Dynamic regulation of functionally distinct virus-specific T cells

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The functional capacities of CD8$^+$ T cells important for virus clearance are influenced by interactions with antigen presenting cells (APCs) and CD4$^+$ T cells during initial selection, subsequent expansion, and development of memory. Recently, investigators have shown that polyfunctional T cells correlate best with long-term protection, however, it is still unknown how to stimulate T cells to achieve these responses. To study this, we examined the phenotypes and functions of CD8$^+$ T cells specific for two different virus antigens stimulated ex vivo using either autologous monocyte-derived dendritic cells (moDCs) or HLA-A2-Ig-based artificial APCs (aAPCs). Although similar numbers of influenza virus and measles virus tetramer-positive cells were generated by stimulation with peptide-loaded moDCs and aAPCs, T cell function, assessed by expression of IL-2, IFN-γ, TNF-α, MIP1β, and CD107a, showed that aAPC-generated CD8$^+$ T cells were multifunctional, whereas moDC-generated cells were mostly monofunctional. aAPC-generated cells also produced more of each cytokine per cell than CD8$^+$ T cells generated with moDCs. These phenotypes were not fixed, as changing the culture conditions of expanding T cells from aAPCs to moDCs, and moDCs to aAPCs, reversed the phenotypes. We conclude that CD8$^+$ T cells are heterogeneous in their functionality and that this is dependent, in a dynamic way, on the stimulating APC. These studies will lead to understanding the factors that influence induction of optimal CD8$^+$ T cell function.

antigen presenting cells | CD8 T cells | viral immunity | multifunctional T cells

Viruses cause infections that result either in acute disease of varying severity associated with clearance of the virus or in chronic progressive disease associated with failure of clearance and virus persistence. CD8$^+$ T cells are critical immune effectors for clearance of many viruses (1, 2) and failure of CD8$^+$ T cell effector function has been associated with persistent infection of humans with HIV and hepatitis C virus and of mice with lymphocytic choriomeningitis virus (3–5). Because of the importance of virus clearance in determining the outcome of infection, there are substantial ongoing efforts to understand the process of induction and maintenance of effective antiviral CD8$^+$ T cells and to apply this knowledge to treatment or prevention of persistent virus infection.

The functions of CD8$^+$ T cells known to be important for virus clearance include antigen specificity, avidity, cytotoxic activity, production of effector cytokines, and localization to tissue sites of virus infection (6–9) ultimately leading to development of a sustained memory response. Furthermore, several studies have shown that multifunctionality of antigen-specific T cells at the single cell level is an important predictor of T cell-mediated immune protection and development of memory (10–12). In vivo, CD8$^+$ T cell expansion and functional maturation are determined by interactions with professional antigen presenting cells (APCs), primarily myeloid dendritic cells (DCs) (13, 14), and some of the factors that influence T cell function have been identified (15–17).

The development of bead-based artificial APCs (aAPCs) allows regulation of both the amount and types of signals used to stimulate antigen-specific T cells. In our aAPC system, there are only two well-defined signals. Signal 1, provided by HLA A2-Ig complexes loaded with peptide to engage the clonotypic T cell receptor, and signal 2, provided by anti-CD28, for costimulation. Both signal 1 and signal 2 are covalently bound to a cell-sized bead. In contrast, although DCs are very potent natural stimulators of antigen-specific cells, both positive and negative regulatory signals contribute to T cell stimulation. The interactions of myeloid DCs with T cells involve DC expression of a variety of cell surface molecules, cytokines, and chemokines that determine T cell differentiation and expansion. These interactions can lead to induction of CD4$^+$ and CD8$^+$ T cells with different effector and regulatory functions, T cell tolerance to self-antigens, immune suppression, and anergy (18–21). Furthermore, it is unclear whether these phenotypes are fixed or can change in response to evolving stimulation conditions.

To assess the effect of the stimulus on antigen-specific CD8$^+$ T cell function, virus-specific CD8$^+$ T cells were stimulated with either monocyte-derived DCs (moDCs) or aAPCs loaded with the same A2-restricted dominant or subdominant viral peptides. aAPCs and moDCs were equally efficient at expanding the numbers of influenza virus or measles virus (MV)-specific CD8$^+$ T cells, but aAPC-stimulated cells were more likely to synthesize multiple cytokines and chemokines than moDC-expanded cells. These studies shed light on the APC-T cell interactions necessary for inducing multifunctional T cells and inform further development of vaccination strategies and immunotherapeutic approaches to chronic viral disease.

