

Selective Induction of High Avidity CTL by Altering the Balance of Signals from APC

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High avidity CTL are most effective at clearing viruses and cancer cells. Therefore, understanding the mechanisms involved in induction of high avidity CTL is critical for effective vaccines. However, no vaccine approach to selectively induce high avidity CTL in vivo has been discovered. In a new approach, signals from MHC class I (signal 1) and costimulatory molecules (signal 2) were adjusted by varying Ag dose and by use of recombinant poxvirus expressing a triad of costimulatory molecules (B7-1, ICAM-1, and LFA-3), respectively. Independent of CTL avidity, a strong signal 1 resulted in an increased frequency of CD8⁺ CTL. However, a strong signal 2 was necessary for the induction of high avidity CD8⁺ CTL that killed target cells more efficiently, and signal 2 played a more crucial role in the absence of a strong signal 1. Only CTL induced with strong signal 2 killed tumor cells endogenously expressing low levels of Ag. Signal 2 contributed to the induction of high avidity CD8⁺ CTL in both primary and secondary responses. Thus, although signal 2 has been known to increase the quantity of CTL response, in this study we show that it also improves the quality of CTL response. Our data also suggested that dendritic cells play an important role in induction of high avidity CD8⁺ CTL in vivo. This strategy to selectively induce higher avidity CTL may lead to more effective vaccines for viruses and cancer. *The Journal of Immunology*, 2003, 170: 2523–2530.

Antigen-specific CD8⁺ CTL play a pivotal role in antiviral and antitumor immunity. However, in both viral infection (1, 2) and tumor (3–9) models, only high avidity, and not low avidity, CD8⁺ CTL mediated protective immunity. Therefore, selective and preferential induction of Ag-specific high avidity CD8⁺ T cells, along with the consequent increase in their precursor frequency in the memory pool, could be the best way to enhance the quality of immunity to both viral infection and cancer. However, no vaccine strategy to achieve this goal in vivo has been discovered previously.

Ag-specific T cell responses during viral infection are highly diverse (10–12) in terms of T cell repertoire as well as TCR recognition of different densities of Ag. Based on studies of T cell signaling (13–15), the biological consequences of the interaction between TCR and MHC-peptide complex are primarily determined by the duration of interaction as well as the amount of signal through MHC-peptide. Nevertheless, a single Ag or peptide can induce CD8⁺ T cells that have a variety of affinities for the same MHC-peptide complex. Unlike B cells, T cells cannot take advantage of the mechanism of somatic mutation during the course of the immune response. Therefore, it is possible that affinity of Ag-specific CD8⁺ T cells and the distribution of CD8⁺ T cells bearing different TCR affinities could be predetermined in the course of initial contact with APCs. Once activated, high avidity CD8⁺ T

cells can be selectively expanded in vivo by low density of Ag during viral infection (16), so-called T cell affinity maturation, corresponding to the Ag density-dependent high and low avidity CD8⁺ CTL expansion first demonstrated in vitro (1). A similar T cell expansion mechanism for memory responses has been reported (16, 17). In a recent study, Slifka and Whitton (18) reported that CD8⁺ T cells could also undergo avidity maturation without changing TCR affinity, but it is not clear whether this mechanism can exclude the selective expansion of high avidity CD8⁺ CTL during infection as described by ourselves (1) and others (16, 17). Likewise, Alexander-Miller and coworkers (19) reported that factors other than TCR affinity can influence the avidity of CD8⁺ T cells, and our laboratory (20) found complementary evidence in a series of CTL clones.

Our goal was to design a vaccine strategy that would preferentially induce or expand high avidity CD8⁺ CTL in vivo. Simply lowering the immunization dose failed to achieve this result (data not shown), because below a certain threshold dose, no detectable response was obtained. In considering the importance of the initial stage of CD8⁺ T cell activation, we postulated that the induction and primary distribution of Ag-specific CD8⁺ T cells having different avidities could be regulated by both signal 1 and signal 2 provided by the APC. Therefore, we hypothesized that increasing signal 2 might compensate for a low signal 1 and result in the induction of higher avidity CD8⁺ CTL and better expansion in vivo. To address this hypothesis, we adjusted the strength of signal 1 by using different types of APC as well as different doses of peptide Ag. Signal 2 was controlled by using different types of APC or by using APC infected with recombinant poxvirus expressing a triad of costimulatory molecules (TRICOM),² B7-1, ICAM-1, and LFA-3. During the immune response, the functional avidity of CD8⁺ CTL was measured, and we conclude that a strong signal 2 is necessary for the induction of high avidity CD8⁺

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² Abbreviations used in this paper: TRICOM, triad of costimulatory molecules; DC, dendritic cell; FP, fowlpox virus; VV, vaccinia virus; CEA, carcinoembryonic Ag; Wt, wild type.

CTL, and signal 2 plays a more crucial role in the absence of a strong signal 1 in both primary and secondary immune responses. Our data also suggested that dendritic cells (DC) are critical APC that can induce high avidity CD8⁺ CTL *in vivo*. Although signal 2 is recognized to increase the magnitude of CTL response, the current study reveals an unexpected effect on the quality of the immune response.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice were used at 6–8 wk of age (Animal Production Colonies, Frederick Cancer Research Facility, National Institutes of Health, Frederick, MD). All procedures with animals were conducted in accordance with the institutionally approved protocols.

Cell lines

15-12RM is a transfected, tumorigenic BALB/c 3T3 cell line that expresses the gp160 protein from HIV-1III_B as well as the *ras* and *myc* oncogenes (21). 18neo is a similar control cell line transfected only with the neo vector. Both cells were maintained in T cell complete medium containing 10% FCS and 0.2 mg/ml of geneticin (Sigma-Aldrich, St. Louis, MO). MC38 is a murine colon adenocarcinoma cell line, and MC38-CEA-2 is a tumorigenic clone of MC38 that expresses a truncated form of human carcinoembryonic Ag (CEA) (22). Both MC38 and MC38-CEA-2 were grown in DMEM containing high glucose and 10% FCS. P815 (H-2D^d) and EL-4 (H-2D^b) were maintained in RPMI 1640 complete medium supplemented with 10% FCS, L-glutamine, sodium pyruvate, penicillin, streptomycin, and 5×10^{-5} M 2-ME.

