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Flow cytometric detection of degranulation reveals phenotypic heterogeneity of degranulating CMV-specific CD8+ T lymphocytes in rhesus macaques

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Abstract

Flow-cytometric conditions for detection of lysosomal-associated membrane proteins (LAMPs) on the surface of recently degranulated cells were optimized for rhesus macaques and used to investigate the functional properties of rhesus cytomegalovirus (rhCMV)-specific CD8+ T lymphocytes with regards to cytotoxicity and interferon (IFN)- γ secretion in six asymptomatic CMV-seropositive rhesus macaques. Unlike humans, the rhesus macaque LAMP-1 protein CD107a underwent little or no endocytosis over a six to 18 hour stimulation period. Following in vitro stimulation, rhCMV-specific CD8+ T lymphocytes were heterogeneous with regards to the composition of cells positive for CD107a and/or IFN- γ , time to reach peak degranulation, and kinetics of IFN- γ secretion relative to degranulation. Responder CD8+ T lymphocytes that underwent degranulation without IFN- γ production (CD107a+IFN- γ -) were predominantly composed of terminally differentiated effectors (CD28-CD45RA+). Moreover, they had significantly lower frequencies of effector memory (CD28-CD45RA-) cells compared to the IFN- γ -secreting cells that did or did not undergo degranulation (CD107a+IFN- γ + or CD107a-IFN- γ +). The perforin content of effector CD8+ T lymphocytes was significantly greater than that of effector memory CD8+ T lymphocytes in rhesus macaques, suggesting that they were more cytolytic. Our findings suggest that the composition of rhCMV-specific CD8+ T lymphocytes with regards to CD107a+IFN- γ - responders may be an important determinant of their ability to control CMV replication.

Keywords

Rhesus CMV; CD8+ T lymphocytes; SIV; CD107a; Degranulation; CTL

Introduction

Cytotoxic CD8+ T lymphocytes (CTL) are essential for the immune control of several viral pathogens. CTL recognize cells presenting viral peptides bound to surface MHC class I molecules. Upon TCR engagement with peptide-MHC class I complexes, activated CD8+ T lymphocytes exhibit several effector functions, including cytokine production and cytotoxicity. Cytotoxicity is mediated either by release of pre-formed cytolytic granules, or less frequently by a granule-independent pathway involving Fas/FasL interaction (Kagi et al., 1994; Barry and Bleackley, 2002). Lytic granules consisting of perforin and granzymes are contained in

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membrane-bound lysosomes coated with lysosomal-associated membrane proteins (LAMPs) (Peters et al., 1991). During the process of degranulation, the membrane of the secretory lysosomes fuses with the plasma membrane of the activated CD8⁺ T lymphocyte and the lysosomal granules are then released into the immunological synapse between the CD8⁺ T lymphocyte and its target (Fukuda, 1991). LAMPs, therefore, are not usually present on the surface of T cells but are exposed only during active degranulation. This property has recently been exploited for flow-cytometric identification of CTL at the single-cell level. (Betts et al., 2003)

Simultaneous characterization of cytolytic and cytokine-secreting effector functions at the single-cell level have shed light on the functional heterogeneity of virus-specific CD8⁺ CTL in mice and humans (Betts et al., 2004; Wolint et al., 2004; Lacey et al., 2005; Betts et al., 2006; Lacey et al., 2006; Mongkolsapaya et al., 2006). In this study, we have optimized the flow-cytometric technique for detection of degranulating CD8⁺ T lymphocytes in rhesus macaques, and used it to characterize the functional properties of rhesus cytomegalovirus (rhCMV)-specific CD8⁺ T lymphocytes. Our data show that while anti-human CD107a and CD107b antibodies can reliably detect degranulating T lymphocytes in rhesus macaques, the endocytosis properties of rhesus CD107a differ appreciably from their human counterpart. This difference led to modifications in the technique for detecting LAMPs in rhesus macaques and facilitated optimal detection of concurrent cytokine secretion in activated T lymphocytes. Concurrent detection of degranulation and IFN- γ secretion revealed heterogeneity in the composition of rhCMV-specific CD8⁺ T lymphocytes with regards to the proportion of cells exhibiting either one or both functions. Activated CD8⁺ T lymphocytes displaying evidence of degranulation without IFN- γ production differed in their memory phenotype from cells producing IFN- γ with or without degranulation, suggesting that these represent a functionally distinct population. These data reinforce the importance of using multiple effector functions to evaluate virus-specific CD8⁺ T lymphocytes.

Materials and methods

Animals

T cell responses to superantigen (SAg) and rhCMV CD8⁺ T cell epitopes were analyzed in six SIV-negative, CMV-seropositive rhesus macaques housed in the breeding colony at the New England Primate Research Center (Table I). All animals were maintained in accordance with federal and institutional guidelines of animal care (Anonymous, 1996).

Antibodies

Monoclonal antibodies (MAb) used for costimulation in the intracellular cytokine staining assay consisted of purified, azide-free anti-human CD28 MAb (clone CD28.2, BD Biosciences, San Jose, CA) and anti-CD49d MAb (clone 9F10, BD Biosciences), cross-linked with affinity-purified F(ab')₂ fragments of goat anti-mouse IgG (Kierkegaard and Perry Lab, Inc., Gaithersburg, MD). The following directly conjugated anti-human MAb reagents were obtained from BD Biosciences: anti-CD3 FITC and APC (clone SP34), anti-CD3 PB and APC-Cy7 (clone SP34-2), anti-CD4 PE and PerCP (clone L200), anti-CD8 PerCP (clone SK1) and Alexa Fluor 700 (clone RPA-T8), anti-CD107a FITC and PE (clone H4A3), anti-CD107b FITC (clone H4B4), anti-CD63 FITC (clone H5C6), anti-CD95 APC (clone DX2), anti-CD28 FITC and PE (clone 28.2), anti-CD45RA PerCP and PE-Cy7 (clone L48), anti-IFN- γ APC (clone B27) and PE-Cy7 (clone 4S.B3), and anti-TNF- α FITC (clone Mab11). Anti-CD28 ECD (clone 28.2) was obtained from Beckman Coulter (Fullerton, CA). Anti-perforin FITC (clone Pf-344) was obtained from Mabtech (Mariemont, OH). Anti-granzyme B PE (clone GB12) was obtained from Caltag Labs (Burlingame, CA).

