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Factors Affecting the Efficiency of CD8⁺ T Cell Cross-Priming with Exogenous Antigens

Holden T. Maecker,^{1*} Smita A. Ghanekar,* Maria A. Suni,* Xiao-Song He,[†] Louis J. Picker,[‡] and Vernon C. Maino*

Processing of exogenous protein Ags by APC leads predominantly to presentation of peptides on class II MHC and, thus, stimulation of CD4⁺ T cell responses. However, "cross-priming" can also occur, whereby peptides derived from exogenous Ags become displayed on class I MHC molecules and stimulate CD8⁺ T cell responses. We compared the efficiency of cross-priming with exogenous proteins to use of peptide Ags in human whole blood using a flow cytometry assay to detect T cell intracellular cytokine production. CD8⁺ T cell responses to whole CMV proteins were poorly detected (compared with peptide responses) in most CMV-seropositive donors. Such responses could be increased by using higher doses of Ag than were required to achieve maximal CD4⁺ T cell responses. A minority of donors displayed significantly more efficient CD8⁺ T cell responses to whole protein, even at low Ag doses. These responses were MHC class I-restricted and dependent upon proteasomal processing, indicating that they were indeed due to cross-priming. The ability to efficiently cross-prime was not a function of the number of dendritic cells in the donor's blood. Neither supplementation of freshly isolated dendritic cells nor use of cultured, Ag-pulsed dendritic cells could significantly boost CD8 responses to whole-protein Ags in poorly cross-priming donors. Interestingly, freshly isolated monocytes performed almost as well as dendritic cells in inducing CD8 responses via cross-priming. In conclusion, the efficiency of cross-priming appears to be poor in most donors and is dependent upon properties of the individual's APC and/or T cell repertoire. It remains unknown whether cross-priming ability translates into any clinical advantage in ability to induce CD8⁺ T cell responses to foreign Ags. *The Journal of Immunology*, 2001, 166: 7268–7275.

Exogenous protein Ags can be taken up by APC, processed, and Ag-derived peptides presented by class II MHC molecules to CD4⁺ T cells. This has been referred to as the "exogenous" pathway of Ag presentation (1, 2). In contrast, proteins produced intracellularly, including viral proteins, are preferentially processed via proteasomes into peptides that associate with nascent class I MHC molecules in the endoplasmic reticulum. These class I MHC complexes, upon reaching the cell surface, stimulate CD8⁺ T cell responses. This has been referred to as the "endogenous" pathway of Ag presentation (1, 2).

Macrophages and dendritic cells have also been shown to be capable of "cross-priming;" that is, Ag taken up via the exogenous pathway can enter the endogenous pathway for processing and presentation of peptides by class I MHC (3–13). The result of this process is stimulation of CD8⁺ T cells to exogenous Ag. This is a desirable outcome for protein-based vaccines that are intended to stimulate CD8⁺ T cell responses, and increasing the efficiency of this process is thus an area of great interest for vaccine development (14–23). Cross-priming is generally inefficient, but dendritic cells are thought to cross-prime CD8⁺ T cells more readily than other APC (4, 5, 24). In addition, particulate Ags (or apoptotic cells) result in more efficient cross-priming than soluble Ags (3, 7, 25). Finally, cross-priming can be induced by linking Ags to cer-

tain peptides or proteins, such as bacterial toxins or HIV tat, as these proteins or peptide sequences appear to shuttle extracellular proteins into the cytosol (16, 17, 19, 21).

Using a rapid *in vitro* assay to determine frequencies of Ag-specific T cell cytokine production in whole blood (26, 27), we sought to assess the ability of APC to cross-prime CD8⁺ T cells. We show here that cross-priming can indeed occur in this setting and that it is dependent upon proteasomal processing. However, the efficiency of cross-priming varies widely between individuals and is not related to the number of dendritic cells available to process Ags. Instead, there appears to be an intrinsic limit on cross-priming ability that is low ($\leq 10\%$) in most donors. This low efficiency of cross-priming could be due to various properties of the Ag presentation machinery. Alternately, or in addition, the T cell repertoire may contain very few cells that can respond to low-density Ags resulting from presentation of exogenous proteins. To the extent that cross-priming may be useful for combating certain types of infections, this ability may correlate with the ability to protect against such infections.

Materials and Methods

Ag stimulation for cytokine assays

CMV viral lysate was purchased from Advanced Biotechnologies (Columbia, MD), diluted to 1 mg/ml in PBS, aliquoted into single-use vials, and stored at -80°C before use. Recombinant CMV pp65 (gp65) protein was purchased from Austral Biologicals (San Ramon, CA) and diluted, aliquoted, and stored as described above. A HLA-A2-restricted peptide from pp65 (NLVPMVATV) was generously provided by F. Kern (Humboldt-Universität zu Berlin, Campus Mitte, Berlin, Germany) (28). The peptide was dissolved in DMSO at 2 mg/ml and stored in single-use aliquots at -80°C . A peptide mixture consisting of 138 overlapping 15-mers spanning the sequence of pp65 was made at 100 mg/ml per peptide in DMSO, aliquoted, and stored at -80°C . The Ags were used at the doses indicated in the figures. All stimulations, including no Ag controls, also received 1

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$\mu\text{g/ml}$ final concentration of CD28 and CD49d mAbs (BD Immunocytometry Systems, San Jose, CA) to provide costimulatory signals (29). Unless otherwise noted (Fig. 2), 200 μl of whole blood from CMV-seropositive donors was stimulated for a total of 6 h at 37°C in 0.5 ml polypropylene microfuge tubes. A final concentration of 10 $\mu\text{g/ml}$ brefeldin A was added for the last 4 h of incubation by adding 4 μl 0.5 mg/ml brefeldin A stock. The stock was prepared by diluting a frozen aliquot of 5 mg/ml brefeldin A in DMSO (1/10) with sterile PBS just before use.