Results

aAPC Expansion of Virus-Specific Cells. CD8$^+$ T cells with specificity for the immunodominant influenza virus M1 matrix protein peptide (22) or the MV H576 hemagglutinin protein peptide (23) were expanded from peripheral blood mononuclear cells (PBMCs) of three healthy adult donors by 3 weeks of repeated stimulation with HLA A2-Ig-based aAPC loaded with either the immunodominant influenza virus-specific $M1_{58–66}$ peptide (^{M1}aAPCs) or the MV-specific peptide $H_{576–584}$ (^{H576}aAPCs). Tetramer-positive cells were assessed before culture and after each stimulation cycle (Fig. 1 and Table 1). aAPC-stimulated cultures showed a steady expansion of M1-specific (Fig. 1*A*) and H576-specific (Fig. 1*B*) CD8$^+$ T cells. For example, donor 1 started with 10^6 total CD8$^+$ T cells that were less than 0.55% M1 tetramer-positive. After 3 weeks of stimulation by ^{M1}aAPC, cultures had expanded to 10^8 cells of which 81.5% were M1 tetramer-positive, a 10,000-fold increase (Table 1). The same donor had a 1,000-fold expansion of MV H576-specific CD8$^+$ T cells. For all three donors, aAPC stimulation led to expansion of both influenza virus M1-specific cells and MV H576-specific cells.

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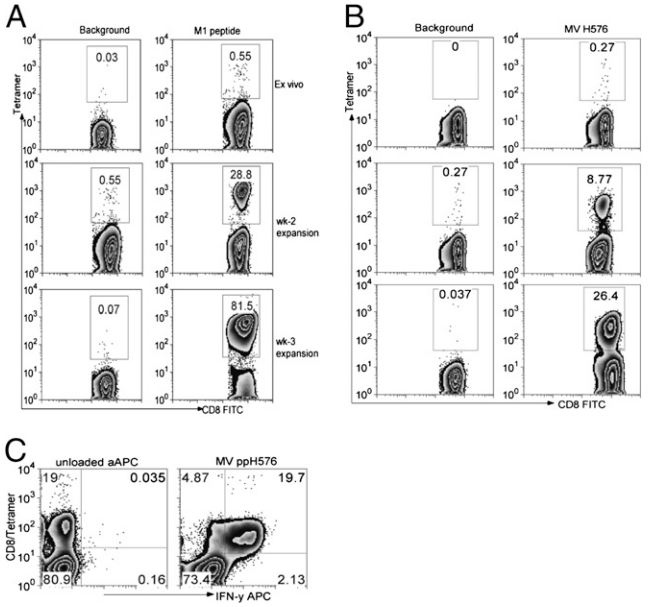

Fig. 1. Expansion of virus-specific CD8⁺T cells by in vitro stimulation with M1- and H576-loaded aAPCs. Enriched CD8⁺ T cells were ex vivo-stimulated with aAPCs loaded with either M1 peptide (*A*) or H576 peptide (*B*) and analyzed weekly by tetramer staining. Plots are gated on CD8⁺ tetramer-positive cells and show representative results for three healthy donors. (*C*) Antigen-specific CD8⁺ T cell function was analyzed after 3 weeks of expansion with ^{H576}aAPC. Frequencies of tetramer-positive, IFN-γ producing cells in response to stimulation with unloaded (*Left*) or peptide-loaded (*Right*) aAPC are indicated in the *Upper Right* quadrants.

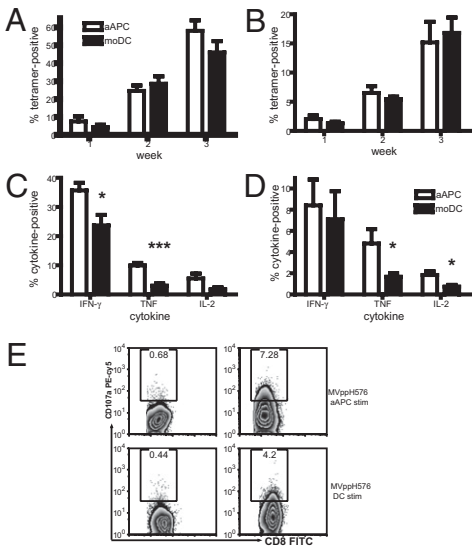

Fig. 2. Comparison of virus-specific CD8⁺ T cells expanded by aAPCs and moDCs. Enriched CD8⁺ T cells from three donors were cultured with (*A*) M1 peptide and (*B*) H576 peptide-loaded aAPCs (open bars) or moDCs (filled bars) for 3 weeks. Tetramer and intracellular cytokine staining was performed at the end of each 7-day stimulation period. Bars represent percentage of CD8⁺ T cells that were tetramer-positive minus background ± SEM averaged from three donors, each analyzed in triplicate. On day 21 expanded (*C*) M1-specific CD8⁺ T cells and (*D*) H576-specific CD8⁺ T cells were incubated with either unloaded or peptide-loaded aAPCs for 6 h, followed by intracellular cytokine staining for IFN-γ, TNF-α, and IL-2. Bars represent percentage of total CD8⁺ T cells expressing the cytokine minus background averaged from three donors ± SEM. Student's unpaired *t* test was used for statistical analysis. * $P < 0.05$, *** $P < 0.0001$. Cytotoxicity was evaluated by staining for granule membrane protein CD107a (*E*).

