T cell clonotype or the virus to which the response is generated. The inherent ability of viruses to induce high or low Ag states may be the primary determinant of the cytokine vs cytolytic nature of the virus-specific CD8+ T cell response. The Journal of Immunology, 2004, 172: 6407–6417.

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D8+ T lymphocytes play a critical role in the immune response to various chronic and acute viral pathogens in humans (1–7). Upon TCR engagement with peptide-MHC class I complexes (pMHCI), CD8+ T lymphocytes elaborate multiple effector functions, including cytokotoxicity and cytokine production (8). Cytotoxicity, mediated by the release of cytolytic granules containing perforin, granzymes, and Fas ligand (9–12), occurs rapidly after CD8+ T cell activation, inducing target cell apoptosis. Newly synthesized cytokines and chemokines, including IFN-γ, TNF-α, macrophage inflammatory protein-1α and -1β, and IL-2, are secreted within a few hours after CD8+ T cell activation (13).

Some chronic viral infections have an inherent ability to induce high viral loads, and their immune responses are characterized by inflammation, chronic activation, and ineffective CD8+ T cell responses. For example, inflammation, probably resulting in part from the massive, yet ineffective, CD8+ T cell response to HIV, causes extensive damage to lymph node architecture in HIV-infected individuals (14). Similar observations have been made in chronic hepatitis C virus (HCV) disease, in which inflammation-induced damage occurs in the liver, possibly due to the presence of HCV-specific CD8+ T cells, leading to cirrhosis (15). Conversely, low or intermittently high viral loads and effective CD8+ T cell responses in the absence of inflammation are found in chronic CMV infection. Recent evidence suggests that HIV may alter the functional and phenotypic characteristics of the resultant antiviral CD8+ T cell-mediated immune response; whether these alterations result from virus-specific effects or generalized effects of chronic exposure to high Ag loads remains to be determined. For example, HIV-specific CD8+ T cells have been shown to be phenotypically immature (16), perforin deficient (17), and replicatively senescent (18), potentially due to chronic activation caused by constant exposure to high levels of Ag. Indeed, chronic activation, as measured by CD38 expression on HIV-specific CD8+ T lymphocytes, is directly correlated with HIV disease progression (19). Taken together, these findings suggest that a relationship may exist between viral Ag load and the effectiveness of the CD8+ T cell response, whereby high Ag states result in a less effective and more inflammatory CD8+ T cell response in vivo.

It has been shown in human CD8+ T cell clones that cytotoxicity, proliferation, and cytokine expression can be triggered by different Ag concentrations depending on the level of TCR occupancy (20–22); however, these analyses were performed by examining the average response from bulk populations and therefore do not represent functional analysis at the single-cell level. These studies showed that the activation threshold of cytotoxic activity in CD8+ T cell clones is lower than that required for proliferation or cytokine production and further suggested that cytotoxic activity can be detected in the absence of significant cell surface TCR down-modulation. Because CD8+ T lymphocytes are capable of sequential recognition and elicitation of effector function (23, 24), down-modulation of cell surface TCR could lead to an impairment of CD8+ T cell-killing ability, decreasing the overall effectiveness

*Immunology Laboratory, †Human Immunology Section, ‡ImmunoTechnology Section, Vaccine Research Center, and ¶Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and †Center for Infectious Medicine, Karolinska Institute, Stockholm, Sweden

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1 Address correspondence and reprint requests to Dr. Michael R. Betts, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 40 Convent Drive, Bethesda, MD 20892. E-mail address: mbetts@mail.nih.gov

2 Abbreviations used in this paper: pMHCI, peptide-MHC class I complex; DC, dendritic cell; HCV, hepatitis C virus; MDC, myeloid dendritic cell; PDC, plasmacytoid dendritic cell.
of the immune response. Importantly, many of these studies used CTL clones selected for cytotoxic activity and ability to be maintained under prolonged in vitro propagation, conditions that leave, through necessity of growth selection, altered basal signaling properties that inevitably affect response patterns to specific Ag. As such, these clones may not represent the full functional heterogeneity of memory antiviral CD8+ T cells in vivo.