Cell preparation and staining for cytokine assay

After incubation of whole blood with Ags and costimulatory mAbs as described above, 2 mM final concentration of EDTA was added to each tube for 15 min at room temperature. For convenience, in some experiments, the activated blood was cooled to 4°C for a period of time before EDTA treatment (27). After treatment with EDTA, the cells were vortexed vigorously. In assays using PE-labeled HLA-A2-pp65 peptide tetramer, the tetramer was added along with the EDTA, and incubation was extended to 1 h at room temperature to allow staining with tetramer before fixation and permeabilization. In all other experiments, the staining mAbs were added only after fixation and permeabilization, as described below. After EDTA treatment, erythrocytes were lysed and leukocytes fixed by addition of the blood to 2 ml FACS Lysing Solution (BD Immunocytometry Systems) for 10 min at room temperature in 5 ml polystyrene tubes (Falcon; BD Biosciences, Bedford, MA). The tubes were centrifuged at 500 $\times g$ for 5 min at room temperature, and the supernatants were decanted. A total of 0.5 ml of FACS Permeabilizing Solution 2 (BD Immunocytometry Systems) was then added to each tube, and the tubes were vortexed and incubated for 10 min at room temperature. A total of 2 ml of PBS + 0.5% BSA + 0.1% NaN_3 was then added to each tube, and the tubes were centrifuged again as described above. The supernatants were again decanted, and staining mAbs were added to the residual volume, vortexed, and incubated for 30 min at room temperature. The staining mAbs were typically CD3-APC, CD4-PerCP-Cy5.5, CD8-PE, and anti-IFN- γ -FITC, unless otherwise noted. All staining reagents were obtained from BD Immunocytometry Systems, except PE-labeled HLA-A2-pp65 peptide tetramer, which was produced by J. Mumm, K. Olson, and M. Davis (Stanford University, Stanford, CA) (30). After staining, cells were washed with 2 ml of PBS + 0.5% BSA + 0.1% NaN_3 , centrifuged, and supernatants decanted as described above. A total of 200 μl of 1% paraformaldehyde in PBS was added to each tube, after which the tubes were vortexed and the samples stored at 4°C for up to 24 h.

Flow cytometry analysis

Stained samples were run on a FACSCalibur flow cytometer (BD Immunocytometry Systems). Typically, 60,000–80,000 CD3⁺ lymphocytes were collected by gating on both forward light scatter vs side light scatter and CD3 APC vs side light scatter. For analysis, gates were set so as to include CD3⁺CD8^{bright} cells or, for comparison, CD3⁺CD4⁺ cells. These were >99% exclusive populations. Percentages were reported as percentage of the gated population expressing cytokine.

Blocking and inhibition studies

Lactacystin was purchased from Sigma (St. Louis, MO), reconstituted in sterile water at 2 mg/ml, aliquoted in single-use vials, and stored at –80°C before use. It was added at a final concentration of 20 $\mu\text{g/ml}$ to blood samples 30 min before addition of Ags and costimulatory mAbs. Anti-HLA A, B, and C mAb (clone G46-2.6) was obtained from BD Pharmingen (San Diego, CA). Another anti-HLA class I mAb, DX17, was a generous gift of L. Lanier (University of California, San Francisco, CA). These mAbs were used at a final concentration of 5 $\mu\text{g/ml}$. Anti-HLA DR (clone L243), DP (clone B7/21), and DQ (clone SK10) mAbs were obtained from BD Immunocytometry Systems and were combined 1:1:1 in a mixture and used at a final concentration of 5 $\mu\text{g/ml}$ total protein. All blocking mAbs were added to blood samples 30 min before addition of Ags and costimulatory mAbs. The assays were then conducted as described above.

Isolation of autologous dendritic cells

PBMC were harvested from blood collected in cell preparation tubes (BD Vacutainer Systems, Franklin Lakes, NJ) following the manufacturer's instructions. Dendritic cells were isolated using a MACS dendritic cell isolation kit and an autoMACS cell sorter (Miltenyi Biotec, Auburn, CA). Briefly, dendritic cells were pre-enriched from PBMC by immunomagnetic depletion of T cells (CD3⁺), monocytes (CD11b⁺), and NK cells (CD16⁺) by using a mixture of hapten-conjugated primary mAb and an anti-hapten mAb coupled to MACS MicroBeads (Miltenyi Biotec). The labeled cells were retained on the column by selecting a depletion program on the

toMACS cell sorter. CD4⁺ dendritic cells were positively selected from the nonmagnetic fraction using direct CD4 MicroBeads and were subsequently enriched on a column using a positive selection program on the autoMACS. The purity of the dendritic cells was always >90% based on surface phenotype (HLA-DR⁺/lineage[–]/CD11c⁺ or CD123⁺).

Preparation of monocyte-derived dendritic cells

Dendritic cells were generated *in vitro* using a modification of the protocol of Romani et al. (31). CD14⁺ dendritic cell precursors were enriched by adherence of PBMC and removal of nonadherent cells by carefully washing the adherent cell layer five times with PBS. The adherent monocytes were cultured in AIM V medium (Life Technologies, Grand Island, NY) with 100 ng/ml IL-4 and 250 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) on days 0, 3, and 5. On day 5, the loosely adherent cells were harvested and cultured in AIM V medium and monocyte-conditioned medium (1:1) with GM-CSF, IL-4, and TNF- α (30 ng/ml) for 2 more days, with or without CMV Ag (10 $\mu\text{g/ml}$). The cells were harvested on day 7, washed twice with PBS, and an aliquot was stained to check purity. The dendritic cells prepared in this way were enriched to ~60–70% based on surface phenotype (HLA-DR⁺/lineage[–]).