More cells were seen in response to stimulation by the immunodominant M1 peptide than the subdominant H576 peptide although initial frequencies were similar (Table 1).

At the end of the 3-week culture period, antigen-specific cell function was assessed by measuring production of IFN-γ after stimulation for 6 h with ^{peptide}aAPCs. Tetramer-positive cells produced IFN-γ in response to stimulation with cognate peptide-loaded aAPCs but not in response to stimulation with unloaded control aAPCs (Fig. 1*C*).

Comparison of Virus-Specific CD8⁺ T Cells Expanded by aAPCs and moDCs. Antigen-loaded moDCs are commonly used to induce and expand antigen-specific CD8⁺ T cells from PBMCs (24–27). To compare the phenotypes and functional capacities of aAPC- and moDC-expanded virus-specific CD8⁺ T cells, cells from three donors were stimulated with either ^{peptide}aAPCs or autologous ^{peptide}moDCs for 3 weeks. Frequencies of antigen-specific cells expanded by aAPCs and moDCs were comparable for both M1 (Fig. 2*A*) and H576 (Fig. 2*B*).

Table 1. In vitro expansion of virus-specific CD8⁺ T cells using M1 and H576 peptide-loaded aAPCs as assessed by tetramer (tet) staining

Donor	Peptide	Day 0		Day 21	
		Cell no.	Percent tet+	Cell no.	Percent tet+
1	M1	10^6	0.55	10^8	81.5
	H576	10^6	0.27	10^7	26.4
2	M1	10^6	0.01	10^8	73.1
	H576	10^6	0.03	10^7	3.2
3	M1	10^6	0.04	10^8	59.1
	H576	10^6	0.07	10^8	20.1

Important functions of virus-specific CD8⁺ T cells are production of effector cytokines and lysis of virus-infected target cells (28, 29). To characterize the effector function of the expanded cells, we compared aAPC- and moDC-expanded T cells from the three donors for production of IFN-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-2 by intracellular cytokine staining (ICS) following M1 peptide stimulation (Fig. 2*C*). Although numbers of tetramer-positive cells were similar (Fig. 2*A*), a higher proportion of ^{M1}aAPC-expanded than ^{M1}moDC-expanded CD8⁺ T cells produced IFN-γ ($P = 0.016$), TNF-α ($P < 0.0001$), and IL-2 ($P = 0.056$).

To determine whether these findings were unique to stimulation with the immunodominant influenza virus M1 peptide, CD8⁺ T cells expanded by stimulation with the subdominant MV H576 peptide were also analyzed (Fig. 2*D*). In previous studies, ex vivo stimulation showed low levels of IFN-γ production by CD8⁺ T cells to this peptide (30). After expansion, a higher proportion of ^{H576}aAPC-stimulated CD8⁺ T cells than ^{H576}moDC-stimulated cells produced TNF-α ($P = 0.04$) and IL-2 ($P = 0.014$). These data suggested that aAPC-expanded cells are more likely to produce cytokines than moDC-expanded cells.

Cytotoxic activity of the aAPC and moDC-stimulated antigen-specific CD8⁺ T cells was evaluated by staining for CD107a, a granule membrane protein that appears on the cell surface after cytotoxic granule release (31). Proportions of CD8⁺ T cells expressing CD107a were somewhat higher (7.3%) after expansion by ^{H576}aAPC than after expansion by ^{H576}moDC (4.2%).

A Higher Proportion of aAPC-Generated CD8⁺ T Cells than moDC-Generated CD8⁺ T Cells Are Multifunctional. To investigate whether individual aAPC-generated CD8⁺ T cells produce more cytokines than moDC-generated cells, we assessed dual func-

tionality (IFN-γ/TNF-α) of ^{M1}aAPC and ^{M1}moDC-stimulated cultures by ICS (Fig. 3). Following 1 week, ^{M1}aAPC stimulation resulted in a higher proportion of dual function CD8$^+$ T cells, 6.1% produced both IFN-γ and TNF-α, than ^{M1}moDC stimulation, where only 1.3% of CD8$^+$ T cells produced both cytokines. Furthermore, when ^{M1}moDC-stimulated cells were restimulated with ^{M1}aAPCs for an additional week, the proportion of cells producing both cytokines increased to 12.7%, and when ^{M1}aAPC-stimulated cells were restimulated with ^{M1}moDCs, the proportion of double cytokine-producing cells dropped from 6.1 to 0.8%, (Fig. 3*A*).

