Direct Presentation Regulates the Magnitude of the CD8⁺ T Cell Response to Cell-Associated Antigen through Prolonged T Cell Proliferation

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Direct Presentation Regulates the Magnitude of the CD8+ T Cell Response to Cell-Associated Antigen through Prolonged T Cell Proliferation

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The magnitude and complexity of Ag-specific CD8+ T cell responses is determined by intrinsic properties of the immune system and extrinsic factors, such as vaccination. We evaluated mechanisms that regulate the CD8+ T cell response to two distinct determinants derived from the same protein Ag, SV40 T Ag (T Ag), following immunization of C57BL/6 mice with T Ag-transformed cells. The results show that direct presentation of T cell determinants by T Ag-transformed cells regulates the magnitude of the CD8+ T cell response in vivo but not the immunodominance hierarchy. The immunodominance hierarchy was reversed in a dose-dependent manner by addition of excess naive T cells targeting the subdominant determinant. However, T cell competition played only a minor role in limiting T cell accumulation under physiological conditions. We found that the magnitude of the T cell response was regulated by the ability of T Ag-transformed cells to directly present the T Ag determinants. The hierarchy of the CD8+ T cell response was maintained when Ag presentation in vivo was restricted to cross-presentation, but the presence of T Ag-transformed cells capable of direct presentation dramatically enhanced T cell accumulation at the peak of the response. This enhancement was due to a prolonged period of T cell proliferation, resulting in a delay in T cell contraction. Our findings reveal that direct presentation by nonprofessional APCs can dramatically enhance accumulation of CD8+ T cells during the primary response, revealing a potential strategy to enhance vaccination approaches.

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As a result of their ability to recognize and eliminate individual cells expressing the target Ag, CD8+ T cells are an important component of the immune response to intracellular pathogens and tumors. The magnitude of the CD8+ T cell response has been correlated with control of acute infections (1, 2) and tumor progression (3–5), as well as the level of memory T cells that remain in the host following immunization or infection (6). Therefore, it is imperative to identify mechanisms that regulate the magnitude of the CD8+ T cell response to better understand the immune response to infection and to optimize vaccine strategies.

Previous studies highlighted several factors that influence the magnitude of a given epitope-specific CD8+ T cell response, including recent results demonstrating a correlation between the frequency of naive T cell precursors and the number of cells that accumulate at the peak of the response (7–10). In addition, CD8+ T cell competition for available APCs can limit the frequency of CD8+ T cells generated during an immune response (11–14), although this mechanism may not always contribute to the observed response (8, 15). The amount of Ag available to prime CD8+ T cells can also influence the magnitude of a CD8+ T cell response, with high Ag load shown to expand the size of the primary T cell response (16, 17), perhaps by prolonging the duration of Ag presentation in vivo (18).

Processing and presentation of Ag play important roles in initiating an effective immune response and can also contribute to immunodominance, a phenomenon in which the magnitude of T cells simultaneously targeting unique peptide/MHC class I complexes forms a predictable hierarchy (19). Direct presentation and cross-presentation of Ag by professional APCs (pAPCs) can contribute to the induction of an effective CD8+ T cell response (20–23). In the case of tumor Ags, several reports indicate that direct presentation by tumor cells localized to the lymphoid organs can induce T cell activation directly (24–26). An additional role for direct Ag presentation by non-pAPCs may be to promote CD8+ T cell accumulation (27). Such a phenomenon was observed following DNA vaccination; the combination of cross-presenting pAPCs and directly presented Ag on transfected cells enhanced the magnitude of the CD8+ T cell response (28–30). A similar observation was made by Thomas et al. (31): Ag presentation by nonhemopoietic cells significantly contributed to the CD8+ T cell response toward lymphocytic choriomeningitis virus (LCMV), suggesting that nonhemopoietic cells can further drive proliferation during virus infection. More recently, Pavelic et al. (32) found that direct presentation of the dominant LCMV epitope gp33 on tumor cells increased the frequency of responding CD8+ T cells relative to cross-presentation alone. However, the basis for this enhanced response remains unknown.

We investigated how the magnitude of the CD8+ T cell response is shaped toward the two most dominant determinants from SV40 T Ag (T Ag) following cellular immunization. The oncogenic protein SV40 T Ag has four well-defined H-2b-restricted determinants:

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Materials and Methods

Mice

C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Milton S. Hershey Medical Center (Hershey, PA) animal facility under specific-pathogen-free conditions. B6.SIL mice were obtained from Taconic Farms (Germantown, NY). TCR-1 mice on the C57BL/6 background were described previously (34); they are available from The Jackson Laboratory as line B6.Ctg-Tcra1, Tcra1b1416Tcrlv1. Transgenic TCR-1 progeny were identified by staining PBLs with FITC-labeled anti-Vβ7 Ab (BD Pharmingen, San Diego, CA) and anti-CD8 Ab (53-6.7), as described (22). C57BL/6 mice received 200 μl brefeldin A added to each culture after 2 h. Cells were then stained for CD8a and intracellular IFN-γ using the Cytofix/Cytoperm kit (BD Biosciences), per the manufacturer’s instructions, and analyzed by flow cytometry, as above.