Preparation of splenocytes and DC

T cell-depleted splenocytes were prepared by deletion of both CD4⁺ and CD8⁺ T cells. Cells were depleted by using Ab-coated magnetic beads (Dyna, Oslo, Norway). These preparations were >90% anti-B220 positive and <0.5% anti-CD11c positive. DC were prepared from bone marrow as previously described (23). Briefly, bone marrow cells were depleted of lymphocytes and cultured overnight in RPMI 1640 complete medium supplemented with 10% FCS, L-glutamine, antibiotics, and 2-ME in 6-well plates at 5×10^6 cells/well. Cells were replated on day 1 at 3×10^6 cells/well with 10^3 U each of GM-CSF (PeproTech, Frederick, MD) and IL-4 (BD Pharmingen, San Diego, CA). On days 2, 4, and 6, half of the medium was replaced with fresh medium, and loosely attached cells were harvested on day 8 and characterized by flow cytometric analysis.

Peptides and immunization

BALB/c mice were immunized s.c. in the tail base with 1×10^5 DC or 3×10^5 T cell-depleted splenocytes pulsed with P18-I10 peptide (RGPGRFVTI), the immunodominant peptide epitope within HIV-1III_B gp160 in H-2D^d mice (21). C57BL/6 mice were immunized with the same number of syngeneic DC or splenocytes pulsed with CEA₅₂₆₋₅₃₃ peptide (EAQNTTYL), the immunodominant epitope in CEA in H-2D^b mice. For a boost, animals received peptide-pulsed syngeneic APCs at 3–4 wk after the first immunization. In some experiments, H-2D^b-restricted vesicular stomatitis virus N₅₂₋₅₉ (RGYVYQGL) was used as a control peptide. All peptides were synthesized commercially (Multiple Peptide Systems, San Diego, CA).

Recombinant poxviruses and infection

Recombinant fowlpox virus (FP) expressing the TRICOM murine B7-1, ICAM-1, and LFA-3 (designated as FP-TRICOM), was made by using POXVAC-TC (Schering-Plough, Kenilworth, NJ) strain of FP as described elsewhere (24). The recombinant vaccinia virus (VV) expressing the TRICOM (VV-TRICOM), B7-1 (VV-B7-1), or ICAM-1 (VV-ICAM-1) was previously described (25). Wild-type (Wt) VV (VV-Wt) was Wyeth strain. All recombinant poxviruses were generously supplied by D. Panicali (Theion Biologies, Cambridge, MA). To infect cells with viruses, both DC and T cell-depleted splenocytes were resuspended in serum-free RPMI, and then incubated with 25 multiplicities of infection of virus at 37°C at 5% CO₂ incubator. After a 5-h incubation, peptides were pulsed and then used for immunization. The expression levels of costimulatory molecules were analyzed by flow cytometric analysis, and the data showed that both DC and splenocytes infected with FP-TRICOM expressed at least 5–10 times higher levels of costimulatory molecules compared with those of cells infected with FP-Wt (Fig. 1). FP-TRICOM-infected splenocytes expressed

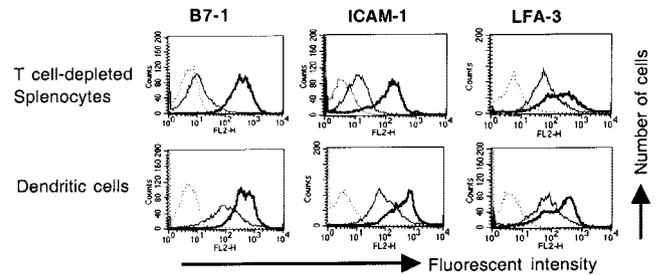


FIGURE 1. Expression of costimulatory molecules (B7-1, ICAM-1, and LFA-3) on T cell-depleted splenocytes and DC. Cells were infected where indicated at a multiplicity of infection of 25 with FP expressing the TRICOM (FP-TRICOM) or Wt virus (FP-Wt) for 18 h. Dotted, thin, and thick lines represent the histograms for control, FP-Wt-infected, and FP-TRICOM-infected cells, respectively.

costimulatory molecule levels between those of DC infected with Wt and TRICOM-expressing vectors. The control vector did not alter the expression levels of any of the 13 phenotypic markers (25).

Abs, tetramer, and flow cytometry

FITC-labeled anti-mouse CD4, CD8 (53-6.7), CD45 (B220), CD11c, CD11b, H-2D^d, H-2D^b, CD80 (B7-1), CD54 (ICAM-1), and CD48 (LFA-3) were used for the staining of cell surface molecules. For intracellular IFN- γ staining, cells were stained with PE-labeled anti-mouse IFN- γ . All Abs and reagents were purchased from BD Pharmingen. PE-labeled H-2D^d-P18-I10 tetramer was provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). For flow cytometric analysis of cell surface, 2×10^5 cells were washed and resuspended in PBS containing 0.2% BSA and 0.1% sodium azide. Cells were incubated on ice with the appropriate Ab for 30 min and then washed. Samples were analyzed on a FACScan (BD Biosciences, Mountain View, CA). Background staining was assessed by use of an isotype control Ab. Intracellular IFN- γ staining was conducted by following manufacturer's protocol (BD Pharmingen), using a 12-h stimulation with peptide to induce IFN- γ expression. For P18-I10-H-2D^d tetramer staining, cells were incubated with FITC-labeled anti-CD8 for 10 min, and then P18-I10-H-2D^d tetramer (200:1 to 300:1 for fresh spleen cells and 50:1 for *in vitro*-restimulated cells) was added, and the cells were further incubated for 30 min on ice.

In vitro restimulation of peptide-specific CD8⁺ CTL and CTL assay

Spleen cells (5×10^6) from immunized mice were cultured with gamma-irradiated (3000 rad) syngeneic splenocytes, 2×10^6 /well in 24-well plates (Costar, Corning, NY) for 1 wk. RPMI 1640 complete medium supplemented with 10% FCS, L-glutamine, antibiotics, 2-ME, and 10% rat T-stim (Collaborative Biomedical, Bedford, MA) was used, and all peptides were added to the wells in a soluble form. Cells were harvested on day 7 to test for CTL activity by 5-h ⁵¹Cr-release assay. The percent specific lysis was calculated as follows: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. Maximum release was determined from supernatants of cells that were lysed by the addition of 5% Triton X-100.

CD8⁺ T cell proliferation

CD8⁺ T cells from the spleens of immunized mice were purified positively by using Ab-coated magnetic beads (Miltenyi Biotec, Auburn, CA) following the manufacturer's instruction. Purified cells were plated at 5×10^4 /well in a 96-well round-bottom microtiter plate (Costar). Irradiated (3000 rad) syngeneic splenocytes with soluble peptides were added at 1×10^5 /well. On day 3, cells were pulsed with 1 μ Ci of [³H]thymidine and further incubated for 18 h before harvesting. Cells were harvested and counted by Microbeta plate counter (Wallac, Gaithersburg, MD).