We recently developed a highly sensitive assay that measures the ability of human CD8+ T cells to degranulate in response to Ag-specific stimulation (25). By measuring the mobilization of cytolytic granule membrane proteins (CD107a and CD107b, lysosome-associated membrane proteins 1 and 2, respectively) to the cell surface, which occurs as the granule membrane merges with the cell membrane during degranulation (9), it is possible to identify and quantify Ag-specific CD8+ T cell degranulation by flow cytometry. The ability of CD8+ T cells to degranulate is directly correlated with cytolytic activity of effector CD8+ T cells (25, 26).

In this study we have used measurement of cytokine production and degranulation to examine functional heterogeneity within primary human virus-specific CD8+ T cells to human CMV, a virus to which the CD8+ T cell response is characterized by prolonged viral control and lack of inflammation, and HIV, a virus to which the T cell response is characterized by ineffective control and extensive activation and inflammation. We also studied the influence of Ag concentration and costimulation on modulating the effector CD8+ T cell responses to these viruses. Finally, we addressed whether the observed functional diversity is attributable to heterogeneity within or between individual HIV- and CMV-specific CD8+ T cell clonotypes.

Materials and Methods

Patient samples

PBMC were obtained by apheresis from anonymous CMV+ donors (donors 1 and 2) at the National Institutes of Health. PBMC from HIV-1-infected patients (donors 3 and 4) were obtained from clinics at the National Institutes of Health. HIV viral load was quantified using the bDNA assay (Chiron, Emeryville, CA). PBMC were isolated using Hypaque-Ficoll (Pharmacia Biotech, Uppsala, Sweden) or sodium diatrizoate (Organon-Teknika, Durham, NC) density centrifugation. PBMC were frozen (90% FCS/10% DMSO) at −140°C until use. Donors 3 and 4 were extensively characterized recently (patients 35 and 34, respectively) (27).

Abs and reagents

The mAbs were obtained from BD PharMingen (San Diego, CA; purified). CD8+ T cells were stained with the appropriate phenotype were sorted directly into RNAlater (Ambion, Austin TX). For single-cell sorting, individual cells of the appropriate phenotype were sorted directly into single wells of a 96-well PCR plate containing 10 µl of cell lysis buffer/well.

TCRB sequencing

TCRB sequencing was performed as previously described (28). Briefly, sorted peptide-specific cells (between 4,000 and 10,000 from each stimulation) were lysed, and mRNA was extracted (Oligotex kit; Qiagen, Valencia, CA). Anchored RT-PCR was performed using a modified version of the SMART (switching mechanism at the 5’ end of the RNA transcripts) procedure (29) and a TCRβ constant region 3’ primer for the PCR to obtain TCRB PCR products from the 5’ end to the start of the TCRβ constant region. The PCR product was ligated into the pGEMT Easy vector (Promega, Madison, WI) and used to transform Escherichia coli. Colonies were selected, PCR-amplified with M13 primers, and sequenced. Between 50 and 95 sequences were analyzed for each subject. Full details of all primers, components, and cycling temperatures are available upon request.

Single-cell IFN-γ mRNA quantification

After the single-cell sort, the cells were lysed in 10 µl of proteinase K to cDNA II buffer (Ambion) at 75°C for 10 min. Five microliters of lysate were transferred to a second plate resulting in two 96-well plates with 5 µl...
of lysate in each well. Two different master mixes were prepared for an IFN-γ/H9253 reaction and a GAPDH reaction. All PCRs contained 500 nM concentrations of each primer and 200 nM concentrations of each corresponding probe (sequences available upon request), Superscript/Platinum Taq polymerase (one step RT-PCR kit; Invitrogen, Carlsbad, CA), and 250 nM Blue-636 reference dye. One-step quantitative RT-PCR was performed with the following conditions: 30 min at 40°C, 5 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. The data were collected on the last stage of each cycle with the ABI 7700 and ABI 7900 Sequence Detection Systems (PE Applied Biosystems, Foster City, CA). Wells in which no GAPDH mRNA was detected were excluded from the analysis.