To determine whether CD8$^+$ T cell function was set by the initial stimulation or responsive to each round of stimulation, we investigated the effect of stimulus on the cytokine production profile of M1-specific CD8$^+$ T cells by switching APCs back and forth over a 3-week period (Fig. 3 *B* and *C*). At the end of each 7-day culture period, the T cell cultures were divided and restimulated with either moDCs or aAPCs and the proportions of cytokine-producing cells were determined by ICS. After the first round of stimulation, ^{M1}aAPC and ^{M1}moDC-stimulated cells had similar numbers of IFN-γ-producing cells (0.7% and 0.3%) (Fig. 3*B*). In the second week, the ^{M1}aAPC cultures were restimulated with either ^{M1}aAPC or ^{M1}moDC (Fig. 3*B Left*). When restimulated with ^{M1}aAPC, the percentage of IFN-γ producing cells increased to 23.2%. However, when the cultures were stimulated with ^{M1}moDC, only 5.4% of the cells produced IFN-γ. If these cultures were then stimulated with ^{M1}aAPC for the third cycle, the percentage of IFN-γ producing cells increased from 5.4 to approximately 42.3%.

A similar impact of stimulus on cytokine production was seen in cultures that were initially induced by ^{M1}moDC stimulation (Fig. 3*B Right*). In the second cycle switching from ^{M1}moDC to ^{M1}aAPC increased the percentage of IFN-γ-producing cells from 0.3% up to 48.6%. In the third stimulation cycle, a switch from ^{M1}aAPC to ^{M1}moDC resulted in decreased IFN-γ-producing cells from 48.6 to 6.9%.

A similar trend was observed with TNF-α, MIP-1β, and CD107a expression (Fig. 3 *C–F*). In contrast, for all stimulation conditions, IL-2 production reached a peak after two rounds of stimulation and then declined to baseline levels by week 3 (Fig. 3*G*), potentially due to negative feedback regulation by the IL-2 present in the culture (32, 33). IL-4 expression was not detected under either stimulation condition. Taken together, our results suggested that moDCs are less likely to stimulate cytokine production by in vitro cocultured antigen-specific CD8$^+$ T cells than aAPCs.

To determine the multifunctionality of individual ^{M1}aAPC and ^{M1}moDC-expanded CD8$^+$ T cells, the frequency of subpopulations of cells expressing one or more functional characteristics (IFN-γ,

