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J Immunol 2003; 170:2523-2530; doi: 10.4049/jimmunol.170.5.2523
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Selective Induction of High Avidity CTL by Altering the Balance of Signals from APC

SangKon Oh,*‡ James W. Hodge,† Jeffrey D. Ahlers,* Donald S. Burke,‡ Jeffrey Schlom,† and Jay A. Berzofsky1*

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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1 Address correspondence and reprint requests to Dr. Jay A. Berzofsky, Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Institutes of Health, Bethesda, MD 20892; and 2 Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-1578. E-mail address: berzotik@helix.nih.gov

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

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0022-1767/03/$02.00

The Journal of Immunology
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-1IIIB as well as the ras and myc oncoproteins (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 genetin (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\(^b\)) and EL-4 (H-2D\(^d\)) were maintained in RPMI 1640 complete medium supplemented with 10% FCS, l-glutamine, sodium pyruvate, penicillin, streptomycin, and 5 \(\times\) 10\(^{-5}\) M 2-ME.

Preparation of splenocytes and DC

T cell-depleted splenocytes were prepared by depletion of both CD4\(^+\) and CD8\(^+\) T cells. Cells were depleted by using Ab-coated magnetic beads (Dynal, 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 \(\times\) 10\(^5\) cells/well. Cells were replated on day 1 at 3 \(\times\) 10\(^5\) cells/well with 10\(^5\) 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 \(\times\) 10\(^5\) DC or 3 \(\times\) 10\(^5\) T cell-depleted splenocytes pulsed with P18-I10 peptide (RGYPVYQGL) and CEA526 (EAQNTTYL), the immunodominant epitope in CEA in H-2D\(^b\) mice. 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\(^b\)-P18-I10 tetramer was provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). For flow cytometric analysis of cell surface, 2 \(\times\) 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\(^b\) tetramer staining, cells were incubated with FITC-labeled anti-CD8 for 10 min, and then P18-I10-H-2D\(^b\) 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 \(\times\) 10\(^5\)) from immunized mice were cultured with gamma-irradiated (3000 rad) syngeneic splenocytes, 2 \(\times\) 10\(^5\) 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 (Coy Laboratories, Grass Valley, CA). All recombinant poxviruses were generously supplied by D. Panicali (The-
Reduced a measurable number of P18-I10-specific splenocytes and DC, either FP-Wt- or FP-TRICOM-infected, in BALB/c mice. FP-TRICOM-infected splenocytes (3 × 10^6 /mouse) were immunized s.c. in tail base with peptide-loaded T cell-depleted 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 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 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 that signal 2 is more crucial when T cells receive a weak signal 1.

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.
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 μM 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 spleen 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 μM peptide-pulsed FP-TRICOM-infected cells achieved a similar percentage of maximum proliferation at an almost 10-fold lower concentration of peptide compared with those induced with FP-Wt-infected cells (Fig. 4a). Although the difference in peptide concentration to produce a similar level of proliferation was <10-fold when mice were immunized with 10 μM-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. 4b). 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 μM CEAl533, 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 μM-pulsed splenocytes (Fig. 5a), 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 μM 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. 5b). 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 μM P18-I10-pulsed DC instead of splenocytes (Fig. 5c). However, the additive contribution of costimulatory molecules provided by FP-TRICOM was not seen in the mice immunized with 10 μM P18-I10-pulsed DC (Fig. 5d), 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 CEAl533-pulsed splenocytes (Fig. 5e) or DC (Fig. 5f) infected with FP-TRICOM.

**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 × 10⁶ CD8⁺ T cells from the pooled spleens of three to four mice in each group were cultured with 1 × 10⁶ syngeneic splenocytes in 96-well plates. Different concentrations of soluble peptides, either P18-I10 or CEAl533, were added into each well. On day 2, 1 μCi 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.

**SELECTIVE INDUCTION OF HIGH AVIDITY CTL**

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⁺-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 μM, rather than at 1.0 and 0.1 μM P18-I10, and the biggest enhancement in the number of P18-I10-specific CD8⁺ CTL by FP-TRICOM was observed at 0.0001 μM 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...
lower density of Ag. This avidity shift was also observed in experiments using DC without FP. Both 1.0 and 10 μM 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 μM. However, after second immunization, the largest number of CD8+ CTL was observed at 0.001 μM 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 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 μM 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 CEA\(_{26-533}\)-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 \(\mu\)M peptide-pulsed 18neo cells (Fig. 9, a and b) than CD8\(^+\) CTL induced with FP-TRICOM, both CD8\(^+\) T cells induced 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 CEA\(_{26-533}\)-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 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 naïve 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 1 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 naïve 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
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 affinity 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, Anderson 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 affinity 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 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 Lanzevecchia (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
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.

Acknowledgments

We thank Drs. Pierre Henkart, Doug Grosenbach, and Maryna Eichelberger for critical reading of the manuscript and helpful suggestions, and Lisa Smith for assistance in preparation of the manuscript.

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