Isolation of myeloid (MDC) and plasmacytoid (PDC) dendritic cells (DC)

PDC and MDC were isolated from donor 1 PBMC using magnetic beads (Miltenyi Biotec, Auburn, CA) specific for BDCA-4 and CD1c expression, respectively. The DC were separated using AutoMacs (Miltenyi Biotec). This isolation procedure resulted in >85% pure populations of DC. The contaminating cells were monocytes. To maintain viability, the PDC and MDC were cultured in the presence of IL-3 (1 ng/ml; R&D Systems, Minneapolis, MN) and GM-CSF (2 ng/ml; PeproTech, Rocky Hill, NJ), respectively. To obtain a mature phenotype of the DC, both PDC and MDC were exposed to the imidazoquinoline R-848 (4-amino-2-ethoxymethyl-α, α-dimethyl-1H-imidazoquinoline-1-ethanol; GLSynthesis, Worcester, MA) at 1 μg/ml (2.8 × 10⁻⁷ M) for 24 h before use as APCs in T cell experiments.

Purified DC were pulsed with various concentrations of CMV-A2 peptide for 1 h at 37°C. The cells were washed twice in medium, and PBMC were added in the presence of αCD107a/b-allophycocyanin and monensin as described above at a 1:10 DC to PBMC ratio. The cells were incubated for 5 h, then stained for cell surface markers and intracellular cytokine production as described above. As a control, PBMC from donor 1 were CD8-depleted by magnetic bead separation (Miltenyi Biotech), pulsed with varying amounts of CMV-A2 peptide, washed twice, and then added back to unsorted PBMC at a 1:10 target cell to PBMC ratio. The cells were then incubated and treated as described above.

Results

Effector function in HIV- and CMV-specific CD8⁺ T lymphocytes

We examined the HLA-A2-restricted response to the CMV pp65.495–503 NLVPVMATV peptide (CMV-A2) in donors 1 and 2, and the HLA-B57-restricted response to the HIV Gag p24.163–174 KAFSPEVPIMF peptide (HIV-B57) in donors 3 and 4 (Table I).
Substantial CD8^+ T cell populations reactive to either the CMV-A2 or HIV-B57 tetramer were detectable in these donors (Fig. 1A, left columns).

We next examined the functionality of the tetramer^+ cells in these donors using intracellular IFN-γ production and the cell surface mobilization of CD107a and b after peptide stimulation. As shown in Fig. 1A, right columns, a large proportion of the tetramer^+ cells in each donor responded to exogenous peptide by producing cytokine or degranulating. For example, in donor 1, ~77% of the tetramer^+ cells degranulated, and 75% of the tetramer^+ cells produced IFN-γ after stimulation.

There was no inherent difference between the functional capabilities of the CD8^+ T cells specific to CMV or HIV (Fig. 1B). The majority of responding cells in each donor both degranulated and produced IFN-γ. A variable, but detectable, number of cells degranulated without producing IFN-γ in each donor. In donor 3, this population accounted for up to 50% of the response. There was never a considerable percentage of tetramer^+ cells that produced IFN-γ without degranulation. Therefore, differential effector functions can be elicited from peripheral blood CD8^+ T cells responding to the same Ag.

Effect of Ag concentration on CD8^+ T cell effector function

We next examined the ability of the CMV-specific CD8^+ T cells from donor 1 to recognize decreasing concentrations of CMV-A2 peptide, ranging from 2 μM to 20 PM (Fig. 2A). At the highest peptide concentration, the majority of responding cells exhibited dual effector function by both degranulating and producing IFN-γ. However, as the peptide concentration decreased, more cells degranulated than produced detectable IFN-γ. This is particularly evident at the lowest concentrations used (Fig. 2A; 200 and 20 PM), at which little or no IFN-γ production was detected despite a substantial degranulation response. Similar profiles were also observed in donors 2–4 (Fig. 2B). The most notable separation of effector function was observed in donor 2, in whom at 2 nM peptide, 1.6% of total CD8^+ T cells responded by degranulating, whereas only 0.26% of CD8^+ T cells produced IFN-γ. Importantly, for both CMV- and HIV-specific CD8^+ T cells, the inflammatory vs cytolytic profiles were similar over a range of peptide concentrations.