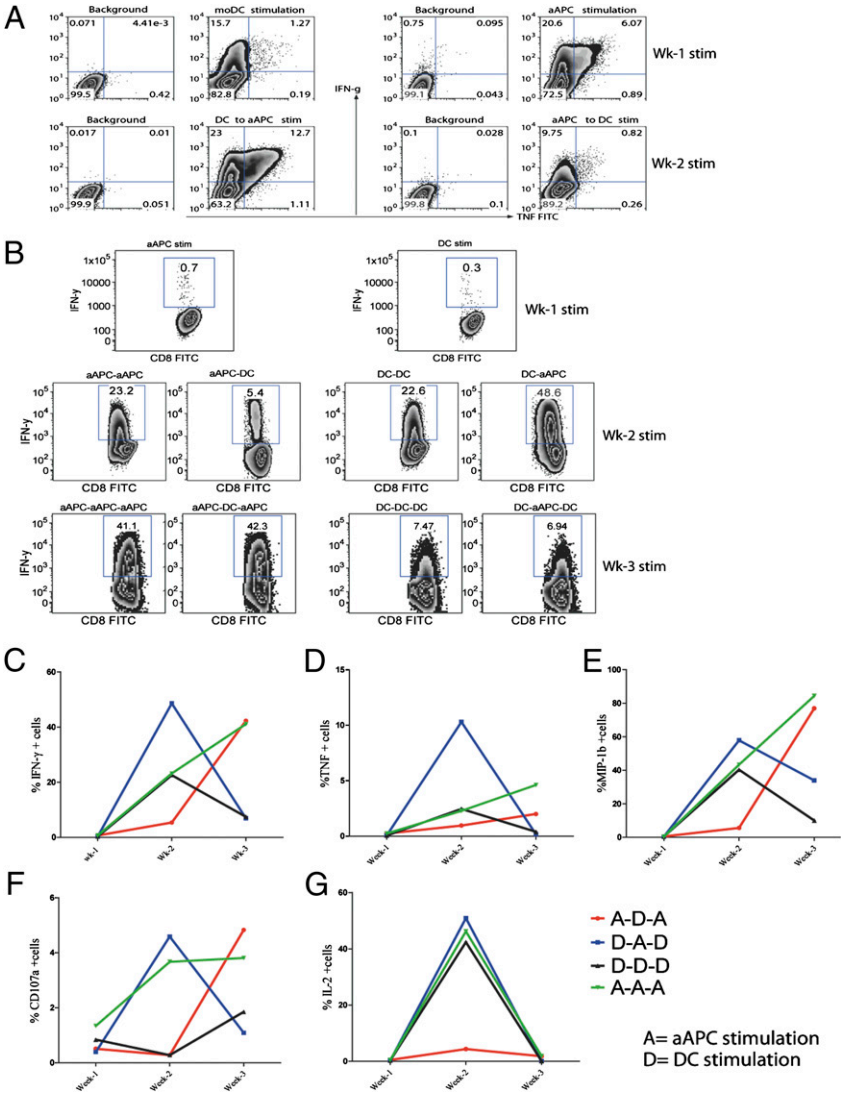

Fig. 3. Cytokine production by CD8$^+$ T cells is determined by the type of APC used for stimulation. (*A*) To assess the effect and reversibility of APC stimulation on cytokine production, CD8$^+$ T cells stimulated with ^{M1}aAPCs for 7 days were restimulated with ^{M1}moDCs for a further 7 days or CD8$^+$ T cells initially stimulated with ^{M1}moDCs were restimulated with ^{M1}aAPCs in week 2. The proportion of cells producing two cytokines (IFN-γ and TNF-α) was assessed by intracellular cytokine staining on day 7 and day 14 (before and after switching APCs). The upper right quadrant represents the proportion of cells expressing IFN-γ and TNF-α. (*B*) To assess the effect of switching APCs on the cytokines produced, enriched CD8$^+$ T cells were cultured for 3 weeks changing the type of APCs (aAPCs or moDCs) at the end of each 7-day stimulation cycle. The gated cells represent the proportion of cells expressing IFN-γ out of total CD3/CD8$^+$ T cells. (*C–G*) The effect of switching the type of APC on cytokine, chemokine, and cytotoxicity is shown by a trend line that shows the proportion of cytokine-producing cells at the end of each 7-day stimulation cycle.

TNF-α, IL-2, MIP-1β, and CD107a), as well as cells expressing none of the functions tested, was calculated (Fig. 4*A*). Most of the $CD8^+$ T cells expanded with ^{M1}aAPC produced multiple effector cytokines, whereas most $CD8^+$ T cells expanded with ^{M1}moDCs expressed a single cytokine.

The mean fluorescence intensity (MFI), measured by ICS, correlates directly with the amount of cytokine secreted by cells (10). Therefore, we compared the quantities of cytokine production and CD107a mobilization of $CD8^+$ T cells expanded by ^{M1}aAPCs and ^{M1}moDCs by calculating the MFI for each parameter. $CD8^+$ T cells expanded by ^{M1}aAPCs produced more IFN-γ, MIP-β, and IL-2 per cell than $CD8^+$ T cells expanded by ^{M1}moDCs (Fig. 4*B*).

To determine whether inhibitory receptors were differentially induced, cells were stained for PD-1. A higher proportion of aAPC-expanded cells (52.8%) expressed PD-1 than moDC-expanded cells (21.2%) (data not shown).

Discussion

In this study, we compared expansion, from very low precursor frequency to high frequency, of virus-specific $CD8^+$ T cells using HLA-Ig-based aAPCs and autologous moDCs loaded with the immunodominant M1 influenza virus peptide or the subdominant H576 MV peptide. The two types of APCs expanded similar numbers of $CD8^+$ T cells, but cells expanded by aAPCs produced greater numbers and larger amounts of effector molecules than cells expanded by moDCs. Furthermore, the phenotypes were not fixed, but could be shifted from being mostly monofunctional to multifunctional or vice versa by changing the type of APCs. We conclude that stimulation of memory $CD8^+$ T cells exclusively through the TCR and CD28 by aAPCs stimulates a large expansion of multifunctional cells, whereas stimulation with moDCs does not. This system should allow further characterization of the factors that regulate production of functionally distinct effector T cells.