In vitro culture of T cells and 1H3/Crel assay

Spleen cells from B6/K-TagI–immunized mice were restimulated in vitro at 1 × 10^7 cells/well of a 12-well plate with 5 × 10^5 gamma-irradiated (100 Gy) B6/K-TagI cells in 4 ml complete RPMI 1640 medium. Cultures were passed 1:2 on day 7 and provided with fresh B6/K-TagI stimulators and 10 μM recombinant human IL-2 (kindly provided by Amgen, Thousand Oaks, CA). Cytoxicity assays were performed on day 5 poststimulation, and the percentage of specific target cell lysis was determined using a previously described approach (36, 38).

In vivo cytotoxicity assay

Splenocytes from sex-matched B6.SIL mice were incubated with 1 μM peptide for 1 h at 37°C in complete RPMI medium. Cells were then washed three times and resuspended at 10^7/ml in PBS containing 0.1% BSA. Peptide-pulsed targets were differentially labeled with the following concentrations of CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C: Flu NP pulsed (0.25 μM), site I pulsed (0.5 μM), and site IV pulsed (5 μM). Cells were washed twice and resuspended at 4 × 10^7/ml in PBS. Each mouse received 2 × 10^7 cells of each target group in 150 μl by i.v. injection. The percentage of target cell elimination was assessed at 1, 4, or 16 h postadoptive transfer by gating on CD45.1+ cells. The percentage of in vitro stimulated and in vitro rested targets was determined using the following formula: [(ratio of CFSEFluo [site IV] or CFSEmed [site I] cells to CFSEFluo [control]) − (ratio of CFSEFluo [site IV] or CFSEmed [site I] cells to CFSEFluo [control cells])]/(ratio of CFSEFluo [site IV] or CFSEmed [site I] cells to CFSEFluo [control unimmunized mice]).

In vivo BrdU incorporation assay

Mice received two 1-mg doses of a 1-mg/ml BrdU solution (diluted in PBS) 12 h apart by i.p. injection at the indicated times postimmunization. Splenocytes were harvested within 12 h of the last injection and stained for BrdU incorporation using a modified intracellular staining protocol (BD Biosciences). B6.SIL mice were incubated with 1 μM peptide for 1 h at 37°C in complete RPMI medium. Cells were then washed three times and resuspended at 10^7/ml in PBS containing 0.1% BSA. Peptide-pulsed targets were differentially labeled with the following concentrations of CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C: Flu NP pulsed (0.25 μM), site I pulsed (0.5 μM), and site IV pulsed (5 μM). Cells were washed twice and resuspended at 4 × 10^7/ml in PBS. Each mouse received 2 × 10^7 cells of each target group in 150 μl by i.v. injection. The percentage of target cell elimination was assessed at 1, 4, or 16 h postadoptive transfer by gating on CD45.1+ cells. The percentage of in vitro stimulated and in vitro rested targets was determined using the following formula: [(ratio of CFSEFluo [site IV] or CFSEmed [site I] cells to CFSEFluo [control]) − (ratio of CFSEFluo [site IV] or CFSEmed [site I] cells to CFSEFluo [control cells])]/(ratio of CFSEFluo [site IV] or CFSEmed [site I] cells to CFSEFluo [control unimmunized mice])

Adoptive transfers and immunizations

TCR-1 T cells were enriched from spleen and lymph nodes of TCR-1 mice by AutoMACS sorting using CD8α microbeads (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. CD8α-enriched cells were stained with anti-CD8 and DiI tetrimer to determine purity, which ranged between 85 and 90%. C57BL/6 mice received 10^6 “magnetically sorted” TCR-1 CD8+ T cells i.v. in 0.2 ml HBSS, unless otherwise noted. Where indicated, CD11c+ cells were depleted using CD11c microbeads (Miltenyi Biotec) and an AutoMACS sorter, according to the manufacturer’s instructions, prior to CD8 enrichment. For immunizations, mice received an i.p. injection of 5 × 10^7 live T Ag-transformed cells.

Lymphocyte isolation and flow cytometry

Lymphocyte populations were isolated from spleens according to previously described protocols (38) and stained with PE-labeled MHC class I tetramers corresponding to the control Db/Flu (NP366-374), DiI, and Kb/IV at a 1:200 dilution in conjunction with anti-CD8, as previously described (4). Stained cells were fixed with 2% paraformaldehyde prior to flow-cytometric analysis using a FACScan, FACSCalibur, or FACSCan flow cytometer (BD Biosciences, San Jose, CA) housed within the Penn State Hershey Flow Cytometry Core Facility. Data were analyzed using FlowJo software (Tree Star, Ashland, OR). The percentage of epitope-specific CD8+ T cells was determined by subtracting the percentage of cells that stained positive with the control Db/Flu tetramer from the percentage of cells that stained positive with the T Ag epitope tetramer. The following Abs were purchased from eBioscience (San Diego, CA): PE-labeled anti-CD45.1 (clone A20), FITC- and eFluor450-labeled anti-CD8 (clone 53-6.7), FITC-labeled anti-CD11c (clone N418), FITC-labeled anti–IFN-γ (clone XMG1.2), and FITC-labeled anti-BrDU (clone PRB-1). PerCp-Cy5.5-labeled anti-CD8 (clone 53-6.7), PE-labeled anti-CD11c (clone HL3) and PE-labeled anti-VβTCCR (clone H57-597) Abs were purchased from BD Pharmingen.