Stimulating antigens

Staphylococcus enterotoxin A (SEA) and enterotoxin B (SEB) were used together or alone at a final concentration of 100 ng/ml (Sigma, St. Louis, MO) for superantigen (SAg) stimulation. rhCMV-specific CD8⁺ T lymphocytes were stimulated with their cognate peptides (Table I). CD8⁺ T lymphocyte epitopes in the rhCMV immediate early 1 (IE1), immediate early 2 (IE2), phosphoprotein 65 (pp65), and interleukin-10 (IL10) proteins were mapped by the IFN- γ ELISPOT and intracellular cytokine staining assay using 8 to 15 amino acid (aa) long overlapping peptides (Table I and unpublished data). With the exception of IE1#83L and IE2#46F, epitopes were mapped to the 15 aa level. Peptides were synthesized by F-moc chemistry at the Massachusetts General Hospital peptide core facility (Charlestown, MA) using an automated peptide synthesizer (MBS 396, Advanced Chemtech). Individual lyophilized peptides were resuspended at 100 mg/ml in 100% DMSO (Sigma) and used at 1-2 μ g/ml with a final DMSO concentration of <0.05%.

Isolation of PBMC

Blood collected in vacutainer tubes containing the anticoagulant heparin was subjected to centrifugation over a Ficoll Lymphocyte Separation Medium (MP Biomedicals, Aurora, OH) density gradient, washed twice in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Sigma), 10 mM HEPES (Mediatech, Inc.; Herndon, VA), 2 mM L-glutamine (Invitrogen), 50 U penicillin/ml and 50 μ g streptomycin/ml (Invitrogen) (henceforth referred as R-10 medium). Assays were performed either on freshly isolated peripheral blood mononuclear cells (PBMC) used within 2 to 4 hours of cell preparation, or on PBMC cryopreserved in 90% FCS and 10% DMSO.

Intracellular cytokine staining (ICS) assay

PBMC were stimulated with the appropriate antigen in the presence of purified anti-CD28 and anti-CD49d costimulatory antibodies for all four-color flow cytometry experiments as previously described (Kaur et al., 2002). In experiments that included CD28 as a cell surface marker for delineating the memory phenotype of rhCMV-specific CD8⁺ T lymphocytes, only anti-CD49d was used for costimulation during the period of in vitro stimulation. For optimal stimulation, costimulatory antibodies were cross-linked in 12 $\times$ 75-mm polystyrene tubes precoated with 2.5 μ g/ml of affinity purified F(ab')₂ fragments of goat anti-mouse IgG suspended in 50 mM Tris pH 8.6 and kept overnight at 4°C. 1×10^6 PBMC were stimulated with SAg or rhCMV peptides for six hours or overnight (14-18 hours). Brefeldin A (Golgiplug, 1 μ g/ml; BD Biosciences) was added one hour after the start of stimulation. In some experiments, brefeldin A was replaced with monensin (GolgiStop, 2 μ M; BD Biosciences) for the duration of stimulation. PBMC were stimulated in tubes incubated at a 5° slant above horizontal at 37°C in a humidified 5% CO₂ atmosphere. At the end of the stimulation period, cells were washed with PBS and incubated with 0.02% EDTA solution in DPBS (Sigma) for 15 min at 37°C. Subsequent washes were in PBS/2% FCS. Cells were washed and surface stained with two or more of the fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD45RA, CD95 and CD28 at 4°C for 30 min. After surface staining, cells were washed and fixed using 100 μ l of Fixation Medium A (Caltag) at RT for 15 min. After being washed, cells were incubated with 100 μ l of Permeabilization Medium B (Caltag) and simultaneously stained with fluorochrome-conjugated antibodies specific for intracellular markers: IFN- γ , TNF- α , or perforin at RT for 30 min. After a final wash, cells were resuspended in 1% paraformaldehyde in PBS. Four-parameter flow cytometry samples were acquired on a FACSCalibur flow cytometer (BD Biosciences), while seven-color polychromatic flow cytometry samples were acquired on a LSRII flow cytometer (BD Biosciences). A minimum of 200,000 lymphocyte events was collected for analysis. Flow cytometry data files were analyzed using FlowJo

software version 8.1.1 (Tree Star, San Carlos, CA). Flow cytometry gates to identify positive cytokine signal were based on unstimulated control tubes. Responses towards stimulating antigen were calculated after subtraction of background responses in unstimulated (medium alone) PBMC. The surface markers CD95, CD28 and CD45RA were used to characterize the memory phenotype of IFN- γ -secreting or degranulating CD8⁺ T cell populations. The Boolean gate platform was used to create the full array of possible combinations amongst all CD95⁺ memory cells. These equated to four phenotype patterns when using CD28 and CD45RA.

Flow cytometric detection of degranulation

In initial experiments, the protocol used for identification of degranulating CD8⁺ T lymphocytes in humans (Betts et al., 2003) was followed. Thus, fluorochrome conjugated antibodies to the LAMP proteins CD107a, CD107b, and CD63 were added at the beginning of stimulation along with monensin. At the end of the stimulation period, cells were washed, surface stained with antibodies to CD3, CD4, and CD8 as described previously, and fixed in 1% paraformaldehyde. Flow cytometry gates for positive CD107a and CD107b expression were based on unstimulated control tubes (Fig. 1). Modifications to this protocol for detecting degranulating cells in rhesus macaques are described under the results section.

Statistical analysis

Statistical analysis was performed using PRISM version 4.0 (GraphPad Software Inc., San Diego, CA). Correlation between surface CD107a and perforin expression was analyzed by the nonparametric Spearman Rank correlation test. The nonparametric Wilcoxon signed rank test was used for all paired comparisons. These included comparison of surface CD107a expression under different experimental conditions, and comparison of granzyme B and perforin content in effector and effector memory CD8⁺ T lymphocytes. Differences in the memory phenotype of the three responder populations, CD107a+IFN- γ ⁻, CD107a-IFN- γ ⁺ and CD107a+IFN- γ ⁺, were analyzed by the repeated measures ANOVA and the Bonferroni post-hoc test.