Results

Exogenous subunit Ags derived from CMV were used to stimulate CD4⁺ and CD8⁺ T cell responses using an intracellular cytokine flow cytometry assay. Because cross-priming is a relatively inefficient process, we reasoned that higher doses of Ag and/or longer incubation times than those required for detection of CD4⁺ T cell responses might induce significant CD8⁺ T cell responses to exogenous Ags.

Titration and kinetics of CD4 and CD8 T cell responses to CMV

We used both a whole-virus preparation (CMV lysate; Advanced Biotechnologies) and a purified recombinant protein from CMV (pp65, also known as gp65; Austral Biologicals) to test the dose response of cytokine production from CD8⁺ T cells (Fig. 1). Whole blood from CMV-seropositive donors was incubated with varying doses of Ag for 6 h with 10 $\mu\text{g/ml}$ brefeldin A added during the last 4 h of incubation to inhibit cytokine secretion and to allow intracellular detection. Under these conditions, most donors displayed maximal numbers of cytokine-producing CD4⁺ T cells at doses between 1 and 10 $\mu\text{g/ml}$ (Fig. 1A, donor 1). For CD8⁺ T cell responses, most donors showed lower numbers of cytokine-producing cells that did not reach a plateau until 10–100 $\mu\text{g/ml}$. This CD8⁺ T cell response would be completely missed with typical doses of 1–5 $\mu\text{g/ml}$ CMV Ag used to detect CD4⁺ T cell responses. An example of the high-dose CD8⁺ T cell response from one such donor is shown in Fig. 1B for both whole CMV viral lysate and recombinant pp65 protein.

Similar results could also be obtained by longer incubation with CMV Ags. As seen in Fig. 2, incubation for 16 h in the presence of 5 $\mu\text{g/ml}$ CMV lysate or 2 $\mu\text{g/ml}$ recombinant pp65 protein also induced a significant response of CD8⁺ IFN- γ -producing cells. However, higher doses did not improve these responses at 16 h, indicating that a combination of high dose and long incubation time was no better than high dose alone (data not shown).

In addition to higher doses or longer incubation times, we could also detect CD8⁺ T cell responses in these assays by coating the CMV Ag onto 0.3- μm polystyrene beads (Idexx, Westbrook, MA). However, the maximum CD8⁺ T cell response obtained with such particulate Ags was not higher than that obtained with soluble CMV lysate (data not shown). The particulate Ag simply increased the efficiency of detecting both CD4⁺ and CD8⁺ T cell responses by shifting the dose-response curves by almost 1 log (i.e., equivalent responses could be detected with ~10-fold less particulate Ags compared with soluble Ags).

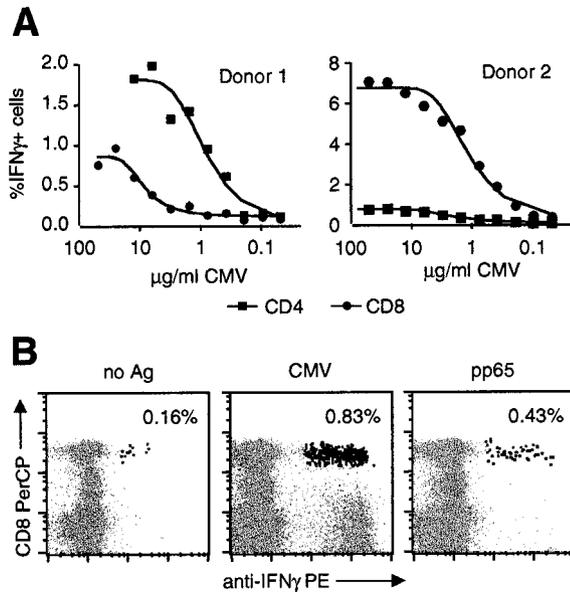


FIGURE 1. A, Titration of exogenous Ag for stimulation of CD4⁺ and CD8⁺ T cell responses. Whole blood from CMV-seropositive donors was incubated with 1 $\mu\text{g/ml}$ costimulatory mAbs (CD28 and CD49d) and the indicated concentrations of CMV viral lysate. Cells were incubated at 37°C for 6 h with 10 $\mu\text{g/ml}$ brefeldin A added for the last 4 h. Cells were then processed and stained as described in *Materials and Methods*. The percentage of CD3⁺CD4⁺ cells (■) or CD3⁺CD8^{bright} cells (●) producing IFN- γ was plotted. Results are representative of eight different experiments using a total of four different donors. B, Example of raw data from donor 1 using 25 $\mu\text{g/ml}$ CMV or 5 $\mu\text{g/ml}$ recombinant pp65 protein. The dot plots were gated on CD3⁺ cells. Note the response in bold from CD3⁺CD8^{bright} cells.

Relationship of dose-response profile to cross-priming ability

A few donors examined showed a radically different dose-response profile for CD8⁺ T cells (Fig. 1A, donor 2). These donors displayed high-level CD8⁺ T cell responses to CMV Ags, even at relatively low doses of 1–10 $\mu\text{g/ml}$. In the donor shown, this CD8 response to exogenous Ags was actually much higher than the CD4 response. This pattern of high CD8 and low CD4 responses was occasionally seen in other CMV⁺ donors. However, no significant inverse correlation was noted between the number of CMV-specific CD4 and CD8 cells among 11 donors tested (data not shown).