Although aAPC- and moDC-induced cultures produced largely similar amounts of cytokines during the early phases of stimulation (week 1), the amounts of cytokines produced by moDC-expanded $CD8^+$ T cells did not increase in the later phases of culture, as they did in aAPC-expanded cultures. There are a variety of potential mechanisms that can explain why moDC-induced cultures did not show increased cytokine production after multiple rounds of stimulation. First, the number or density of peptide-MHC complexes on aAPCs may be more optimal than on moDCs. Second, "cross talk" between DCs and T cells may involve both suppressive as well as stimulatory signals (34, 35), whereas aAPCs provide unidirectional continuous stimulation. In fact, higher levels of expression of PD-1 on aAPC-expanded $CD8^+$ T cells than on moDC-expanded cells may be related to this continuous stimulation (36). Third, conditions used for maturation and activation of moDCs, or the ratio of $CD8^+$ T cells to APCs in culture, may not be ideal for stimulation of multifunctional cells. Fourth, activated T cells can kill DCs in culture (37), thereby reducing the number of DCs available for stimulation and the accumulation of apoptotic cells in the moDC cultures can further suppress DC function (20, 21, 38). Lastly, $CD4^+$ T cells, which were not present in the cultures, provide crucial help for T cells in vivo through activation of DCs and production of IL-2 (39,