Costimulation augments the responding CD8^+ T cell frequency

It was recently shown that costimulation augments the frequency of responding CMV-specific CD4^+ T cells at submaximal peptide concentration (37). We therefore examined how costimulation affected the sensitivity of the CMV-A2-specific CD8^+ T cell response in donor 1. The presence of costimulatory Abs (αCD28/CD49d) enhanced the frequency of responding CD8^+ T cells at low peptide concentrations (200–200 PM CMV-A2; Fig. 4A). Approximately 8% of the CMV-A2 tetramer^+ cells responded to 20 PM CMV-A2 when costimulatory Abs were present compared with only 3.4% in their absence (data not shown). We further examined this effect by using autologous CMV-A2 peptide-loaded
Individual virus-specific CD8+ T cell clonotypes exhibit functional heterogeneity

The primary human CD8+ T cell response to a specific antigenic peptide may be composed of multiple CD8+ T cell clonotypes (28, 31–33). One potential explanation for the differential effector function observed at low peptide concentrations could be the presence of CD8+ T cell clonotypes with differing TCR affinities and differing functional capacities for the pMHC1 complex. We therefore sequenced the TCRB, CDR3 region to examine the clonality of the CD8+ T cells that degranulate in response to low peptide concentration compared with the nonresponding tetramer+ cells. PBMC from each donor were stimulated with either CMV-A2 or HIV-B57 peptides at the concentrations shown in Figs. 2B and 5A to induce degranulation without IFN-γ production and TCR down-regulation, and were viably sorted into tetramer+ CD107+ (non-responding) and tetramer+CD107+ (responding) populations for TCR sequencing. Donors 1–3 had highly oligoclonal responses to the peptides with a dominant T cell clonotype (Fig. 6). Donor 4 had a more polyclonal response to the HIV-B57 peptide. The majority, if not all, of the peptide specific clonotypes identified in the tetramer+ CD107+ cell population of each donor (except donor 4) shared the same TCRB CDR3 sequences with the tetramer+ CD107+ population. To confirm these results, we stimulated donor 1 with 2 μM CMV-A2 peptide, a concentration at which the majority of the cells respond by both degranulating and producing IFN-γ, and repeated the clonotypic analysis (data not shown). The same clonotypes found previously were present in both the tetramer+ CD107+ and CD107− populations, indicating that even at high peptide concentrations, tetramer+ CD107+ and tetramer− CD107− cells can be composed of the same clonotypes.

In contrast, seven different clonotypes were identified in donor 4, five of which responded to peptide at low concentration. Three clonotypes in donor 4 were only found in the responding population; these clonotypes may be of particularly high affinity and therefore more sensitive to low peptide concentrations. Conversely, the nonresponding cells observed in donors 3 and 4 may be lower affinity clonotypes. Although it is possible that the TCRα-chains could still differ within a particular clonotype, as defined by the TCRβ-chain, it is highly unlikely that in the same person with the same TCRβ-chain against the same Ag there will be a different TCRαβ combination with the same specificity in a mature response. For simplicity, we therefore define clonotypes exclusively by the TCRB VDJ rearrangement. These data indicate that TCR clonotype can affect the efficiency at which CD8+ T cells in the peripheral blood respond to varying concentrations of cognate peptide on stimulator cells (exemplified by donor 4), but that there are additional unknown factors that influence the ability of an individual CD8+ T cell clonotype to degranulate or produce cytokine (exemplified by donors 1–3).