Results

LAMPs are expressed on the surface of degranulating CD8⁺ T lymphocytes in rhesus macaques

We first tested whether antibodies to three human LAMP proteins, CD107a, CD107b and CD63 recognize their rhesus counterparts. CD107a and CD107b molecules were detected on the surface of rhesus macaque CD8⁺ and CD4⁺ T lymphocytes stimulated with SAg, but not on resting T lymphocytes (Fig. 1A). Unlike CD107a and CD107b, the LAMP protein CD63 was consistently detected at a high level on the surface of unstimulated CD8⁺ T lymphocytes (Fig. 1A) and thus, was excluded from further experiments. Although SAg stimulation resulted in some upregulation of CD107 on CD4⁺ T cells, stimulation with a CD8-specific rhCMV peptide resulted in surface expression of CD107a and CD107b exclusively on CD8⁺ T lymphocytes (Fig. 1B). Stimulation with an unrelated peptide did not mobilize CD107a to the cell surface (data not shown). Thus, surface expression of CD107 molecules was specific and confined to responding antigen-specific T lymphocytes.

In humans, the appearance of CD107a and CD107b on the surface of CD8⁺ T lymphocytes was associated with a concurrent loss of intracellular perforin, consistent with its association with degranulation (Betts et al., 2003). In order to verify that this also applies to LAMPs on rhesus macaque CD8⁺ T lymphocytes, we simultaneously measured surface CD107a and intracellular perforin. Resting CD8⁺ T lymphocytes contained intracellular perforin but did not express surface CD107a (data not shown). In SAg-stimulated cells, cells expressing surface CD107a contained little or no intracellular perforin, while perforin-positive cells remained

CD107a-negative (Fig. 2A). Kinetic analysis showed a reciprocal relationship between CD107a and perforin (Fig. 2B-C). Surface expression of CD107a on CD8+ T lymphocytes was detected as early as one hour after stimulation and increased over a four-hour stimulation period (Fig. 2B). A concurrent decline in perforin-positive CD8+ T lymphocytes (Fig. 2B) and an inverse correlation between surface CD107a and intracellular perforin ($Rho=0.83$, P -Value=0.015, Spearman Rank Correlation test; Fig. 2C) was observed. Thus, similar to humans, measurement of surface CD107a is a sensitive marker for identifying recently degranulated cytolytic T cells in rhesus macaques.

Optimal conditions for detection of degranulating CD8+ T cells in rhesus macaques

Although human CD107a and CD107b molecules are encoded on different chromosomes, they are coordinately expressed on activated T cells in humans, and both proteins are rapidly endocytosed following externalization (Fukuda, 1991; Betts et al., 2003). Due to the rapid endocytosis, optimal detection of degranulated CD8+ T lymphocytes in humans requires the presence of anti-CD107a and anti-CD107b MAb along with monensin throughout the stimulation period (Betts et al., 2003). Monensin neutralizes the acidic pH of endosomes and thereby prevents degradation of fluorochrome conjugates of endocytosed anti-CD107 MAbs (Betts and Koup, 2004). We investigated whether similar experimental conditions are required for optimal detection of degranulating T cells in rhesus macaques.

In contrast to humans, the presence of anti-CD107a MAb throughout the stimulation period ("during"), or its addition at the end of a six-hour stimulation period ("post"), did not alter detection of surface CD107a on activated CD8+ T lymphocytes (Fig. 3A). This did not hold true for CD107b, which similar to humans, was rapidly endocytosed as evidenced by a two- to three-fold reduction in surface CD107b signal when anti-CD107b MAb was added at the end of stimulation (Fig. 3B). Consistent with the observation of differential endocytosis of CD107a and CD107b in rhesus macaques, these molecules were coordinately expressed only when both antibodies were present throughout ("during") the stimulation period (Fig. 3C). If the antibodies were added at the end of the stimulation period ("post"), the frequency of CD107a+ cells far exceeded the frequency of CD107b+ cells (Fig. 3C). Repeated analysis of SAg and rhCMV peptide stimulation in six rhesus macaques confirmed a significant reduction in surface CD107b, but not CD107a, by addition of antibodies at the end ("post") of a six-hour stimulation period (Fig. 3D).

We next asked whether CD107a underwent endocytosis, albeit at a slow rate. Compared to six hours, a greater and significant reduction of surface CD107a was observed if the antibody was added at the end of an 18-hour stimulation period (Fig. 3D). However, the extent of endocytosis of CD107a (maximum 1.4-fold reduction) continued to be considerably lower than that of CD107b. A two- to three-fold reduction in surface CD107b at the end of 18 hours was similar to that observed at six hours. These results suggest that in rhesus macaques, endocytosis of CD107a exposed on the surface of recently degranulated T cells starts late and occurs slowly, while endocytosis of CD107b occurs rapidly and reaches near maximal levels by the end of six hours. Thus, unlike their human counterparts, rhesus macaque CD107a and CD107b molecules are differentially endocytosed in CD8+ T lymphocytes. Moreover, measurement of CD107a alone accounts for all CD107b-positive cells, although the converse is not true (Fig. 3C). Hence, future experiments to identify degranulating T lymphocytes in rhesus macaques were performed only with the anti-CD107a antibody.

Monensin impairs detection of intracellular IFN- γ and TNF- α in rhesus macaque T cells

In the previous experiments, monensin was included whenever the anti-LAMP antibodies were present during the stimulation period. Since monensin also blocks protein transport from the endoplasmic reticulum to the Golgi complex, we examined whether its use was sufficient for

simultaneous detection of intracellular cytokines in activated rhesus macaque CD8⁺ T lymphocytes. Monensin does not completely block secretion of TNF- α from activated human T cells (O'Neil-Andersen and Lawrence, 2002). Hence, a combination of monensin and brefeldin A are used for concurrent detection of degranulation and TNF- α secretion in human T cells (Betts and Koup, 2004).