Cytokine ELISA

Splenocytes (5×10^5) from the immunized mice were mixed with 2×10^5 irradiated syngeneic splenocytes and then cultured in 10% rat T-stim medium containing peptides. After 48-h incubation, culture supernatants were analyzed for IFN- γ by ELISA. The cytokine ELISA used Dynatech Immunolon I plates (Dynatech, Chantilly, VA) and Abs were purchased from

BD PharMingen. Sandwich ELISA was conducted as described in the manufacturer's protocol.

Results

Total number of Ag-specific CD8⁺ T cells induced is similarly regulated by both signal 1 and signal 2

To determine the role of signal through costimulatory molecules (signal 2) at different levels of signal 1, the number of CD8⁺ T cells specific for P18-I10 in mouse spleen was measured by staining with H-2D^d-P18-I10 tetramer (Fig. 2). Both T cell-depleted splenocytes and DC, either FP-Wt- or FP-TRICOM-infected, induced a measurable number of P18-I10-specific CD8⁺ T cells. The number of CD8⁺ T cells induced correlated with the amount of peptide on the APCs, a larger number of CD8⁺ T cells being induced by a higher dose of peptide. However, at all three different concentrations of P18-I10 (0.1, 1.0, and 10 μM), FP-TRICOM-infected splenocytes elicited a significantly greater number of P18-I10-specific CD8⁺ T cells than did FP-Wt-infected splenocytes, and the number of CD8⁺ T cells induced by splenocytes provided with the TRICOM was similar to the number of P18-I10-specific CD8⁺ T cells induced by FP-Wt-infected splenocytes pulsed with even a 10-fold higher amount of peptide. FP-TRICOM-infected DC also induced an increased number of Ag-specific CD8⁺ T cells at low dose (1.0 μM) of peptide, but less enhancement was observed at 10 μM P18-I10, suggesting that signal 2 is more crucial when T cells receive a weak signal 1.

Signals through the TRICOM facilitate the induction of high avidity CD8⁺ CTL

For a given peptide (P18-I10) but at different levels of signal 1, the role of signal 2 in the induction of both high and low avidity CD8⁺ CTL was tested. To quantify the number of P18-I10-specific

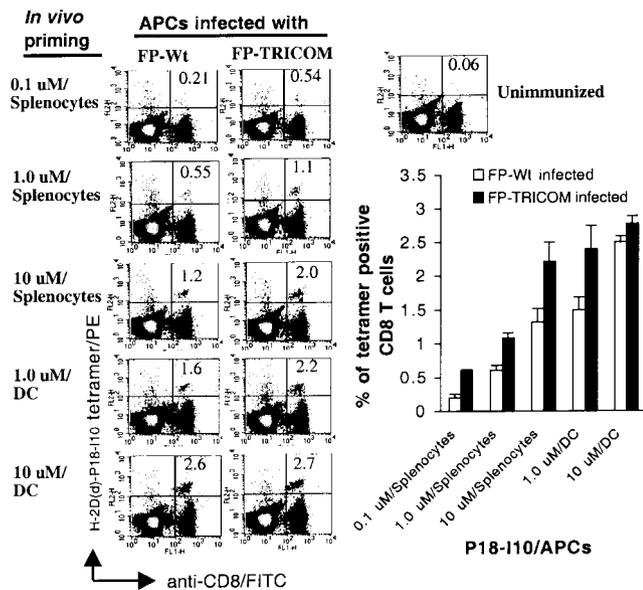


FIGURE 2. Contribution of costimulatory molecules in the induction of Ag-specific CD8⁺ T cells at different levels of TCR occupancy. BALB/c mice were immunized s.c. in tail base with peptide-loaded T cell-depleted splenocytes (3 × 10⁵/mouse) or DC (1 × 10⁵/mouse). After 2–3 wk, spleen cells from the immunized mice were stained with both FITC-anti-mouse CD8 and PE-H-2D^d-P18-I10 tetramer. Each group had three or four mice pooled and the numbers in the quadrant indicate the percentage of H-2D^d-P18-I10 tetramer-positive cells among total CD8⁺ T cells. Unimmunized mice were used as a control. Data are representative of two repeated experiments which showed consistent results.

CD8⁺ CTL that had different avidities, CD8⁺ T cells were expanded with different concentrations of peptide, and then counted by staining with anti-CD8α and H-2D^d-P18-I10 tetramer (Fig. 3). Without an enhanced signal 2, 1.0 μM P18-I10-pulsed T cell-depleted splenocytes (FP-Wt-infected) induced a small number of peptide-specific CD8⁺ T cells, and the largest number of CD8⁺ T cells was observed at the highest concentration of P18-I10 (1.0 μM) for restimulation. However, T cell-depleted splenocytes infected with FP-TRICOM induced significantly greater numbers of P18-I10-specific CD8⁺ T cells at all three different concentrations (55, 237, and 135% at 1.0, 0.1, and 0.001 μM P18-I10, respectively), and the peak number was observed at 0.1 μM P18-I10 for restimulation, a 10-fold lower concentration than in the case of FP-Wt-infected splenocytes. Compared with 1.0 μM-pulsed splenocytes, splenocytes, infected with either FP-Wt or FP-TRICOM, pulsed with 10 μM P18-I10 resulted in a greater number of Ag-specific CD8⁺ T cells as observed in Fig. 2. At the 10 μM immunization dose, the number of CD8⁺ T cells at high peptide concentration (1.0 μM) was similar in both FP-Wt- and FP-TRICOM-infected splenocytes. However, the biggest difference between FP-Wt- and FP-TRICOM-infected splenocytes was observed only when a low concentration of peptide was used for restimulation (155% increase at 0.001 μM; 2.55 fold), suggesting