We wondered whether these varying dose-response profiles represented differences in cross-priming or just differences in overall levels of CD4⁺ and CD8⁺ T cell responses in these donors. To examine this question, we turned to a more defined CMV Ag, pp65. For this protein, we have produced an overlapping peptide library of 15 amino acid peptides (with 11 amino acid overlaps) spanning the entire pp65 protein sequence. This peptide mix can be used to efficiently stimulate both CD4⁺ and CD8⁺ T cell responses, presumably without requiring extensive Ag processing (Ref. 32 and our unpublished observations). The 15-aa peptide mix stimulates CD8 responses nearly as efficiently as 9-aa peptides but without regard to HLA type (data not shown). We compared the CD8 response of 11 donors to this peptide mix vs their CD8 response to recombinant pp65 protein. By examining the ratio of response to intact protein vs peptide mix, we could determine the efficiency of cross-priming of CD8⁺ T cells by recombinant protein. The results are shown in Fig. 3. Of the 11 donors, 3 showed a relatively high “response ratio,” meaning that their response to recombinant pp65 protein was at least 50% of their response to

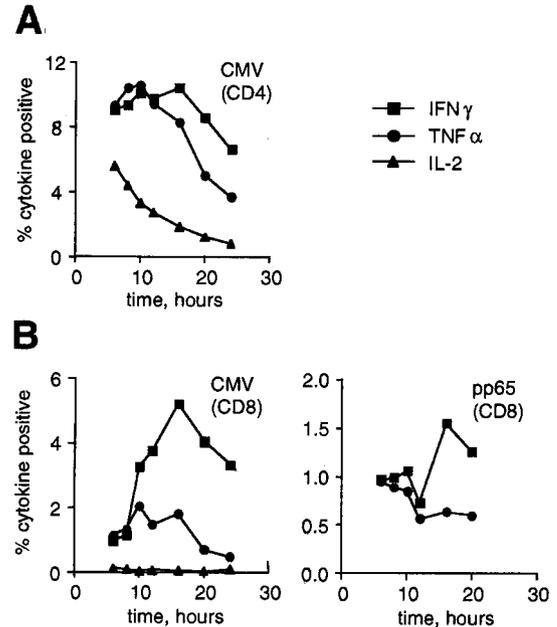


FIGURE 2. Time course of CD4 and CD8 responses. Whole blood from a CMV-seropositive donor (donor 1 of Fig. 1A) was incubated with 1 $\mu\text{g/ml}$ costimulatory mAbs (CD28 and CD49d) and either 5 $\mu\text{g/ml}$ CMV lysate (A and B, left panels) or 5 $\mu\text{g/ml}$ recombinant CMV pp65 protein (B, right panel) for the indicated times. CD4 responses to pp65 were too low to determine accurate kinetics, so this panel is not shown. A total of 10 $\mu\text{g/ml}$ brefeldin A was added for the last 4 h of each incubation. Cells were processed and stained as described in *Materials and Methods*, and the percentage of CD3⁺CD4⁺ cells (A) or CD3⁺CD8^{bright} cells (B) producing cytokine was plotted. A distinct peak of IFN- γ -producing CD8 cells was seen at 16 h for both CMV lysate and pp65 protein, whereas IFN- γ production from CD4 cells was essentially high from 6–16 h. Kinetics for CD4 cells were similar to those previously reported for these cytokines (27). Similar results were obtained in two different experiments using a total of three different donors.

pp65 peptide mix. A fourth donor had a response ratio of 30%. The remaining 7 donors had very low response ratios, meaning that their response to intact protein was $\leq 10\%$ of their response to peptide mix. These responses were independently tested three times with similar results.

By contrast, CD4⁺ T cell responses to recombinant pp65 protein tended to more closely match CD4⁺ T cell responses to pp65 peptide mix (Fig. 3, right column). The ratios tended to be < 1 , indicating that peptides are still more efficient at inducing CD4⁺ T cell responses than processed protein. Nevertheless, the mean response ratio (recombinant protein:peptide mix) was significantly higher for CD4⁺ responses than for CD8⁺ responses ($p \leq 0.025$).

Efficiency of recruiting a known epitope

Another way to examine whether most donors' CD8 response to whole protein represented incomplete cross-priming was to examine the response to a known CD8 epitope when stimulating with CMV lysate. To do this, we used an MHC-peptide tetramer specific for T cells recognizing a class I-restricted epitope of CMV pp65 presented on HLA-A2. Whole blood from an HLA-A2⁺ CMV-seropositive donor was stimulated with either the same HLA-A2-restricted peptide or with a high dose of CMV lysate (25 $\mu\text{g/ml}$). CD3⁺CD8⁺ cells were then costained for intracellular IFN- γ production and binding to the pp65 tetramer. As seen in Fig. 4A, 1.3% of CD3⁺CD8^{bright} cells were stained with the pp65 tetramer in the absence of stimulation. Upon stimulation with the