Following a six to 18 hour period of stimulation with SEB, the use of monensin resulted in a significantly lower frequency of both IFN- γ - and TNF- α -secreting cells compared to brefeldin A (Fig. 4A-C). The blunting effect of monensin on intracellular IFN- γ and TNF- α signal was more pronounced at the end of 18 hours (Fig. 4B). In contrast to intracellular cytokines, detection of surface CD107a did not significantly differ between brefeldin A and monensin (Fig. 4A-B, bottom panel and Fig 4D). These data are consistent with the delayed kinetics of endocytosis of CD107a in rhesus macaques.

We next asked whether addition of monensin to brefeldin A would enhance detection of surface CD107a in experiments that combined intracellular cytokine staining with CD107a staining. Titration experiments were performed by addition of monensin at concentrations ranging between 2 μ M and 0.2 μ M to 1 μ g/ml of brefeldin A. Data on SEB- and rhCMV peptide-stimulated PBMC in two macaques after a six hour stimulation period are shown (Fig. 4E to J). Consistent with our previous experiment, intracellular IFN- γ detection in the presence of monensin alone was always lower than that with brefeldin A alone while CD107a was unchanged or slightly higher with brefeldin A alone (Fig. 4E to J). On addition of monensin to brefeldin A in the titration experiments, surface expression of CD107a either remained unchanged (Fig. 4F-G), or in some instances, resulted in a small increment (19-36%) in CD107a detection (Fig. 4E, H, I, J). Since the increment of CD107a detection by addition of monensin was minimal when present, future experiments combining IFN- γ and CD107a staining were performed with only brefeldin A as the secretory inhibitor for intracellular cytokine staining and monensin was not added.

Heterogeneity in degranulation and IFN- γ secretion in rhCMV-specific CD8⁺ T lymphocytes

It is now well recognized that measurement of IFN- γ secretion alone does not provide a complete assessment of the number and function of virus-specific CD8⁺ T lymphocytes (De Rosa et al., 2004; Betts et al., 2006). Having established optimal conditions for detection of degranulating and IFN- γ -secreting CD8⁺ T lymphocytes in rhesus macaques, we next evaluated the functional composition of rhCMV-specific CD8⁺ T lymphocytes targeting individual epitopes. First, the kinetics of degranulation and IFN- γ production in rhCMV-specific and SAg-stimulated CD8⁺ T lymphocytes were evaluated in three healthy CMV-seropositive rhesus macaques (Mm247.87, Mm212.87, and Mm105.93; Table I) using four-color flow cytometry.

Three responding CD8⁺ T cell populations consisting of I) cells degranulating but not secreting IFN- γ (CD107a+IFN- γ -), II) cells secreting IFN- γ without degranulation (CD107a-IFN- γ +), and III) degranulated cells concurrently secreting IFN- γ (CD107a+IFN- γ +) were detected (Fig. 5A). The relative proportion of these three populations was dynamic and changed with the duration of stimulation (Fig. 5B). In the first hour after SAg or rhCMV peptide stimulation, 70-85% of the responding CD8⁺ T lymphocytes had degranulated but not produced IFN- γ (Fig. 5B). In these experiments, brefeldin A was added from the beginning of stimulation. Thus, the relative scarcity of IFN- γ -secreting CD8⁺ T cells at this early time-point was not due to the absence of a secretory inhibitor in the first hour of stimulation. By two hours, all three responding populations were detected, and the relative proportions of each varied with animal and stimulating antigen (Fig. 5B and data not shown). By four hours, the pattern of responding CD8⁺ T cells was relatively stable and dominated by population III (CD107a+IFN- γ +; Fig. 5B and data not shown). When the kinetics of surface CD107a were compared with intracellular

IFN- γ , three patterns (denoted as A, B and C) were evident (Fig. 5C). In pattern A illustrated by Mm247.87 PBMC stimulated with SAg, responding CD8⁺ T cells had degranulated to near maximal levels by two hours post-stimulation (Fig. 5C). IFN- γ production was <40% of its maximal value at two hours, and peaked later at 4-6 hours post-stimulation (Fig. 5C). In pattern B illustrated by rhCMV IE1#83L-specific CD8⁺ T lymphocytes in Mm247.87, both IFN- γ production and degranulation peaked early and simultaneously in the first two hours post-stimulation (Fig. 5C). Pattern C illustrated by rhCMV IE1#83L-specific CD8⁺ T lymphocytes in Mm105.93 resembled pattern B with regards to the kinetics of degranulation and IFN- γ production being concordant. However, peak levels were reached only after six hours of stimulation (Fig. 5C). The patterns were reproducible in separate experiments performed one to four months apart (Fig. 5C). It is noteworthy that the kinetic experiments revealed differences in the rapidity of degranulation and IFN- γ production between different responding CD8⁺ T lymphocytes which are not apparent by examination of single time-points four to six hours post stimulation (Fig. 5B-C). Whether the differences in kinetics of degranulation relative to IFN- γ secretion have a bearing on their functional properties remains to be determined.

Heterogeneity with regards to IFN- γ secretion and degranulation was further examined in all but one specificity of rhCMV-specific CD8⁺ T lymphocytes in six rhesus macaques at the end of an 18-hour stimulation period (Table I and Fig. 5D). One epitope (rhCMV IE1#83L) was recognized by three animals, Mm247.87, Mm105.93, and Mm361.95 (Table I and Fig. 5D). Variations in the proportion of CD107a+IFN- γ -, CD107a+IFN- γ +, and CD107a-IFN- γ + among responding CD8⁺ T lymphocytes were apparent between animals responding to the same stimulus (rhCMV IE1#83L) and in the same animal for different antigen specificities, as illustrated by Mm105.93 (Fig. 5D).