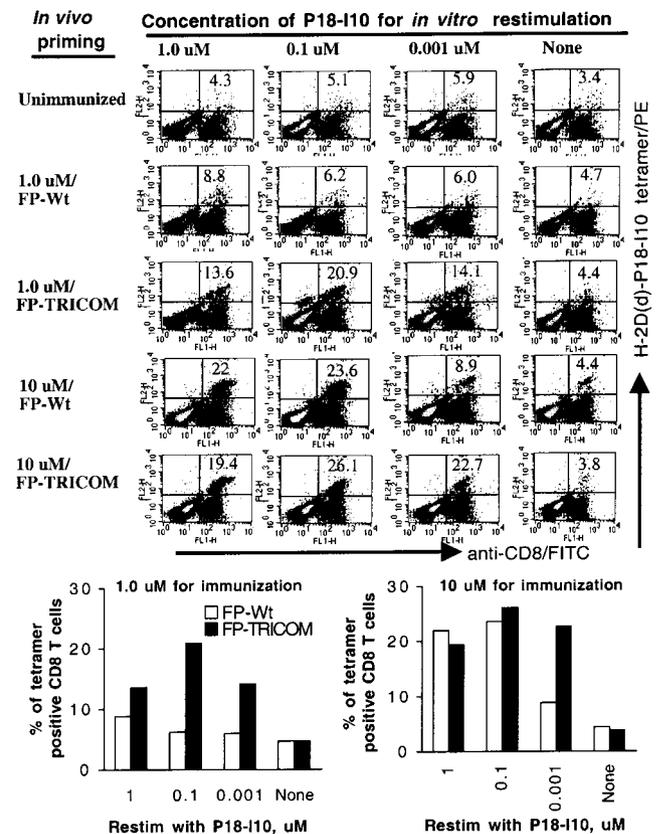


FIGURE 3. TRICOM facilitates the induction of Ag-specific high avidity CD8⁺ T cells. Animals were immunized as described in Fig. 2. Two to 3 wk after immunization, CD8⁺ T cells from the immunized mice were restimulated with different concentrations of P18-I10. On day 7, cells were stained with FITC-anti-mouse CD8α and PE-H-2D^d-P18-I10 tetramer. Individual numbers in the upper right quadrant indicate the percentage of PE-H-2D^d-P18-I10 tetramer-positive cells among the total CD8⁺ T cells. Both histograms summarize the percentage of H-2D^d-P18-I10 tetramer-positive CD8⁺ T cells at different restimulation concentrations of P18-I10. Data are representative of two repeated experiments and similar results were consistently observed.

that the increased signal 2 mediated by TRICOM preferentially facilitates the induction of Ag-specific high avidity CD8⁺ T cells.

Given the lack of effect of TRICOM when DC pulsed with 10 μ M peptide were used for immunization (Fig. 2), the effect of TRICOM infection of T-depleted splenocytes on CTL avidity could not be explained by the small number of DC that might be contaminating the depleted splenocyte preparation, which was >90% B cells and <0.5% DC (see *Materials and Methods*).

CD8⁺ T cells induced with a strong signal 2 show better proliferation and cytolytic activity at lower density of peptide Ags

To test the responses of CD8⁺ CTL induced with either FP-Wt- or FP-TRICOM-infected splenocytes to various densities of peptide Ags, CD8⁺ T cell proliferation was measured. As shown in Fig. 4, *a* and *b*, P18-I10-specific CD8⁺ T cells induced with FP-Wt-infected splenocytes showed maximum proliferation at 100 nM P18-I10. However, CD8⁺ T cells induced with FP-TRICOM showed maximum proliferation at 10 nM, suggesting 10-fold higher avidity. CD8⁺ T cells induced with 1 μ M peptide-pulsed FP-TRICOM-infected cells achieved a similar percentage of maximum proliferation at an almost 10-fold lower concentration of peptide

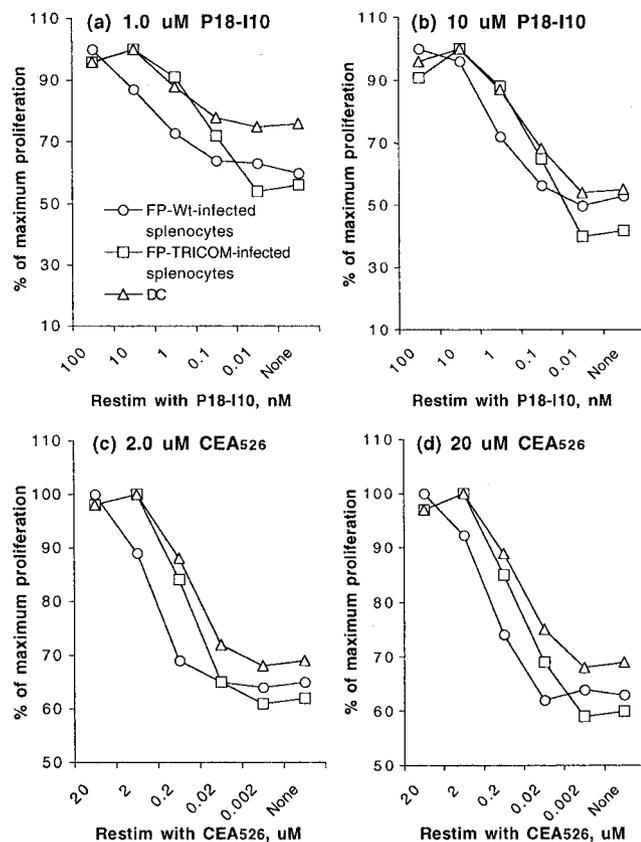


FIGURE 4. Ag-specific CD8⁺ T cells induced with a strong signal 2 show better proliferation at lower density of peptide Ags. Mice were immunized as described in Fig. 2. After 3 wk, 5×10^4 CD8⁺ T cells from the pooled spleens of three to four mice in each group were cultured with 1×10^5 syngeneic splenocytes in 96-well plates. Different concentrations of soluble peptides, either P18-I10 or CEA₅₂₆₋₅₃₃, were added into each well. On day 2, 1 μ Ci of [³H]thymidine was added into each well, and further cultured overnight before harvesting the cells. In each experiment, triplicate assays were done and SD was <10%. To facilitate comparison of dose-response curves, results are normalized as the percentage of maximum proliferation. Data are representative of three repeated experiments and consistent results were observed.

compared with those induced with FP-Wt-infected cells (Fig. 4*a*). Although the difference in peptide concentration to produce a similar level of proliferation was <10-fold when mice were immunized with 10 μ M-pulsed splenocytes, CD8⁺ T cells induced with FP-TRICOM still responded to lower concentrations of P18-I10 better than did CD8⁺ T cells induced by FP-Wt-infected splenocytes (Fig. 4*b*). Consistent with P18-I10-specific CD8 T cells, CEA₅₂₆₋₅₃₃-specific CD8 T cells induced with FP-TRICOM-infected splenocytes showed a better proliferation at lower dose of CEA₅₂₆₋₅₃₃ peptide (Fig. 4, *c* and *d*). The proliferation of CD8⁺ T cells induced with DC was also compared, and the responses to different concentrations of peptides were relatively similar to CD8⁺ CTL induced with FP-TRICOM-infected splenocytes (Fig. 4, *a* and *b*). However, unlike the case of splenocytes, CD8⁺ T cells induced with peptide-pulsed DC showed a higher background, which may represent a response to the FCS in which the DC were prepared, as has been observed by others (26, 27).