Discussion

CD8+ T lymphocytes are important in the clearance and control of viral infection (1–7, 34–36) and elaborate multiple effector mechanisms after activation. Therefore, delineating the nature of the CD8+ T cell response to a viral infection cannot be fully accomplished by examination of a single effector function. In this study we show simultaneous measurement of cytokine and cytolytic effector functions at the single-cell level in human antiviral CD8+ T lymphocytes ex vivo, and how these responses are influenced by Ag density, costimulation, and clonal heterogeneity.

Our results indicate that primary CMV- and HIV-specific CD8+ T cells both degranulate and produce IFN-γ in response to specific viral peptides. There is no inherent difference in the ex vivo CD8+ T cell response to these viruses despite the profound difference in the in vivo effectiveness of each response. At high peptide concentration, both cytokine production and degranulation were observed in the majority of responding CD8+ T cells, but at lower
peptide concentrations fewer cells produced cytokine, whereas many of those same cells continued to degranulate. This relationship was observed regardless of the presence of costimulation. At the lowest peptide concentrations at which a response was detected by degranulation, no cytokine expression was detectable. Additionally, at these lower peptide concentrations, responding CD8\(^+\) T cells did not down-modulate surface TCR. Finally, we found that the ability of CD8\(^+\) T cells to respond at low peptide concentrations is due to both interclonal and intraclonal functional heterogeneity among individual Ag-specific CD8\(^+\) T cells.

Separation of effector function in response to decreasing peptide concentration has been observed in human CD8\(^+\) T cell clones (20) and more recently in ex vivo human CD4\(^+\) T lymphocytes (37), but no such observations have previously been made in primary human CD8\(^+\) T lymphocytes. Separation of CD8\(^+\) T cell effector function can also be observed in mouse lymphocytic choriomeningitis virus-specific CD8\(^+\) T lymphocytes, where at low peptide concentrations degranulation (as measured by CD107a and -b mobilization) occurs in the absence of detectable IFN-\(\gamma\) production (P. Wolint, M. Betts, R. Koup, and A. Oxenius, manuscript in preparation). Even at high peptide concentrations, we observed differential CD8\(^+\) T cell effector function. This is particularly noticeable for HIV-specific CD8\(^+\) T cell responses (donors 3 and 4 and data not shown), where a substantial proportion of HIV-specific CD8\(^+\) T cells degranulate without producing cytokine. If and how this finding pertains to the CD8\(^+\) T cell immune response to HIV remains to be elucidated.

It is unclear whether there may be different signaling requirements for degranulation and cytokine expression, but these data suggest that at the very least, a more potent signal is required for IFN-\(\gamma\) production. It was recently shown that the addition of Abs to CD28 and CD49d augments the frequency of responding cells at low peptide concentrations in ex vivo CMV-specific human CD4\(^+\) T lymphocytes (37). We also observed increased responses in the presence of \(\alpha\)CD28 and \(\alpha\)CD49d or using peptide-loaded dendritic cells as stimulators. Importantly, only the frequency of responding cells changed in the presence of costimulation, and the nature of the CD8\(^+\) T cell effector response (degranulation vs cytokine) was not dramatically affected.

Internalization of the TCR complex is known to occur after activation of CD8\(^+\) T cells, an observation sometimes used to estimate CD8\(^+\) T cell activation level (20). We found that CD8\(^+\) T cells responding at low peptide concentrations do not significantly down-modulate cell surface TCR. These data definitively