Memory phenotype of responding CD8⁺ T lymphocytes subsets

Heterogeneity in composition of IFN- γ -secreting and degranulating cells could be related to differences in the memory phenotype of different rhCMV-specific CD8⁺ T lymphocyte populations. We next examined whether the three subsets of rhCMV-specific CD8⁺ T lymphocytes identified on the basis of co-expression of surface CD107a and IFN- γ secretion differed in their memory phenotype. rhCMV-specific ($n=12$) CD8⁺ T lymphocyte responses were analyzed in six rhesus macaques (Table II) by seven-color flow cytometry at the end of an 18 hour stimulation period. The memory phenotype of rhCMV-specific and total CD8⁺ T lymphocytes were analyzed by surface expression of CD95, CD28, and CD45RA. As previously described in rhesus macaques (Pitcher et al., 2002), naïve CD8⁺ T lymphocytes were phenotypically homogenous and consisted of CD95-CD28+CD45RA+ cells, while CD95+ memory cells displayed phenotypic heterogeneity (Fig. 6A). Based on co-expression of CD95 and CD28, memory CD8⁺ T lymphocytes in rhesus macaques have been broadly designated as central memory (CD95+CD28+) and effector memory (CD95+CD28-) cells (Pitcher et al., 2002). In humans, CD27-CD28-CCR7- memory CD8⁺ T lymphocytes that express CD45RA have been shown to be functionally different from those that are CD45RA- in having increased cytolytic activity and poor proliferative ability, and are thus commonly referred to as “terminally differentiated effectors” (Hamann et al., 1997; Sallusto et al., 1999). In this study, four phenotypically distinct populations of memory (CD95+) CD8⁺ T lymphocytes could be identified based on co-expression of CD28 and CD45RA. These included a CD95+CD28-CD45RA- population designated as effector memory (EM), a CD95+CD28-CD45RA+ population designated as terminally differentiated effectors (Eff), a CD95+CD28+CD45RA- population designated as central memory 1 (CM1) cells, and a minor (<5%) CD95+CD28+CD45RA+ population designated as central memory 2 (CM2) (Fig. 6A). While the designations of EM, Eff and the dominant central memory (CM1) T lymphocytes conforms to previous phenotypic definitions of memory CD8⁺ T lymphocytes (Hamann et al.,

1997; Sallusto et al., 1999; Pitcher et al., 2002), the minor CD95+CD28+CD45RA+ population, which hitherto has not been reported, was arbitrarily designated as CM2 in this study.

rhCMV-specific CD8+ T lymphocytes were predominantly effector memory (CD95+CD28–CD45RA–) and/or terminally differentiated effector (CD95+CD28–CD45RA+) cells (Table II). Differences in memory phenotype between the degranulating alone and IFN- γ -secreting subsets of rhCMV-specific CD8+ T lymphocytes were observed (Table II and Fig. 6B). The CD107a+IFN- γ – subset of rhCMV-specific CD8+ T lymphocytes had significantly higher frequencies of effector as compared to effector memory cells (Fig. 6B). In contrast, the two IFN- γ -secreting subsets of rhCMV-specific CD8+ T lymphocytes had comparable frequencies of effector and effector memory cells (Fig. 6B). As a result, CD107a+IFN- γ – rhCMV-specific CD8+ T lymphocytes contained significantly higher frequencies of effectors and significantly lower frequencies of effector memory cells compared to the dual functional CD107a+IFN- γ + or the single functional CD107a–IFN- γ + rhCMV-specific CD8+ T cell population (Table II).

In humans, memory CD8+ T lymphocytes (including CMV-specific CD8+ T cells) with a terminally differentiated effector phenotype (CD28–CD45RA+) have been shown to be cytolytic and have a high granzyme B and perforin content compared to effector memory cells (Baars et al., 2000; Appay et al., 2002). Since the enrichment of effectors in the CD107a+IFN- γ – subset of rhCMV-specific CD8+ T lymphocytes suggested a higher cytolytic capability of this population, we investigated whether the cytolytic granule content of effectors differed from that of effector memory cells in rhesus macaques. The intracellular granzyme B and perforin content of different memory populations (CD95+) of CD8+ T lymphocytes was examined in nine rhCMV-seropositive rhesus macaques (Fig. 7). Central memory CD8+ T lymphocytes consistently had low (<5%) to undetectable intracellular granzyme B and perforin (Figure 7A and data not shown). The cytolytic granule content of effectors and effector memory CD8+ T lymphocytes varied considerably between animals (Fig. 7B). The frequency and content of perforin granules, but not granzyme B, were found to be significantly higher in the effector as compared to effector memory CD8+ T lymphocytes (Fig. 7B–C). These data suggest that similar to humans, a terminally differentiated effector phenotype of CD8+ T lymphocytes in rhesus macaques may represent CD8+ T cells with greater cytolytic ability.

Discussion

In this study the flow-cytometric technique used for detection of degranulating cells in humans was adapted to rhesus macaques, and used to evaluate the IFN- γ -secreting and cytolytic properties of rhCMV-specific CD8+ T lymphocytes in immunocompetent CMV-seropositive animals. CD8+ T lymphocytes recognizing at least twelve rhCMV epitopes in six macaques were analyzed at the single-cell level. Following a 6–18 hour period of *in vitro* stimulation, rhCMV-specific CD8+ T lymphocytes were composed of a dominant population that had secreted IFN- γ and simultaneously undergone degranulation, and two minor populations that showed evidence of one of either two effector functions. We show that the proportion of each of the three responder populations was heterogeneous, varying between different rhCMV-specific CD8+ T lymphocyte populations in the same animal, as well as varying between responder CD8+ T lymphocytes recognizing the same rhCMV epitope in different animals. We also show that the memory phenotype of the three responding populations of rhCMV-specific CD8+ T lymphocytes was not uniform. Responder CD8+ T lymphocytes that underwent degranulation without IFN- γ secretion were predominantly composed of terminally differentiated effector type (CD28–CD45RA+) cells. In contrast, the two IFN- γ -secreting populations consisted of comparable frequencies of effector memory (CD28–CD45RA–) and effector cells, regardless of the presence or absence of concurrent degranulation. Since effector CD8+ T lymphocytes in rhesus macaques were found to have significantly greater perforin content compared to effector memory CD8+ T lymphocytes, these findings raise the possibility

that the composition of rhCMV-specific CD8⁺ T lymphocytes with regards to CD107a+IFN- γ ⁻ responders may be an important determinant of their ability to control CMV replication.