In addition to better proliferation at lower density of Ag, high avidity CD8⁺ CTL lyse target cells that express a low density of peptide. After 1-wk restimulation with 1.0 nM P18-I10 or 0.2 μ M CEA₅₂₆₋₅₃₃, cytolytic activity of CD8⁺ CTL was measured by using target cells pulsed with different concentrations of peptides. Fig. 5, *a* and *b*, shows that CD8⁺ CTL induced with FP-TRICOM-infected splenocytes have a greater cytolytic activity against targets pulsed with all concentrations of P18-I10 tested, but the most critical difference was in the lowest peptide concentration at which lysis could be detected. In the case of 1.0 μ M-pulsed splenocytes (Fig. 5*a*), CD8⁺ T cells from only the mice immunized with FP-TRICOM-infected splenocytes lysed target cells pulsed with <10 nM P18-I10. Similarly, for CD8⁺ CTL from mice immunized with 10 μ M P18-I10-pulsed cells, the lowest concentration of peptide on target cells at which lytic activity was detected was 1 nM P18-I10 for CD8⁺ CTL induced without FP-TRICOM but 0.1 nM for those induced with FP-TRICOM (Fig. 5*b*). Lytic activity also reached a peak at a lower peptide concentration when FP-TRICOM was used. Similar enhancement by FP-TRICOM was also observed in mice immunized with 1.0 μ M P18-I10-pulsed DC instead of splenocytes (Fig. 5*c*). However, the additive contribution of costimulatory molecules provided by FP-TRICOM was not seen in the mice immunized with 10 μ M P18-I10-pulsed DC (Fig. 5*d*), suggesting that this high concentration of peptide on DC, which already express higher levels of costimulatory molecules (see Fig. 1), produced a maximal signal. A similar increase in the number of high avidity CD8⁺ CTL induced by increasing the contribution of costimulatory molecules was also observed in the mice immunized with CEA₅₂₆₋₅₃₃-pulsed splenocytes (Fig. 5*e*) or DC (Fig. 5*f*) infected with FP-TRICOM.

Signals through costimulatory molecules play more important roles in the induction of high avidity CD8⁺ CTL in boosted animals

To test the role of signal 2 in the induction of high avidity CD8⁺ CTL in the secondary immune response, spleen CD8⁺ T cells from animals given a second immunization were restimulated with different concentrations of peptides (P18-I10), and then the number of H-2D^d-P18-I10 tetramer-positive CD8⁺ T cells was counted (Fig. 6). Compared with the situation observed in the primary response (Fig. 3), the enhancement by FP-TRICOM was seen at even lower concentrations, 0.001 and 0.0001 μ M, rather than at 1.0 and 0.1 μ M P18-I10, and the biggest enhancement in the number of P18-I10-specific CD8⁺ CTL by FP-TRICOM was observed at 0.0001 μ M P18-I10 (330% increase compare to FP-Wt; 4.3-fold). Comparing the data in Figs. 3 and 6 also suggests that boosting with same Ag resulted in CD8⁺ CTL that responded better to

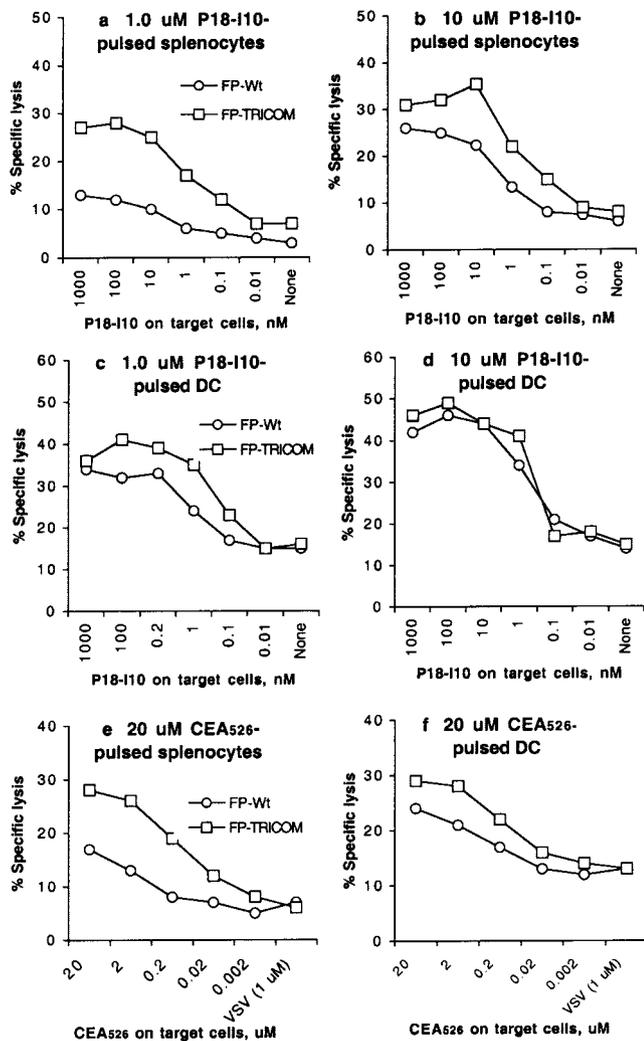


FIGURE 5. CD8⁺ T cells induced with a high signal through costimulatory molecules (B7-1, ICAM-1, and LFA-3) have an increased cytolytic activity to target cells loaded with low density of peptides. Mice were immunized as described in Fig. 2. After 2–3 wk, CD8⁺ T cells from the pooled spleens of three mice in each group were restimulated in vitro with peptides, 0.001 μ M P18-I10 or 0.2 μ M CEA_{526–533}, for 1 wk, and then tested in a 5-h ⁵¹Cr-release assay. E:T ratios for P18-I10- and CEA_{526–533}-specific CD8⁺ CTL were 50:1 and 80:1, respectively. Data are from one experiment and three repeated experiments showed similar results. In each experiment, triplicate assays were done, and the SD of triplicates was <10% of the mean.