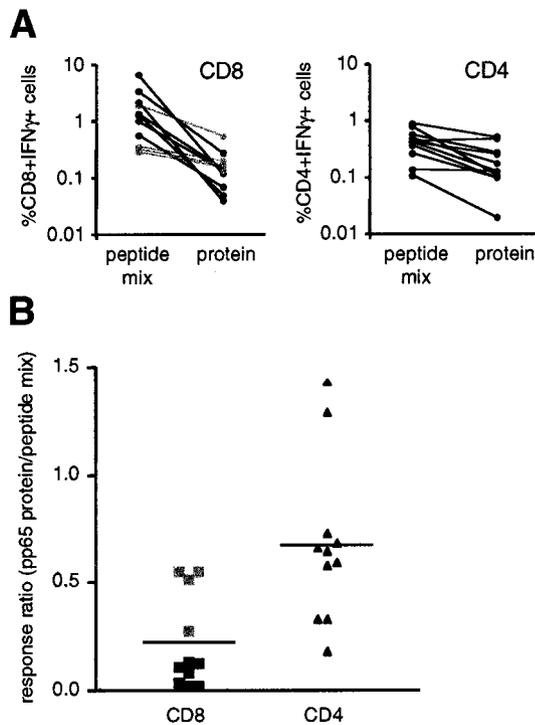


FIGURE 3. Assessing cross-priming ability in multiple donors. Whole blood from 11 CMV-seropositive donors was incubated for 6 h with either 0.5 $\mu\text{g/ml}$ recombinant pp65 protein or a mixture of 138 overlapping 15-aa peptides spanning the sequence of pp65 (used at 1.8 $\mu\text{g/ml}$ per peptide). The CD4⁺ and CD8⁺ T cell responses were determined by intracellular cytokine staining as described in *Materials and Methods*. *A*, CD8 (left panel) and CD4 (right panel) responses of the 11 donors. A similar response between peptide mix and protein for a given donor is seen as a line with shallow slope. Most of the donors showed similar CD4 responses between peptide mix and whole protein (right panel), whereas their CD8 responses tended to be dissimilar, seen as a line with steep slope (left panel). However, note the four donors with similar CD8 responses to peptide mix and protein (gray lines with shallow slope in left panel). *B*, Response ratios. The ratio of the response to recombinant protein divided by the response to peptide mix was plotted for the data shown in *A*. Because the peptide mix presumably does not require intracellular Ag processing, this ratio provides a measure of the ability of these donors to process exogenous Ag for presentation to either CD8⁺ or CD4⁺ T cells. The mean ratio for CD4⁺ cells was significantly higher than that for CD8⁺ cells ($p \leq 0.025$). Furthermore, 7 of the 11 donors had very low ratios for CD8 cross-priming (≤ 0.1), whereas 4 of the donors (■) had ratios more similar to those seen for CD4⁺ cells (these are the four donors denoted by gray lines in *A* above).

relevant peptide, nearly all of the tetramer-reactive cells produced IFN- γ , and tetramer staining was significantly down-modulated, along with CD3, as a result of activation. A small fraction of tetramer-reactive cells appeared to be lost, most likely because they down-modulated tetramer to such an extent that they appeared tetramer-low or -negative without producing IFN- γ . However, when CMV lysate was used to stimulate the cells in the same experiment, <10% of the tetramer-reactive cells produced IFN- γ (0.1 of 1.3%). There was also much less down-modulation of tetramer staining, indicating less efficient stimulation of these cells. Thus, only a small fraction of the cells specific for this particular epitope was able to respond to exogenous Ag via cross-priming. As expected, stimulation with CMV lysate resulted in IFN- γ production from additional tetramer-negative cells (1.5%), indicating that CD8⁺ T cells specific for additional epitopes of CMV were stimulated by cross-priming. Fig. 4*B* shows a second example, in

which 7.3% of CD3⁺CD8^{bright} cells were stained with the pp65 tetramer in the absence of stimulation. Peptide was again quite efficient at stimulating tetramer-reactive cells to produce IFN- γ . As in Fig. 4*A*, <10% of tetramer-positive cells produced IFN- γ in response to CMV lysate (0.4 of 7.3%).

From both the experiments described in Fig. 3 (using pp65 protein vs peptide mix stimulation) and Fig. 4 (using tetramer to assess efficiency of responses to CMV lysate), it appeared that the majority of donors actually have high levels of CD8⁺ T cells capable of responding to CMV Ags. However, these are inefficiently detected when stimulating with exogenous Ag, presumably due to limitations of cross-priming.

Class I restriction of CD8 responses

To determine whether cross-priming was truly occurring in our experiments using exogenous Ag stimulation, we wanted to demonstrate that the CD8⁺ T cell responses measured were: 1) dependent upon proteasomal processing and 2) MHC class I restricted.

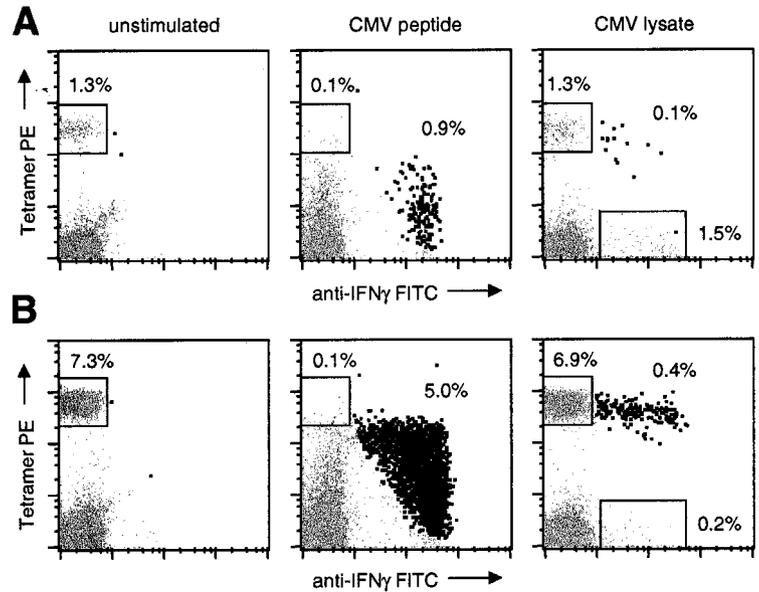
To determine whether the CD8 responses being detected using soluble Ags were dependent upon proteasomal processing, inhibition experiments were performed using lactacystin or chloroquine. Lactacystin is a drug that inhibits the class I MHC processing pathway by preventing TAP-dependent transport. As seen in Fig. 5*A*, lactacystin dramatically inhibited the CD8 response while having little effect upon the CD4 response. Similar data were also obtained with the class I inhibitor *N*-acetyl-leu-leu-norleucinal (data not shown). In contrast, chloroquine is a drug that prevents acidification of endocytic vesicles, inhibiting the class II MHC processing pathway. Chloroquine had no effect on CD8 responses, while it decreased CD4 responses as expected. Thus, the CD8 response to CMV lysate requires Ag processing via the endogenous (class I MHC) Ag processing pathway, consistent with the occurrence of cross-priming. Because inhibition of CD8 responses with lactacystin was incomplete, it remained possible that pre-existing peptides contributed to the CD8 response seen with CMV viral lysate. However, dialysis of CMV lysate with a 10,000 m.w. cut-off membrane did not significantly change the CD8 response measured (data not shown), suggesting that such peptides, if present in CMV lysate, did not contribute significantly to the response seen.