Using a combination of the CD107 mobilization assay and intracellular cytokine staining, there have been limited reports of heterogeneity in the degranulating and cytokine producing properties of human CD8⁺ T lymphocytes responding to epitopes in HIV, CMV, and dengue virus (Betts et al., 2004; Lacey et al., 2005; Lacey et al., 2006; Mongkolsapaya et al., 2006). Similar to our observation in rhCMV-specific CD8⁺ T lymphocytes, human CMV-specific CD8⁺ T lymphocytes targeting different epitopes showed variation in their degranulating and cytokine producing capacity (Lacey et al., 2005; Lacey et al., 2006). Interestingly, IFN- γ -secreting CD8⁺ T lymphocytes recognizing epitopes in the human CMV IE1 protein contained fewer cells with degranulating ability compared to pp65-specific CD8⁺ T lymphocytes (Lacey et al., 2005; Lacey et al., 2006). In this study, the analysis of rhCMV-specific CD8⁺ T lymphocytes was confined to those recognizing epitopes in the immediate early 1 and 2 proteins, and hence a comparison with pp65-specific CD8⁺ T lymphocyte response cannot be made. However, it is noteworthy that unlike human CMV-CD8⁺ T cells (Lacey et al., 2006), there did not appear to be a defect in degranulating ability of IE-specific rhCMV-specific CD8⁺ T cells. The functional implication of the differential degranulating ability of IE1-specific and pp65-specific CD8⁺ T lymphocytes in humans is not clear, particularly in light of a recent report on the efficacy of IE1-specific but not pp65-specific CTL, to protect heart and lung transplant recipients against CMV disease (Bunde et al., 2005). It is of interest that unlike IE1-specific CTL in humans which showed low levels of degranulation (Lacey et al., 2005; Lacey et al., 2006), rhCMV IE1-specific CTL showed degranulation in the majority of responding cells.

It is not known whether the cells that mobilize CD107 with or without cytokine production differ in their cytolytic potential. In this study, we observed that CD107a+IFN- γ ⁻, but not CD107a+IFN- γ ⁺, rhCMV-specific CD8⁺ T lymphocytes were enriched for memory cells with a terminally differentiated effector phenotype (CD28⁻CD45RA⁺). Since viable cell sorting of CD107a+IFN- γ ⁻ and CD107a+IFN- γ ⁺ cells is not possible in rhesus macaques, we compared the cytolytic granule content of effector and effector memory CD8⁺ T lymphocytes in rhesus macaques. Although both memory subsets contained variable levels of granzyme B and perforin, the effectors contained a significantly higher proportion of perforin⁺ cells. The observation that CD107a+IFN- γ ⁻ cells were enriched for effectors raises the possibility that rhCMV-specific CD8⁺ T lymphocytes undergoing degranulation without cytokine production may be more cytolytic than the dominant dual effector population. Data from humans and mice support this possibility. Memory CD8⁺ T lymphocytes (including CMV-specific CD8⁺ T lymphocytes) of a differentiated effector phenotype (CD28⁻CD27⁻CD45RA⁺) in humans have been shown to be cytolytic and have a higher perforin content compared to those with an effector memory (CD28⁻CD27⁺CD45RA⁻) phenotype (Baars et al., 2000; Appay et al., 2002). In mice, lymphocytic choriomeningitis virus-specific CD8⁺ T lymphocytes with an effector phenotype were found to be more cytotoxic than those with an effector memory phenotype, even though both memory subsets showed similar CD107 mobilization (Wolint et al., 2004). The recent report of CD45RA^{bright} and CD45RA^{dim} subsets of CD107a-positive HIV-specific CD8⁺ T lymphocytes that are differentially lost in progressive HIV infection (Jones et al., 2006) is further evidence for the presence of distinct phenotypic and functional populations of degranulating cells within antigen-specific CD8⁺ T lymphocytes.

It is noteworthy that studies that have used granzyme B or perforin ELISPOT, or the Lysispot assay to measure cytolytic function at the single-cell level, have shown a marked discordance between the frequencies of IFN- γ -secreting and cytolytic cells for SIV-, HIV-, and human CMV-specific CD8⁺ T lymphocytes (Snyder et al., 2003; Kleen et al., 2004; Calarota et al., 2006; Snyder-Cappione et al., 2006). In most instances, the frequency of IFN- γ -secreting cells

has far exceeded the frequency of cytolytic cells. The reason for the discrepancy from flow cytometric-based assays where the majority of responding CD8+ T lymphocytes tend to be CD107a+IFN- γ +, may lie in the fact that cytolytic granule content is not taken into consideration when CD107 mobilization alone is used as a surrogate marker of cytotoxicity. Even though CD107a mobilization on the surface of activated CD8+ T lymphocytes or natural killer cells directly correlates with the extent of cytotoxicity evaluated by a traditional chromium release assay (Betts et al., 2003; Rubio et al., 2003; Alter et al., 2004), studies in mice have elegantly demonstrated that cytolytic ability is dependent on the granule content, and CD107a mobilization alone is not a measure of cytolytic ability (Wolint et al., 2004).

The kinetic experiments confirmed the onset of degranulation within one hour of stimulation, results that are similar to those described previously for activated CD8+ T lymphocytes in humans and mice (Betts et al., 2003; Wolint et al., 2004) and for human NK cells (Alter et al., 2004). In this study the analysis of kinetics of degranulation and IFN- γ secretion following SEB and rhCMV peptide stimulation in three animals (three SEB responders and six rhCMV-specific CD8+ T lymphocytes) illustrated an interesting finding. Virus-specific CD8+ T lymphocytes displaying similar co-expression profiles of CD107a and IFN- γ at the end of 6-18 hours of in vitro stimulus, could differ with regards to the rapidity to reach peak degranulation and the rate at which IFN- γ secretion “caught up” with degranulation (Figure 5C). The differential kinetics of degranulation and IFN- γ secretion may be related to the differences in memory composition of the different responding populations. Effectors degranulate at a faster rate compared to effector memory cells while the rate of cytokine production is comparable in both (Bachmann et al., 1999). If the rapidity of degranulation relative to cytokine secretion is a determinant of effective immune control, kinetic experiments may be required to tease out functional differences between two seemingly similar antigen-specific responses. Additionally, the dynamics of degranulation and IFN- γ kinetics also demonstrate that the duration of antigen stimulation can significantly affect the composition of virus-specific CD8+ T lymphocytes and thus comparative studies need to take the duration of stimulus into consideration.