lower density of Ag. This avidity shift was also observed in experiments using DC without FP. Both 1.0 and 10 μ M P18-I10-pulsed DC induced a large number of peptide-specific CD8⁺ CTL in the primary response (Fig. 7a), and the peak number of CD8⁺ CTL was observed at 0.1 μ M. However, after second immunization, the largest number of CD8⁺ CTL was observed at 0.001 μ M P18-I10. Although the overall number of CD8⁺ T cells responding to different concentrations of P18-I10 was higher in the mice immunized with DC than in those immunized with splenocytes, the peptide concentrations inducing the greatest number of CD8⁺ CTL resembled those for FP-TRICOM-infected splenocytes and were lower than those in the case of FP-Wt-infected splenocytes (compare Fig. 7 with Figs. 3 and 6). Unlike the case of splenocytes, relatively higher numbers of P18-I10-specific CD8⁺ CTL were observed after 1-wk culture without peptide when DC were used for immunization. This higher background was also observed in the proliferation and CTL assays, and may reflect a response to

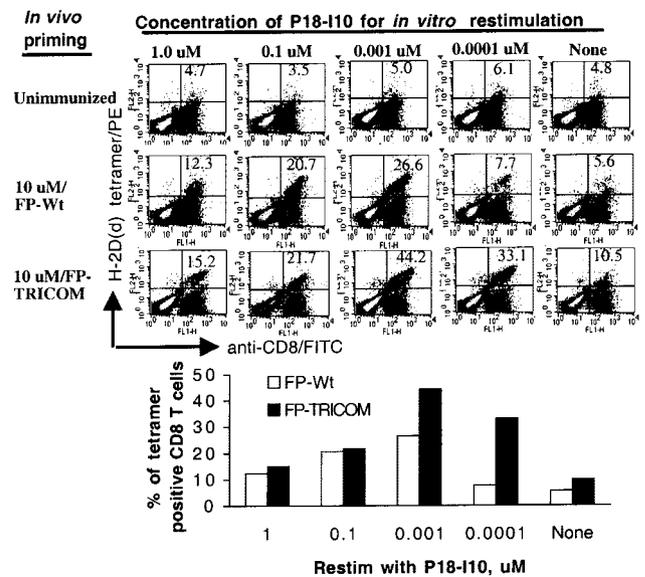


FIGURE 6. Amplification of the number of Ag-specific high avidity CD8⁺ T cells by boosting animals with a strong signal through costimulatory molecules. Three to four mice in each group were immunized. After 3 wk, mice were boosted in the same way as in the first immunization. After 3–4 wk, CD8⁺ T cells from the spleens pooled from each group were restimulated with different concentrations of P18-I10 indicated across the top for 1 wk, and then cells were stained with FITC-anti-mouse CD8 and PE-H-2D^d-P18-I10 tetramer. Numbers in the upper right quadrant indicate the percentage of tetramer-positive cells among total CD8⁺ T cells. Data are representative of two repeated experiments and similar results were consistently observed.

the FCS used in preparation of the DC, as has been reported previously (26, 27).

Signal through the TRICOM results in the increased production of IFN- γ from CD8⁺ CTL at low peptide concentration

The number of CD8⁺ T cells producing IFN- γ in response to brief (12-hr) stimulation with peptide was measured directly ex vivo, without in vitro expansion (Fig. 8a). Consistent with our proliferation and CTL data, CD8⁺ T cells induced with FP-TRICOM-infected splenocytes or DC had a higher number of IFN- γ -producing cells when they were restimulated with the low concentration of peptide (0.001 μ M P18-I10) used in Fig. 8a. The fraction of cells producing IFN- γ was similar to the fraction staining with peptide-MHC tetramer ex vivo (Fig. 2), indicating that most of the tetramer-positive cells can make IFN- γ . FP-B7-1-infected splenocytes also induced an increased frequency of IFN- γ -positive CD8⁺ T cells. Although the magnitude of IFN- γ production determined by ELISA was not directly proportional to the number of secreting cells from intracellular staining, CD8⁺ CTL induced with a strong signal 2, FP-TRICOM or FP-B7-1, produced a greater amount of IFN- γ than CD8⁺ T cells induced with FP-Wt (Fig. 8b) when they were restimulated with low concentration of peptide (1.0 nM P18-I10). With regard to avidity, CD8⁺ T cells induced by all the cell preparations produced high levels of IFN- γ in response to a high concentration of peptide (100 nM P18-I10), but only in the case of those induced by FP-Wt-infected splenocytes did the IFN- γ response fall significantly at the lower, 1.0 nM, concentration of peptide, indicating that these latter CD8⁺ T cells were of low avidity.

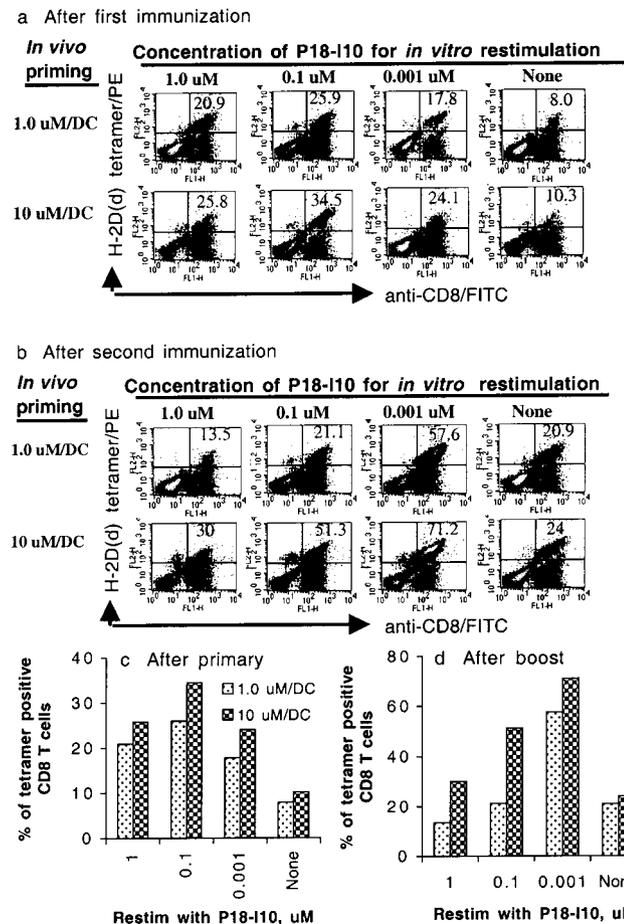


FIGURE 7. DC play an important role in the induction of high avidity CD8⁺ T cells in both primary and secondary immune responses. BALB/c mice were immunized as described in Fig. 2. Two to 3 wk after immunization, CD8⁺ T cells of spleens pooled from the immunized mice were restimulated with different concentrations of P18-I10, shown across the top. On day 7, cells were stained with FITC-anti-mouse CD8 and PE-H-2D^d-P18-I10 tetramer. For the secondary response, mice were boosted at 3 wk after primary injection. Numbers in the upper right quadrant indicate the percentage of tetramer-positive cells from the total CD8⁺ T cells. Both histograms summarize the percentage of H-2D^d-P18-I10 tetramer-positive CD8⁺ T cells at different concentrations of P18-I10 for restimulation. Data are representative of two repeated experiments and similar results were consistently observed.