FIGURE 4. Contribution of costimulation to CMV-A2-specific CD8\(^+\) T cell responses. A, PBMC from donor 1 were stimulated with varying concentrations of CMV-A2 peptide in the presence or the absence of Abs to CD28 and CD49d. The CD8\(^+\) T cell response observed at a 20 pM peptide concentration is shown. The background expression of CD107a/b in unstimulated cells (regardless of the addition of costimulatory antibodies) was 0.48% of the total CD8\(^+\) T cells. Background IFN-\(\gamma\) expression was <0.02% of the total CD8\(^+\) T cells. B, MDC-, PDC-, and CD8-depleted PBMC from donor 1 were loaded with CMV-A2 peptide at concentrations ranging from 2 \(\mu\)M to 200 fM and were used as APCs. Plots depict the percentage of IFN-\(\gamma\) CD107\(^+\) (pink), IFN-\(\gamma\)-CD107\(^+\) (blue), and IFN-\(\gamma\) CD107\(^+\) (green) responses from the total CD8\(^+\) CMV-A2 tetramer\(^+\) cells observed from 2 \(\mu\)M to 200 pM; below this peptide concentration, no response was observed. The leftmost point of each plot shows the background response (no peptide added).
show at the single-cell level what had been presumed from results obtained in bulk responses and in CD8\(^+\) T cell clones (20). We observed a relationship between peptide concentration, surface TCR down-modulation, and IFN-\(\gamma\) production; once the activation level was sufficient to detect IFN-\(\gamma\) production, TCR down-modulation was observed. These results suggest that antiviral CD8\(^+\) T cells responding by only degranulation should be capable of sequentially recognizing multiple infected cells, because they retain high surface TCR levels. CD8\(^+\) T cells activated sufficiently to produce cytokine may be limited in their short term potential to recognize multiple targets without a refractory period. This could lead to less effective antiviral activity while simultaneously inducing greater inflammation.

The ability of some CD8\(^+\) T cells to respond at low peptide concentrations could be explained by the presence of high affinity CD8\(^+\) T cell clonotypes within the total peptide-specific CD8\(^+\) T cell population. We show, however, that this threshold difference is not solely due to a mixture of high and low affinity T cell clonotypes at the population level, but that individual CD8\(^+\) T cell clonotypes may or may not respond to peptide at any given peptide concentration. This suggests that the signaling pathways and/or triggering sensitivity within individual Ag-specific CD8\(^+\) T cell clonotypes may not be identical, similar to recent observations from Ag-specific CD4\(^+\) T cell clonotypes (37). It is unlikely that such heterogeneity within a clonal CD8\(^+\) T cell population could be discerned using in vitro-derived CD8\(^+\) T cell clones, because these cells probably have different signaling properties and response patterns due to extended propagation. We also found that there are individual CD8\(^+\) T cell clonotypes likely to be of higher affinity in some individuals. Thus, different triggering thresholds exist within and between different CD8\(^+\) T cell clonotypes specific for the same viral peptide. Importantly, we did not find an inherent difference in the clonotypic profiles of the CD8\(^+\) T cells that responded to either CMV or HIV with different effector functions. Thus, it appears to be the level of viral Ag, not the specific CD8\(^+\) T cell clonotype, that dictates the nature of the CD8\(^+\) T cell response.