This study is the first detailed analysis of functionality of rhCMV-specific CD8+ T lymphocytes recognizing several different epitopes in rhesus macaques, an animal model that is valuable for studies on AIDS pathogenesis including immunopathogenesis of CMV infection. Unlike humans in whom both pp65 and IE are immunodominant, (Sylwester et al., 2005) the rhCMV-specific CD8+ T lymphocyte response is characterized by a dominant response to the IE1 and IE2 proteins, with pp65 being recognized to a lesser degree both in terms of magnitude and frequency of responders (Kaur A; unpublished data). Consistent with the memory phenotype of human CMV-specific CD8+ T lymphocytes (Gillespie et al., 2000; Champagne et al., 2001; Gamadia et al., 2001; Appay et al., 2002; Ellefsen et al., 2002; Wills et al., 2002), rhCMV-specific CD8+ T lymphocytes as a whole had lower frequencies of central memory cells (CD28+CD45RA-) and higher frequencies of effector memory (CD28-CD45RA+) and effector cells (CD28-CD45RA+) compared to the total CD8+ T lymphocyte population. The concurrent use of multiple surface markers has added to the complexity and phenotypic heterogeneity of memory T lymphocyte populations within previously defined categories. Thus, a hitherto unreported minor population of CD28+CD45RA+ memory CD8+ T lymphocytes (designated as CM2) was also identified; but its functional significance remains unknown.

Finally, results of our optimization experiments revealed a difference in endocytosis of LAMP proteins, specifically CD107a, between rhesus macaques and humans. Variability in cytoplasmic tail sequences which are involved in endocytosis (Chang et al., 2002) may account for this difference from human CD107a/b as well as from macaque CD107b. Our finding of absent to marginal endocytosis of CD107a during the usual in vitro stimulation period has

important technical implications. Since the presence of monensin during stimulation was found to significantly decrease IFN- γ and TNF- α signal by intracellular cytokine staining, a non-requirement of monensin for CD107a detection would be expected to facilitate concurrent detection of IFN- γ and TNF- α without undue loss of sensitivity. In practice, we found variability in the requirement of monensin for optimal detection of surface CD107a in rhesus macaques. In some instances, addition of monensin to brefeldin A resulted in up to a 30% increase in CD107a detection without appreciably compromising IFN- γ detection. Doses as low as 0.2 μ M of monensin (one-tenth the recommended dose) were sufficient to observe this effect. Thus, the requirement for addition of low doses of monensin to brefeldin A for optimal detection of CD107a and IFN- γ may need to be determined on a case by case basis. In conclusion, the establishment of optimal conditions for flow-cytometric detection of degranulation in rhesus macaques will facilitate studies on simultaneous evaluation of multiple effector functions of antigen-specific CD8⁺ T lymphocytes in the rhesus macaque animal model of AIDS.

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Abbreviations

APC	allophycocyanin
APC-Cy7	allophycocyanin-Cy7
Alexa700	Alexa Fluor 700
BFA	brefeldin A
CTL	cytotoxic T lymphocyte
CM	central memory
ECD	energy coupled dye
Eff	effectors
ELISPOT	enzyme-linked immunospot

EM	effector memory
FITC	fluorescein isothiocyanate
ICS	intracellular cytokine staining
IE1	immediate early 1 protein
IE2	immediate early 2 protein
IFN-γ	interferon-gamma
IL10	interleukin-10 protein
LAMP	lysosomal associated membrane protein
Mon	monensin
PB	Pacific Blue
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PE-Cy7	phycoerythrin-Cy7
PerCP	peridinin chlorophyll protein
pp65	phosphoprotein
rhCMV	rhesus cytomegalovirus
SAg	superantigen
SEA	staphylococcus enterotoxin A
SEB	staphylococcus enterotoxin B
TNF-α	tumor necrosis factor alpha

Figure 1. Expression of LAMPs on T lymphocytes in rhesus macaques

PBMC were stimulated for six hours with (A) superantigen (SAg) and (B) rhCMV peptide IE1#83L in the presence of monensin and antibodies to CD107a (FITC), CD107b (PE) or CD63 (FITC) antibodies. Lymphocytes gated on CD8+ and CD4+ T cells are shown. Numbers denote frequency of the gated population expressing the corresponding LAMP.

Figure 2. Surface expression of CD107a is associated with decline in intracellular perforin

(A) Representative contour plot of SEB-stimulated CD8⁺ T lymphocytes at four hours post-stimulation. (B) Kinetics of surface CD107a expression and intracellular perforin following SEB stimulation. The percent maximal response at each time point is shown. Mean values of two experiments shown. Error bars show standard error of mean (SEM). (C) Inverse correlation between surface CD107a expression and intracellular perforin. Rho and P -Values calculated by the nonparametric Spearman Rank correlation test.

Figure 3. Optimal conditions for detecting degranulating CD8+ T lymphocytes in rhesus macaques PBMC were stimulated with SEB and stained with antibodies to (A) CD107a (PE), (B) CD107b (FITC), or (C) both CD107a and CD107b, present throughout the stimulation period (“during”), or added at the end of the stimulation period (“post”). Gated CD8+ T lymphocytes are shown. (D) Comparison of surface LAMP expression when antibodies were added “during” or “post”. Data following 6 or 18 hours of SEB and rhCMV-CD8 peptide stimulation are shown. P-Values were calculated by the nonparametric Wilcoxon Signed Rank test.