CD8⁺ CTL induced with a strong signal through costimulatory molecules lyse tumor cells more effectively

To compare the cytolytic activity of CD8⁺ CTL against tumor cells, both P18-I10- and CEA₅₂₆₋₅₃₃-specific CD8⁺ T cells induced with either FP-TRICOM- or FP-Wt-infected splenocytes were restimulated *in vitro*, and then the cytolytic activity was measured. Although CD8⁺ CTL induced with FP-Wt showed lower magnitudes of lytic activity to 0.01 μM peptide-pulsed 18neo cells (Fig. 9, *a* and *b*) than CD8⁺ CTL induced with FP-TRICOM, both CTL showed measurable levels of lytic activity against this target cell. However, in the lysis of the tumor line 15-12RM, only CD8⁺ CTL induced with FP-TRICOM showed significant lytic activity. Similar results were observed in the experiment that measured the lytic activity of CEA₅₂₆₋₅₃₃-specific CD8⁺ CTL. As shown in Fig. 9, *d* and *e*, only CD8⁺ CTL induced with FP-TRICOM showed significant lytic activities against MC38-CEA-2 as well as peptide-pulsed control MC38 cells at different E:T ratios. CD8⁺ CTL induced with DC were also tested in this experiment, and their lytic

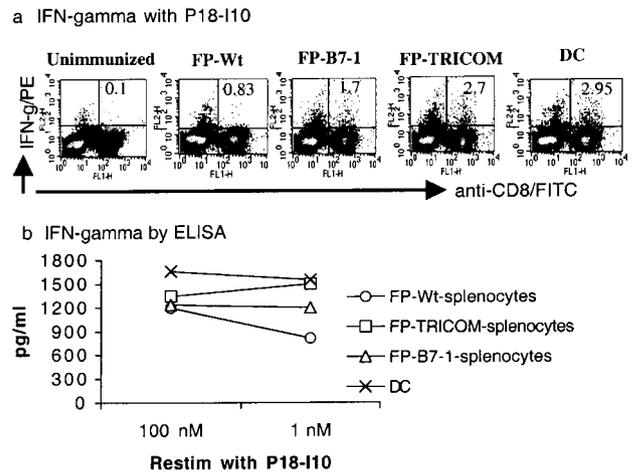


FIGURE 8. Providing the TRICOM (B7-1, ICAM-1, and LFA-3) induces the increased number of CD8⁺ T cells that produce IFN-γ in response to low density of peptide Ag. *a*, BALB/c mice were immunized with 10 μM P18-I10-pulsed T cell-depleted splenocytes. After 3 wk, ex vivo CD8⁺ T cells, without expansion *in vitro*, were stimulated for 12 h with 1.0 nM P18-I10, and then stained for intracellular IFN-γ. *b*, To determine the amount of IFN-γ produced, CD8⁺ T cells were stimulated with 100 or 1.0 nM P18-I10, and the amount of IFN-γ in culture supernatant was determined by ELISA. Data are representative of two repeated experiments and similar results were consistently observed.

activities to parental tumors were similar to the activity observed by CD8⁺ CTL induced with FP-TRICOM-infected splenocytes (Fig. 9, *c* and *f*).

Discussion

It is becoming clear that functional avidity of CD8⁺ T cells, defined as the ability of CD8⁺ CTL to respond to different densities of Ag expressed on MHC class I molecules, is a major determinant for CD8⁺ T cell-mediated immunity, and therefore it is now crucial to understand the mechanism by which high avidity vs low avidity CD8⁺ CTL can be preferentially induced during the immune response. In this study, we first report that signal 2 through costimulatory molecules plays an important role in the induction of high avidity CD8⁺ CTL and that the proportion of high avidity CD8⁺ CTL of total CTL induced is largely determined during the initial contact between TCR and MHC-peptide complex.

Different degrees of TCR occupancy result in different grades of CD8⁺ CTL activation (28). Our data also show that the magnitude of P18-I10-specific CD8⁺ CTL induced was primarily correlated with the degree of TCR occupancy. It has been well known that signal 2 is required not only for a naive T cell activation but also for the increased frequency of Ag-specific CD8⁺ T cells. In addition to this, our data showed that the contribution of signal 2 was greater when T cells received a weak signal 1 than when T cells received a strong signal 1, suggesting that the overall magnitude of P18-I10-specific CD8⁺ T cells induced may be equally dependent on both signal 1 and signal 2. Both signals were likely to compensate for each other quantitatively and result in a similar quantity of Ag-specific CD8⁺ CTL.

However, unlike the total magnitude of Ag-specific CD8⁺ CTL, the functional avidity of CD8⁺ CTL induced was not same when naive CD8⁺ T cells received qualitatively or quantitatively different signals. Without changing signal 2, the proportion of high avidity CTL induced was not significantly enhanced by increasing or decreasing signal 1 alone, although a stronger signal 1 resulted in greater magnitudes of Ag-specific CD8⁺ CTL that responded to