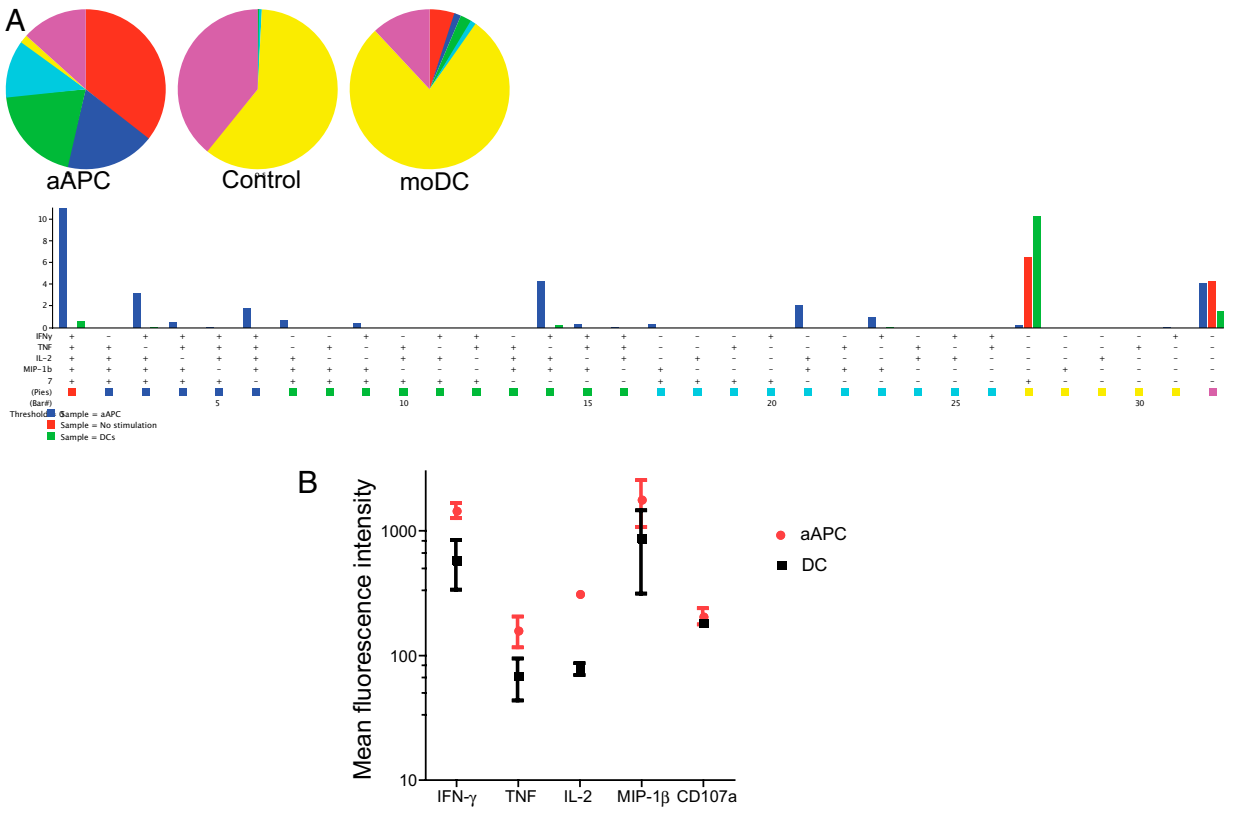

Fig. 4. Generation of virus-specific multifunctional $CD8^+$ T cells by aAPCs and moDCs. The multifunctional capacities of individual cells expanded for 3 weeks by ^{M1}aAPCs and ^{M1}moDCs were analyzed. (*A*) Every possible combination of cytokine production by M1-specific cells is shown on the *x* axis. Bars show the relative proportion of the total response contributed by $CD8^+$ T cells with a given functional response. Blue bars show aAPC-stimulated cells. Green bars show moDC-stimulated cells. Red bars show cells stimulated with unloaded aAPCs. Pie charts (*Left*, aAPC; *Center*, no stimulation; *Right*, moDC) show the proportion of cells positive for different numbers of effectors: lavender, 0; yellow, 1; light blue, 2; green, 3; dark blue, 4; and red, 5. (*B*) Compiled IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a MFI of $CD8^+$ T cells producing cytokines. Dots identify sample means.

40). Expansion of multifunctional $CD8^+$ T cells with moDCs in vitro may also require $CD4^+$ T cell help, as has been shown for induction of HIV-specific $CD8^+$ T cells (24). This system provides the opportunity to investigate these factors.

Previous studies on the functional properties of ex vivo-expanded $CD8^+$ T cells have been limited. Phenotypic characterization of aAPC-generated melanoma antigen Mart-1-specific cytotoxic T lymphocytes (CTLs) showed they were effector memory T cells ($CD45RA^-$, $CD45RO^+$, $CCR7^-$, and $CD62L^{+/-}$) that produced IFN-γ, IL-2, IL-4, and IL-5 upon activation by endogenously processed antigen on tumor cells (41). Limited functional characterization of ex vivo-generated human $CD8^+$ T cells suggests that they can also carry out effector functions in vivo with ex vivo-generated effector memory cell migration to nonlymphoid tissues and selective homing to tumors (41, 42). In addition, investigators have shown that aAPC-generated T cell lines could be maintained in culture up to 1 year without becoming terminally differentiated. Importantly, these cells remained $CD27^+$ $CD28^+$ a phenotype associated with long-term persistence of CTL (43).

There are increasing data to suggest that multifunctional T cells are important for control of infection, for vaccine-induced protection from infection and for eradicating tumor antigen-expressing cells (10, 11, 44, 45). Additionally, aAPC-induced multifunctional T cells adoptively transferred into tumor-bearing mice play a role in vivo. Our aAPC-induced cultures demonstrated that signals through the T cell receptor and CD28 alone were sufficient for successful expansion of competent polyfunctional antigen-specific $CD8^+$ T cells. The beneficial role of additional or alternate costimulatory signals for successful T cell expansion cannot be excluded (46–48), and the differential need for such signals could explain the lesser expansion of H576-specific compared to M1-specific cells. Although our initial experiments were focused on signal 1 and 2, the modular aspect of the aAPC system permits the addition of other stimulatory or inhibitory components that can be used to study the regulation of $CD8^+$ T cell differentiation.

One interesting finding is the transitory nature of the difference between aAPC and moDC-stimulated $CD8^+$ T cells. Even after several weeks of culture, switching the stimulus resulted in T cells that reflected the most recent stimulus, moDC versus aAPC. Thus, the state achieved is not fixed, but dynamic. This appears to be in contrast to Th1 versus Th2 differentiation or induction of anergy in $CD4^+$ T cells (49, 50). This may reflect physiologically relevant differences indicating that it is critically important to rapidly turn on and off $CD8^+$ T cell cytokine responses (51). Recent studies of the chromatin state for cytokine genes has shown retention of bivalent potential at key transcription factor genes that allows $CD8^+$ and some $CD4^+$ T cells to maintain functional flexibility (52–55). Alternatively, this may reflect the specific systems we have used for these studies, and additional factors may serve to "fix" $CD8^+$ T cell responses into a specific differentiation state. In either event, this finding shows a central role of the environment in control of $CD8^+$ T cell function.

In summary, this study provides important insight into the functional capacity of $CD8^+$ T cells stimulated ex vivo by HLA-Ig-based aAPCs. Our results show that in addition to the capacity to support large-scale production of antigen-specific cells, aAPCs stimulated multifunctional antigen-specific $CD8^+$ T cells. The availability of a culture system that can be altered to produce $CD8^+$ T cells with different functional capabilities will facilitate understanding of the interactions between APCs and T cells that lead to different functional states. Knowledge about the functional capacity of in vitro generated antigen-specific cells is not only an essential prerequisite for the potential therapeutic application of these cells, but also shows how a reductionist system can be used to study the regulatory mechanisms controlling effective immune responses.