Next, Abs to class I or class II MHC were used to functionally block the CD4 and CD8 responses as described above. As seen in Fig. 5*B*, two different mAbs to class I MHC diminished the response of CD8 cells to CMV lysate, as expected. In contrast, a mAb mixture to class II MHC inhibited CD4 but not CD8 responses. Thus, the CD8 responses detected in this assay appear to be functionally class I MHC restricted.

Opsonizing effect of serum Abs

The immune response to CMV includes Abs capable of cross-linking and opsonizing viral proteins. To test the contribution of serum Abs to the CD8 response, whole blood was washed free of plasma, and the cells were resuspended in either autologous plasma or heterologous plasma from a CMV-seronegative donor. In such experiments, responses were diminished in the presence of seronegative serum (data not shown). Responses were also diminished by depleting Igs from CMV-seropositive serum (data not shown). This is consistent with an opsonizing role of CMV-specific Abs in stimulating Ag uptake for cross-priming.

FIGURE 4. Recruitment of tetramer-binding cells in the CD8 response to CMV lysate. Whole blood from two HLA-A2⁺ CMV-seropositive donors (A and B) was stimulated for 6 h with costimulatory mAbs (CD28 and CD49d) alone (*left panel*), in the presence of 2 μ g/ml pp65 peptide (*middle panel*), or in the presence of 25 μ g/ml CMV lysate (*right panel*). Cells were then stained with a PE-labeled HLA-A2 tetramer displaying the relevant peptide from CMV pp65, processed as described in *Materials and Methods*, and further stained with CD3-APC, CD8-PerCP-Cy5.5, and anti-IFN- γ -FITC. The dot plots shown are gated on CD3⁺CD8^{bright} cells. Note the more efficient recruitment of tetramer-positive cells using cognate peptide as opposed to CMV lysate stimulation, as well as greater down-modulation of tetramer staining using peptide stimulation. This experiment was repeated four times on two different donors with similar results.



Dendritic cell numbers do not limit cross-priming ability

Because dendritic cells have been shown to most efficiently induce cross-priming in human and mouse systems (4, 5, 24), we wondered whether the differences in cross-priming ability between donors was related to the number and/or function of their dendritic cells. To test whether dendritic cell numbers were a limiting factor in this assay, exogenous autologous dendritic cells were purified from PBMC by MACS (Miltenyi Biotec). Addition of up to 10,000 purified dendritic cells to 200 μ l of blood did not increase responses to CMV or pp65 by >20% (Fig. 6). In fact, in the experiment shown, the donor with a high-level CD8⁺ T cell response actually had lower levels of dendritic cells per milliliter of blood (0.5% HLA-DR⁺/lineage⁻) than the poorly cross-priming donor (2.0% HLA-DR⁺/lineage⁻). Hence, not as many dendritic cells could be isolated to add back to this donor's blood. From this experiment, it is apparent that the number of dendritic cells in whole blood does not appear to be the major limiting factor in eliciting in vitro CD8 responses to intact protein Ags.

Ag-pulsed dendritic cells do not increase cross-priming efficiency

To further test the functional role of dendritic cells in cross-priming, dendritic cells were produced from autologous blood-derived monocytes by a 7-day culture protocol. CMV lysate was added to the dendritic cells for the last 2 days of culture along with TNF- α and monocyte-conditioned medium for maturation of the dendritic cells. Addition of such Ag-pulsed dendritic cells to whole-blood cultures did not induce significantly more cross-priming of CD8⁺ T cells in a donor (donor 1 of Fig. 1A) that demonstrated poor cross-priming to exogenous protein Ag (Fig. 7A). Similarly, Ag-pulsed fresh monocytes did not induce significantly higher cross-priming of CD8⁺ T cells than did Ag alone. In contrast, Ag-pulsed dendritic cells were quite efficient at inducing CD4⁺ T cell responses to CMV, whereas Ag-pulsed monocytes were capable of inducing CD4⁺ T cell responses with only slightly lower efficiency (Fig. 7A, *right panel*). Similar results were seen with two other donors, including one with a relatively high CD8 response to CMV

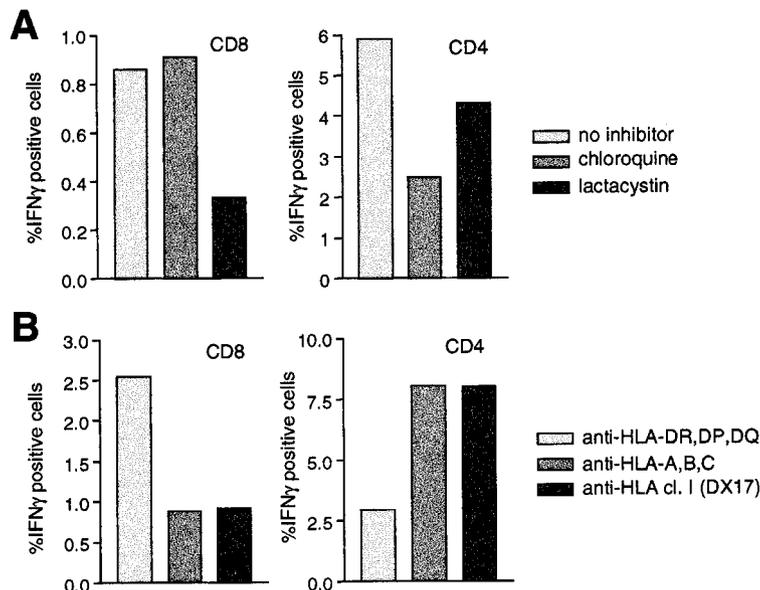


FIGURE 5. Inhibition of CD4 and CD8 responses. Whole blood from a CMV-seropositive donor was stimulated as described in *Materials and Methods* using 5 μ g/ml CMV lysate for 6 h. Lactacystin, an inhibitor of MHC class I-dependent processing, chloroquine, an inhibitor of MHC class II-dependent processing (A), or mAbs to MHC class I or class II (B) were added beginning 30 min before addition of Ags and costimulatory mAbs. The percentage of CD3⁺ cells of the indicated subset (CD4⁺ or CD8⁺) producing IFN- γ was plotted. Results are representative of more than five separate experiments.