Examination of the triggering requirements of human CD8\(^+\) T cell clones have suggested that substantial heterogeneity exists in the effector response elicited by responding CD8\(^+\) T cells depending on the TCR occupancy level (22). Our findings indicate that IFN-\(\gamma\) secretion requires a higher peptide concentration and hence an increased number of pMHC:TCR contacts for such a response to be evoked. Degranulation can be evoked at a lower Ag concentration; thus, fewer pMHC:TCR interactions are required for this response.
Clonal usage alone does not differentiate the responsiveness of cells at low peptide concentrations. PBMC were stimulated with the appropriate peptide at low concentrations known to induce degranulation in the absence of detectable IFN-γ production (donors 1 and 2, 0.0002 μM CMV-A2 peptide; donor 3, 0.002 μM HIV-B57; donor 4, 0.0002 μM HIV-B57). The cells were live-sorted on CD8+ gated, tet+ CD107+ populations as indicated by the gates drawn on each plot. The TCRB CDR3 regions from the cells in each population were then sequenced. Only those CDR3 regions found three or more times or common to the CD107+ and CD107+ populations are shown for each donor. CDR3 regions are depicted as the amino acid sequence of the VDJ region, when each of these regions could be positively identified. The CDR3 region is in bold.
A relevant question that arises is which CD8+ T cell effector function is physiologically predominant in vivo during recognition of a virally infected target cell? This will largely depend on the concentration of antigenic peptide on the target cell surface. It is likely that the actual cell surface viral epitope density presented in context with MHC class I on infected cells is lower than that used for in vitro experiments, where concentrations as high as \(10^{-100}\) M may be used. One study estimated that the cell surface epitope density of an HIV-1 peptide known to prime CTL in some HIV-1-infected patients may be as few as 12 molecules/infected cell, whereas a higher expressed peptide reached levels of 400 molecules/infected cell (38). Some estimations suggest that as little as \(1\)–\(10\) pM complexes are required on a target cell to sensitize it for recognition by CD8+ CTL (39–41), although these estimates are controversial (42, 43). Predicting the number of pMHC complexes present on the surface of a target cell incubated with a certain concentration of either the CMV A2 or HIV B57 epitope is difficult, because this number varies depending on the affinity and avidity of each peptide. However, such analyses have been performed for other peptides in solution, where at a concentration of 1 nM peptide each APC bound \(~500\) peptides, and at 10 pM each APC bound \(~50\) peptides (44). In another study, \(~50\) peptides/APC were predicted to bind at 50 pM (40). In this study we routinely detected CD8+ T cell degranulation occurring at a 200-pM peptide concentration, and in donors 1 and 2 at a 20-pM peptide concentration, whereas, in general, IFN-γ production required 10–100 times more peptide. Thus, the predominant response to a peptide expressed at very low levels may be cytolytic, whereas a peptide expressed at higher density will stimulate a combined cytolysis and cytokine response with resultant TCR down-modulation. It is known from mouse models that epitope density directly correlates with the corresponding size of the memory CD8+ T cell pool (45–47), and our data suggest that epitope density may additionally dictate the functional nature of the CD8+ T cell response. This concept has particular relevance in vaccine models, where the level of Ag produced by a vaccine vector could dictate not only the size of the responding CD8+ T cell pool, but also the type of CD8+ T cell response.

Although each of the multiple CD8+ T cell clonotypes that are expanded in response to a CMV or HIV infection has the potential to carry out cytolysis and cytokine functions, the actual effector mechanism elicited in vivo may be primarily determined by the amount of peptide presented to each CD8+ T cell and not an inherent characteristic of the individual CD8+ T cell clone. In the presence of APCs expressing high viral Ag levels, responding CD8+ T cells would produce cytokines and elaborate cytolytic activity (Fig. 7). However, heightened stimulation would lead to surface TCR down-modulation on the responding CD8+ T cells, causing the CD8+ T cells to enter a refractory period before they could again optimally elaborate effector function. Conversely, presentation of low viral Ag levels would only induce CD8+ T cell cytolytic function, without surface TCR down-modulation. CD8+ T cells responding in this manner could therefore be capable of sequential recognition and lysis of multiple infected cells. Assuming an association between the viral replication level and peptide presentation on APCs or the number of infected cells, viruses that induce high Ag states, such as HIV (48, 49) and HCV (34, 50), may bias the CD8+ T cell response away from effective cytolytic

**FIGURE 7.** Model of the consequences of varying peptide concentration on the CD8+ T cell response. A virus-specific CD8+ T cell clonotype stimulated with APCs expressing high amounts of Ag elaborates cytokine production and cytolytic function. As a result of increased stimulation, however, surface TCR is down-modulated, and the CD8+ T cells enter a refractory period before they can again respond to Ag. Conversely, if the same clonotype of CD8+ T cells is stimulated by APCs expressing low amounts of Ag, the CD8+ T cells only elaborate cytolytic function. The lower level of activation does not cause surface TCR down-modulation, enabling these cells to respond to Ag repeatedly.
function, instead leading to increased production of potentially deleterious inflammatory cytokines. Recently, IFN-γ-mediated inflammation was shown to cause enhanced SIV replication in vaccinated rhesus macaques that were not protected from SIV infection (51), lending support to this model. Indeed, the chronic inflammatory pathological changes observed in lymph node architecture in HIV-infected individuals (14) may reflect the damage initiated by T cells responding to a virus with the capacity for prodigious replication within the site of Ag presentation itself.

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