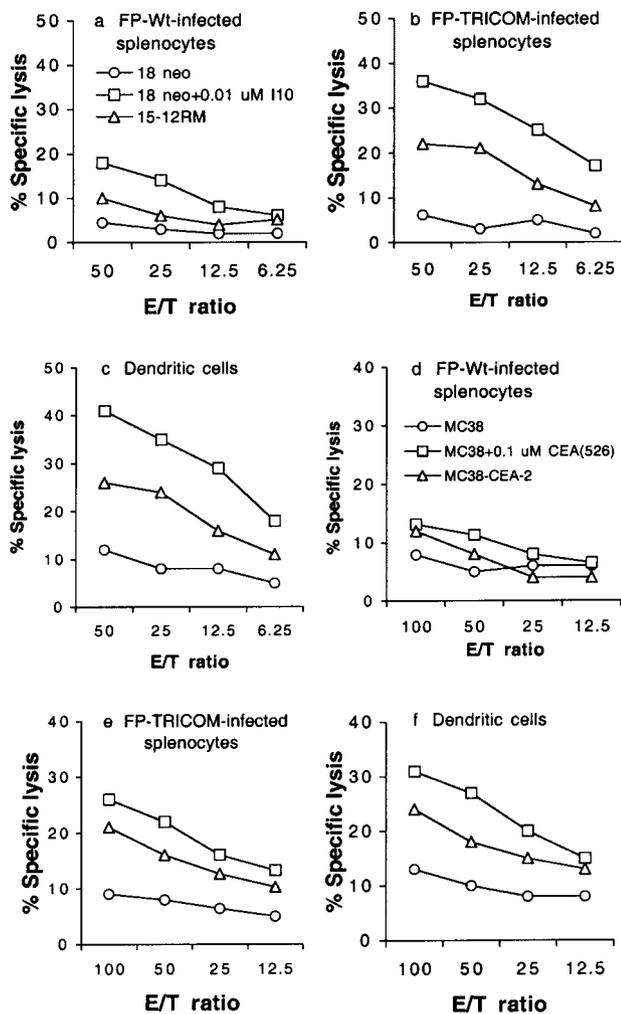


FIGURE 9. CD8⁺ CTL from animals immunized with a strong signal 2 kill tumor cells more efficiently. Both BALB/c and C57BL/6 mice were immunized with 1 μM P18-I10- or 20 μM CEA₅₂₆₋₅₃₃-pulsed T cell-depleted splenocytes. CD8⁺ T cells from the mice immunized were restimulated *in vitro* with splenocytes in medium containing 0.001 μM P18-I10 or 0.2 μM CEA₅₂₆₋₅₃₃. After 1-wk restimulation, 5-h ⁵¹Cr-release assay was carried against tumor cells, 15-12RM and MC38-CEA-2, respectively. Data are representative of three repeated experiments and similar results were consistently observed.

relatively high concentrations of peptide Ag. However, in contrast to signal 1, the increased amount of signal 2 resulted in not only a greater magnitude of total Ag-specific CD8⁺ CTL but also a greater number of high avidity CD8⁺ CTL. This contribution of signal 2 in the induction of high avidity CD8⁺ CTL was observed at both high and low signal 1, but was more significant when T cells received a weak signal 1 than a strong one. Viola and Lanzavecchia (29) and Cai and Sprent (30) reported that CD28-mediated costimulation reduced the number of TCR that need to be triggered by Ag, but these studies addressed the total number of CD8⁺ CTL induced, not their avidity. Moreover, in our experimental system, we could not exclude the synergistic contribution of ICAM-1 and LFA-3 provided with B7-1 in the induction of high avidity CD8⁺ CTL. Both ICAM-1 (31, 32) and LFA-3 (33) have costimulatory function for CD8⁺ T cells, and they also have adhesion properties (34) that may affect the duration of the signal induced in CD8⁺ T cells by APCs. Consistent with those reports (31–34), individual costimulatory signals provided by rVV resulted in both increased magnitude of Ag-specific CD8⁺ CTL and

enhanced responsiveness to low densities of peptide Ag, but no single molecule was as effective as the combination of all three (data not shown).

The role of signal 2 in the enhanced induction of high avidity CD8⁺ CTL was also demonstrated by measuring Ag-specific proliferation and cytolytic activity against different densities of Ags. In particular, only CD8⁺ CTL induced with a high level of signal 2 showed lytic activity against two different tumors tested that express low levels of endogenous Ags. This result is also consistent with induction of higher avidity CD8⁺ CTL, as lysis of parental tumor cells and a better protection against viral infection by high, but not low, avidity CD8⁺ CTL have been demonstrated by ourselves (1, 35) and others (3, 5, 9).

Compared with naive T cells, memory T cells are less dependent on signal 2 for activation (36). Accordingly, APC with a low signal 2 were still capable of increasing the magnitude of total Ag-specific CD8⁺ CTL in a secondary response. Even without a strong signal 2, CD8⁺ CTL in boosted animals showed a better response to lower density of peptide Ag than CD8⁺ CTL in the primary response. It is possible that CD8⁺ CTL which have a higher affinity induced in the primary response may have a selective advantage during *in vivo* expansion. High avidity CD8⁺ CTL that recognize low Ag dose can be activated earlier and faster (35). Moreover, the additional amount of signal 2 provided by FP-TRICOM during the secondary response may need a much smaller amount of signal 1 to activate high avidity CD8⁺ CTL induced in the primary response. Therefore, low Ag dose and high signal 2 during the boost may induce a better selective expansion of high avidity CD8⁺ CTL. Rees et al. (37) reported that there was an inverse relationship between peptide dose and TCR affinity of CD4⁺ T cells in both primary and secondary response. Consistent with this, Anderton et al. (38) reported that immunization with a weaker Ag resulted in more high affinity CD4⁺ T cells in a mouse encephalomyelitis model. However, in our study on CD8⁺ T cells, the induction of high avidity CD8⁺ CTL in both primary and secondary immune response was largely dependent on signal 2, although there was some effect of signal 1 in the induction of CD8⁺ CTL with a low and medium range of avidity in the primary response.

A similar effect of signal 1 and signal 2 was also observed in the experiment using DC as an APC (Fig. 7). Data from this study imply that mature DC, which express high levels of costimulatory molecules, are a major APC that could induce high avidity CD8⁺ CTL *in vivo*. Consistent with our finding, a recent study showed that DC immunization is a potential approach to break tolerance and to induce CD8⁺ CTL-mediated protective immunity against tumor (39), and based on our data, this most likely results from the induction of high avidity CD8⁺ CTL. Others (4, 40) also reported that peptide-pulsed DC induced protective immunity against tumor. Thus, DC-based or DC-targeted immunization may be an effective strategy for an improved quality of CD8⁺ CTL-mediated immunity.

In conclusion, both signal 1 and signal 2 contribute to the total magnitude of CD8⁺ CTL, but in this study we find that signal 2 unexpectedly plays a critical role in altering the quality of CTL response, i.e., in the increased or preferential induction of high avidity CD8⁺ CTL in both primary and secondary immune response. The contribution of signal 2 to the increased number of high avidity CD8⁺ CTL could be partially explained by an enhanced expansion of high avidity CD8⁺ CTL during the immune response, but the data in this study, particularly from the primary response, strongly suggest that different signals provided by APC resulted in different CD8⁺ CTL that have different functional avidity. In addition, our study emphasized the importance of DC as a

major APC for the induction of high avidity CD8⁺ CTL in vivo. Thus, in this study, we have shown how to induce preferentially high avidity CD8⁺ to maximize vaccine efficacy.

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