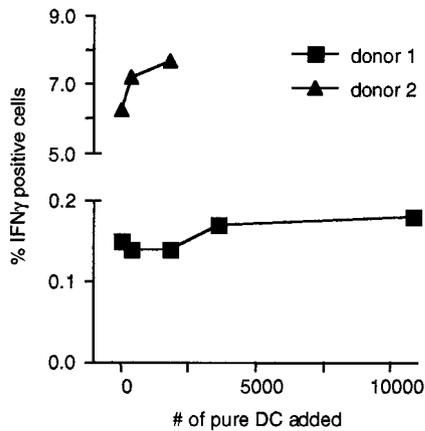


FIGURE 6. Addition of autologous dendritic cells to whole blood. Autologous dendritic cells were isolated from whole blood by magnetic bead separation (Miltenyi Biotec) as described in *Materials and Methods*, counted, and spiked back into 200 μ l samples of whole blood. The blood was then stimulated with CMV lysate as described in the previous figures. Fewer dendritic cells were isolated from the equivalent volume of blood in donor 2 (despite donor 2's higher baseline CD8⁺ T cell response). Addition of autologous dendritic cells to donor 1's blood did not significantly increase the CD8⁺ T cell response to exogenous CMV Ag.

lysate (Fig. 7, *B* and *C*). Thus, donor-dependent differences in cross-priming efficiency could not be overcome by use of Ag-pulsed dendritic cells as stimulators of CD8⁺ T cell responses.

Discussion

In this study, we demonstrate that most individuals have a limited ability to cross-prime CD8⁺ T cell responses using exogenous Ags. These CD8 responses can be improved somewhat by increasing Ag dose or time, but they are not affected by the number of circulating dendritic cells, or even by addition of Ag-pulsed dendritic cells. There is a suggestion that some individuals, at least at certain times, may have an intrinsically higher ability to cross-prime CD8⁺ T cell responses.

We used intracellular cytokine production and flow cytometry to assess cross-priming. This assay allows for a highly quantitative measurement of T cell responses in whole blood with intra-assay and interassay precision of \sim 10 and 20%, respectively (27). In addition, it allows for the simultaneous analysis of both CD4 and CD8 responses in the same sample, thus providing an internal control for measurement of CD8 cross-priming. Because IFN- γ is the major cytokine detected in CD8 cells in this assay (Fig. 2), we focused on this cytokine as a surrogate for the number of T cells stimulated under various conditions. This assay is also positively correlated with proliferation as measured by [³H]thymidine or 5-bromo-2'-deoxyuridine incorporation (Ref. 33, and our unpublished data).²

For CD8 analyses, we focused on CD3⁺CD8^{bright} cells, because these represent MHC class I-restricted $\alpha\beta$ T cells. In fact, we demonstrated that the CD8 responses seen were functionally MHC class I restricted (by Ab blocking), dependent upon proteasomal processing (using inhibitors such as lactacystin), and partially dependent upon serum Ig (consistent with opsonization for Ag uptake). Significant IFN- γ responses to CMV lysate can also be detected in a population of CD3⁺CD8^{dim} cells in some CMV-seropositive donors. Such responses peak at time points earlier