Figure 4. Differential effects of monensin and brefeldin A on detection of CD107a, IFN- γ and TNF- α

PBMC from rhesus macaques were stimulated with SEB in the presence of monensin or brefeldin A for (A) six hours or (B) 18 hours and analyzed by four-color flow cytometry. Numbers denote frequency of gated CD8 $^{+}$ T lymphocytes. Comparison of (C) IFN- γ production and (D) CD107a expression of CD8 $^{+}$ T lymphocytes stimulated with SEB (left panels) or cognate rhCMV peptides (right panels) for six hours in the presence of brefeldin A (BFA) or monensin (Mon). *P*-Values were calculated by the nonparametric Wilcoxon Signed Rank test. (E - J) Effect of addition of different concentrations of monensin to 1 μ g/ml of BFA on CD107a and IFN- γ detection in CD8 $^{+}$ T cells stimulated with SEB or rhCMV peptides in two macaques. Data after six hours of stimulation are shown. Concentrations of monensin ranging between 0.2 and 2 μ M were used.

Figure 5. Heterogeneity in IFN- γ -secreting and cytolytic ability of CD8+ T lymphocytes
 (A) Gating strategy showing responding subsets of SEB-stimulated CD8+ T lymphocytes. Three responding subsets shown; CD107a+IFN- γ - (I), CD107a-IFN- γ + (II), and CD107a+IFN- γ + (III). (B) Representative data on kinetic analysis of responding CD8+ T lymphocyte subsets, and (C) Differential kinetics of surface CD107a expression and IFN- γ production. (D) Composition of eleven rhCMV-specific CD8+ T lymphocytes responses in six rhesus macaques at the end of 18 hours of stimulation. All data obtained by four-color flow cytometry. Mean values are shown. Error bars represent SEM of two to four independent experiments performed one to four months apart.

Figure 6. Memory phenotype of responding CD8⁺ T lymphocyte subsets of rhCMV-specific CD8⁺ T lymphocytes analyzed by seven-color flow cytometry

(A) Delineation of memory populations of CD8⁺ T lymphocytes based on concurrent measurement of CD95, CD28 and CD45RA. Overlay plots of four subsets of CD95⁺ memory cells are shown. (B) Memory phenotype of CD107a+IFN- γ - (I), CD107a-IFN- γ + (II), and CD107a+IFN- γ + (III) functional of rhCMV-specific CD8⁺ T lymphocytes at the end of an 18-hour stimulation period. *P*-Values were determined by repeated measures ANOVA and the Bonferroni post-hoc test. The frequency of CM1 and CM2 cells were significantly lower ($P < 0.05$) than the Eff and EM populations for all three responding subsets (not shown on graph). The flow cytometry panel consisted of CD3 PB, CD8 Alexa700, CD95 APC, CD28 FITC, CD45RA PerCP, CD107a PE, and IFN- γ PE-Cy7 antibodies.

Figure 7. Cytolytic granule content of effector (CD95+CD28-CD45RA+) and effector memory (CD95+CD28-45RA-) CD8+ T lymphocytes in rhesus macaques

(A) Overlay histograms showing granzyme B (left) and perforin (right) content of different memory populations of CD8+ T lymphocytes. (B) Comparison of the frequency of granzyme B+ and perforin+ cells between effector (Eff) and effector memory (EM) CD8+ T lymphocytes. (C) Comparison of the median channel fluorescence (MCF) of granzyme B and perforin content between effectors (Eff) and effector memory (EM) CD8+ T lymphocytes. *P*-Values were calculated by the non-parametric Wilcoxon signed rank test. The eight-color flow cytometry panel consisted of CD3 APC-Cy7, CD4 PerCP, CD8 Alexa700, CD95 APC, CD28 ECD, CD45RA PE-Cy7, perforin FITC, and granzyme B PE antibodies.

rhCMV-specific CD8+ T lymphocyte responses in six CMV-seropositive SIV-negative rhesus macaques

Animal I.D.	Age in years	Target specificity of rhCMV-specific CD8+ T cells		Frequency of rhCMV-specific CD8+ T cells (%) ^d
		rhCMV protein	Peptide nomenclature	
Mm105.93	13	IE1 ^a	73-87	1.2 ± 0.8
		IE1	329-336	4.0 ± 2.3
		IE2 ^b	463-477	6.0 ± 4.4
Mm212.87	19	IE2	186-196	1.5 ± 0.8
		IL10 ^c	121-135	2.2 ± 0.8
		IE1	329-336	4.2 ± 2.2
Mm247.87	19	IE1	245-259	17.1 ± 4.4
Mm361.95	11	IE1	329-336	3.3 ± 0.6
		IE2	199-213	0.3 ± 0.1
		IE2	551-565	0.8 ± 0.7
Mm111.91	15	IE1	217-231	17.7 ± 10.6
Mm543.91	15	IE1	407-421	2.9 ± 0.8
		IE2		

^a immediate early 1 protein.
^b immediate early 2 protein.
^c interleukin-10 protein.
^d mean ± standard deviation of two to five replicates.

Table II
Differences in memory phenotype between the functional subsets of rhCMV-specific CD8+ T lymphocytes

	% CD8+ T cells that are			
	CD95+28-45RA- (EM)	CD95+28-45RA+ (Eff)	CD95+28+45RA- (CM1)	CD95+28+45RA+ (CM2)
Total CD8+ T cells (n=6)	24.9±12.9	38.5±11.4	11.7±4.0	1.8±1.3
rhCMV-specific CD8+ (n=12)				
CD107a+IFN γ -	18.2±13.9 *	61.7±16.3 [#]	3.9±3.4	1.2±0.9
CD107a-IFN γ +	34.8±18.9	48.0±21.9	5.4±4.5	1.3±1.1
CD107a+IFN γ +	36.8±19.0	44.6±24.1	5.4±6.5	1.5±2.5

* *P*-value <0.01 (repeated measures ANOVA and Bonferroni post-hoc test) for difference between CD107a+IFN γ - rhCMV-specific responders and either CD107a-IFN γ or CD107a+IFN γ rhCMV-specific responders.

[#] *P*-value <0.01 (repeated measures ANOVA and Bonferroni post-hoc test) for difference between CD107a+IFN γ - rhCMV-specific responders and CD107a-IFN γ rhCMV-specific responders.