Materials and Methods

Peptides. Peptides representing A2-restricted epitopes from the MV hemagglutinin protein (H 576–584; KLWCRHFCV) (23) and influenza virus matrix protein (M1 58–66; GILGFVFTL) (22) were prepared by the Johns Hopkins University Core facility. The purity (>98%) of each peptide was confirmed by mass-spectral analysis and high performance liquid chromatography.

Cell Isolation and Generation of Peptide-Loaded moDCs. PBMCs from HLA-A*0201 healthy adults were isolated by Ficoll-Paque PLUS gradient centrifugation following the manufacturer's protocol (GE Healthcare). Monocytes were isolated from PBMCs by $CD14^+$ magnetic bead separation (Miltenyi) and the CD14-negative fraction was used to purify $CD8^+$ T cells ($CD8^+$ T cell isolation kit II; Miltenyi). Standard techniques were used to generate moDCs (56–58). Briefly, $CD14^+$ cells were cultured in RPMI supplemented with antibiotics, 2% autologous serum, 100 ng/mL human granulocyte-macrophage colony-stimulating factor, 50 ng/mL IL-4, and 5 ng/mL transforming growth factor β-1. After 6 days of culture, 10 ng/mL TNF-α, 10 ng/mL IL-1β, 1,000 U/mL IL-6 (BD-Pharmingen) and 1 μg/mL prostaglandin E2 (Sigma) were added to immature moDCs for 24 h to induce maturation. For peptide loading, 2×10^6 moDCs were incubated with 50 μg peptide.

Generation of Peptide-Loaded aAPCs. For generation of aAPCs, HLA-A2-Ig and anti-CD28 were coupled to magnetic beads (Dynal), as previously described (58). Briefly, beads were incubated with a 1:1 mixture of HLA-A2-Ig and anti-CD28 monoclonal antibody in 0.1 M borate buffer for 24 h at 4 °C on a rotator and washed twice with bead wash buffer. Peptides were loaded onto aAPC at 10 μg/mL final peptide concentration, washed twice with PBS, and adjusted to 10^7 beads/mL Peptide-loaded aAPCs were stored in the peptide solution at 4 °C.

In Vitro Expansion of MV and Influenza Virus-Specific $CD8^+$T Cells. Enriched $CD8^+$ T cells were cocultured at a concentration of 10^5 cells per well with 10^4 peptide-pulsed aAPCs (^{peptide}aAPC), or with 3×10^4 peptide-pulsed moDCs (^{peptide}moDC) in a 96-well round-bottom plate with RPMI supplemented with 5% autologous plasma and 3% T cell growth factor (58). On day 7 and weekly thereafter, T cells were harvested, counted, and replated at the previous ratios. For a negative control, T cells were cultured with unloaded aAPCs.

HLA-A2*0201 MHC Tetramer Staining. HLA-A2*0201 peptide phycoerythrin (PE) tetrameric complexes for MV H576, influenza virus M1, and a control proprietary peptide were obtained from Beckman Coulter Immunomics Operation. Isolated $CD8^+$ T cells were stained with MHC-tetramers at 2 μg/mL and FITC-labeled anti-CD8. After washing, stained cells were fixed with 0.5% paraformaldehyde and analyzed. Data were acquired on a FACSCalibur flow cytometer and analyzed using FlowJo software. The frequency of tetramer-positive $CD8^+$ T cells was determined by gating on CD3/CD8 double-positive cells.

In Vitro Stimulation and Intracellular Cytokine Expression of T Lymphocytes. To measure antigen-specific $CD8^+$ T cells, a 2× mixture containing Golgiplug, 2 μg/mL CD28/CD49d costimulatory reagent (BD Biosciences) with either ^{peptide} aAPCs (for antigen-specific stimulation) or unloaded aAPCs (nonspecific stimulation) was prepared.100 μL of mixture and10^5 cultured T cells in 100 μL medium were added per well in a 96-well plate (Costar, Corning) and incubated at 37 °C in 5% CO_2 for 6 h. Antibody to CD107a (BD Pharmingen) was added to the cells before stimulation. After stimulation, cells were washed and incubated with 10% human AB serum to block Fc binding and then stained for expression of CD3, CD8, MIP-1β, TNF-α, IFN-γ, and IL-2 (BD Pharmingen) using the BD Cytofix/Cytoperm kit as described by the manufacturer. Cells were resuspended in FACS buffer and data acquired on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software. In some experiments, cells were also stained with tetramers before ICS. Analysis of multicytokine producing cells was done using SPICE 4.1.6 software from the Vaccine Research Center, National Institutes of Health, Bethesda, MD.

Statistical Analysis. Mann-Whitney rank-sum tests were performed using GraphPad 161 Prism version 4 (GraphPad Software).

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