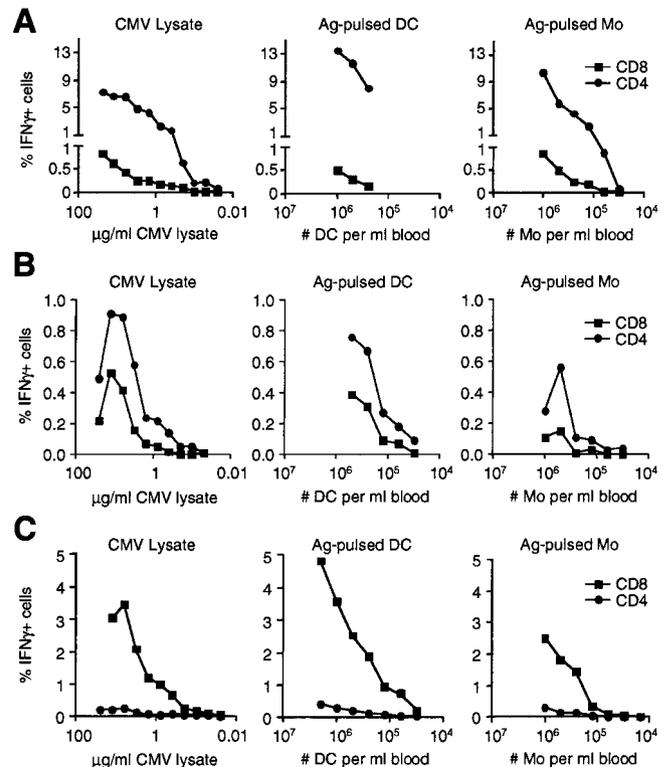


FIGURE 7. Stimulation of whole blood with Ag-pulsed dendritic cells. *A*, Purified monocytes were cultured for 7 days with IL-4 and GM-CSF as described in *Materials and Methods* to produce monocyte-derived dendritic cells. The cells were pulsed for the last 48 h with 2 μ g/ml CMV lysate along with TNF- α and monocyte-conditioned medium to induce maturation. Purified monocytes without culture were also pulsed for 24 h with CMV lysate. The cells were then counted and titrated into whole blood to stimulate CD4⁺ (●) and CD8⁺ (■) T cell responses and measured as described in the previous figures. CMV lysate was also titrated as described in Fig. 1 to allow comparison of responses to exogenous Ag vs Ag-pulsed cells. Ag-pulsed dendritic cells and monocytes (with slightly less efficiency) were both able to induce CD4 responses at least comparable to those induced by exogenous Ag. However, neither Ag-pulsed dendritic cells nor monocytes were able to induce significantly higher CD8⁺ T cell responses than were induced by exogenous Ag alone. *B*, Analogous experiment in a second donor. *C*, As a control, a similar experiment was done in a donor that displayed a relatively high CD8 response to CMV lysate. Results are representative of six independent experiments done on three different donors.

than 16 h and involve cells that produce TNF- α as well as IFN- γ (data not shown). These cells in most donors are also CD4⁺, and they are restricted by class II MHC, not class I MHC, using the same types of experiments as shown in Fig. 5.³ By gating on CD8^{bright} cells in the experiments in this study, we restricted our analysis to only class I MHC-restricted CD8 cells.

Although the observed CD8 responses to CMV and pp65 were shown to be MHC class I restricted, it remained possible that these responses were helped in some way by the presence of CD4 cells. For example, CD4 cell cytokine secretion could provide costimulation to CD8 cells, amplifying their response; alternately, CD4-APC interaction could induce changes in the APC that allow better presentation to CD8 cells. Of course, cytokine secretion and/or induction of new cell surface proteins would not be expected to

² S. A. Ghanekar, S. A. Stranford, J. C. Ong, J. M. Walker, V. C. Maino, and J. A. Levy. Decrease in HIV-1 specific CD4⁺ T cell proliferation in long term HIV-1-infected individuals undergoing antiretroviral therapy. *Submitted for publication.*

³ M. A. Suni, S. A. Ghanekar, D. W. Houck, W. T. Maecker, S. B. Wormsley, L. J. Picker, R. B. Moss, and V. C. Maino. CD4⁺CD8^{dim} T lymphocytes exhibit enhanced cytokine expression, proliferation, and cytotoxic activity in response to HCMV and HIV-1 antigens. *Submitted for publication.*

occur to any great extent in these assays, due to the presence of brefeldin A during most of the incubation period. Nevertheless, to test these possibilities, depletions of CD4 cells from whole blood were done using magnetic beads. Such depletions did not significantly affect the CD8 response to CMV lysate (data not shown). Thus, this response appeared to be largely independent of CD4 cells.

Most of the donors examined in this study were relatively inefficient at cross-priming CD8⁺ T cell responses using exogenous Ag. One way this was demonstrated was using MHC-peptide tetramer to follow the response to one CMV epitope. In such experiments, <10% of the tetramer-positive cells in a given individual responded to stimulation with CMV lysate. It is possible that the tetramer-binding cells are heterogeneous in their avidity and/or threshold for activation, such that some of them require stronger stimulation or additional costimulatory signals for full activation, as has been suggested for CD4 cells (29). Alternately, or additionally, it could be that the amount of MHC class I-presented peptide derived from CMV lysate in this assay is simply insufficient to trigger all of the cells. Nonresponding cells may not differ functionally from responding cells but, by chance, did not encounter their presented peptide in sufficient abundance to induce cytokine production. Additional experiments using alternative ways of presenting Ags and different costimulatory signals will be necessary to help distinguish between these explanations.

It is interesting to consider the information provided by the different assays for Ag-specific CD8 T cells used in this study. Tetramer staining presumably provides a 100% efficient quantitation of T cells specific for a given epitope presented by a single HLA allele. However, it does not provide a comprehensive view of cells responsive to all epitopes of a complex Ag such as CMV, nor does it give any indication of the functional properties of the Ag-specific cells identified. In fact, in some disease states, tetramer-positive cells have been shown to be nonfunctional (34–36). By contrast, cytokine flow cytometry can provide a comprehensive view of T cells responsive to complex Ags, and it gives some indication of cell function in terms of cytokine production. However, the assay is, in most individuals, relatively inefficient in stimulation of CD8⁺ T cells when using whole-protein Ags. Use of overlapping peptide mixes can overcome this limitation and provide efficient stimulation of CD8⁺ T cells.

Two major questions arise as a result of our data showing differences in individual cross-priming ability. First, what is the mechanism of these differences? We have shown in this study that cross-priming ability is not related to dendritic cell numbers (Fig. 6) or to any obvious difference in surface marker phenotype of dendritic cells (data not shown). We were also unable to significantly increase cross-priming by preincubation with recombinant IFN- γ (data not shown), suggesting that IFN- γ -inducible changes are not sufficient to produce efficient cross-priming. However, many factors may be involved, including differences in the responding T cell repertoire, differences in the endocytic machinery, leakiness of endocytic vesicles, HLA type, etc.

Second, do individual differences in cross-priming reflect differing abilities to protect against infection with the corresponding viruses? In other words, is there any clinical correlate of the different cross-priming phenotypes that we have demonstrated? An answer to this question will require much more extensive study and would be aided, for example, by identification of mouse strains that cross-prime with differing levels of efficiency. However, our data would suggest that cross-priming ability is a variable that should perhaps be considered in the assessment of an individual's immune "competence." This may be especially true for vaccines that are designed to elicit CD8⁺ T cell priming using soluble pro-

teins. In this sense, an assay that measures cross-priming ability in addition to T cell function, such as the one described in this study, might be important in assessing an individual's ability to respond to vaccination.

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