Intracellular cytokine stain

One million spleen cells isolated from immunized mice were stimulated ex vivo with 1 μM the indicated synthetic peptides or 5 × 10^7 the indicated T Ag-transformed cells. Cultures were incubated at 37°C, 5% CO2 for 6 h, with 1 μg/ml brefeldin A added to each culture after 2 h. Cells were then stained for CD8a and intracellular IFN-γ using the Cytofix/Cytoperm kit (BD Biosciences), per the manufacturer’s instructions, and analyzed by flow cytometry, as above.

In vivo depletion of CD11c+ and NK1.1+ cells

CD11c+ B6 mice received 2–4 mg/kg body weight of diphtheria toxin (DT; D0564; Sigma-Aldrich, St. Louis, MO), i.p., suspended in PBS, as previously described (22). C57BL/6 mice received 200 μg anti-NK1.1 Ab (clone 2.0 DIRECT PRESENTATION REGULATES CD8+ T CELL PROLIFERATION

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PK-136; BioXCell, West Lebanon, NH) diluted in PBS i.p. 1 d prior to and 3 d postimmunization. Control mice received an injection of rat IgG (Sigma-Aldrich).

Results

The early kinetics of in vivo cytotoxicity to site I and site IV correspond with the T Ag immunodominance hierarchy

We first investigated the relationship between the early kinetics of the T Ag-specific CD8⁺ T cell response and the immunodominance hierarchy previously observed at the peak of the CD8⁺ T cell response (33). Groups of C57BL/6 mice were immunized with B6WT-19 cells expressing wild type T Ag. In vivo cytotoxicity against peptide-pulsed target cells and ex vivo staining of lymphocytes with MHC tetramers were used to evaluate the presence of T Ag-specific T cells at early time points. SJL (CD45.1⁺)-derived splenocytes pulsed with peptides corresponding to site I, site IV, or control Flu NP were injected at 2, 3, 4, 5, or 7 d postimmunization. The presence of CD45.1⁺ target cells in spleens was assessed after 16 h and, thus, correspond to days 3, 4, 5, 6, and 8, respectively (Fig. 1A). No target cell killing was detected on day 3; however, by day 4, 68% of the site IV-pulsed targets had been eliminated, and almost all of the site IV-pulsed targets were eliminated by day 5. In contrast, site I sp. act. lagged by 24 h, with 48% of site I-pulsed targets eliminated by day 5 and complete elimination occurring only beyond 6 d postimmunization. MHC tetramer analysis revealed a small, but detectable, population of site IV-specific CD8⁺ T cells at day 6 postimmunization, and this percentage increased dramatically by day 8 (Fig. 1B). Site I-specific CD8⁺ T cells were only detected above background levels by tetramer analysis at day 8. Thus, the in vivo cytotoxicity assay provided an approach for earlier detection of T Ag epitope sp. act. relative to MHC tetramer analysis. These results demonstrate that the kinetics of early detection correspond to the immunodominance hierarchy observed previously at the numeric peak of the response (33), with activity against site IV detected 24 h prior to activity against site I.

We observed complete elimination of site I- and site IV-pulsed targets by day 8, despite the detection of dramatically different frequencies of CD8⁺ T cells by MHC tetramer staining at this time point (Fig. 1A, 1B). To determine whether the in vivo killing assay could be modulated to better reflect this frequency difference, we determined the efficiency of target cell elimination at 1, 4, and 16 h posttransfer of target cells into immunized mice. Approximately 1 h posttransfer, 71% of site IV-pulsed targets were eliminated, with 91% killed by 4 h (Fig. 1C). In contrast, site I sp. act. was not detected until 4 h posttransfer, with complete elimination of the targets occurring by 16 h. Thus, the efficiency of target cell killing is proportional to the frequency of T Ag epitope-specific CD8⁺ T cells. Overall, these results establish that immunodominance of site IV is apparent very early following immunization.

Altering the naive precursor frequency reverses the immunodominance hierarchy but does not block priming of endogenous T Ag-specific T cells

We next asked whether altering the initial precursor frequency of site I-specific CD8⁺ T cells would change the kinetics and/or magnitude of the T Ag-specific immunodominance hierarchy. One million CD8⁺-enriched naive site I-specific TCR transgenic (TCR-I) T cells were transferred into C57BL/6 mice, followed by immunization with B6WT-19 cells and injection of peptide-pulsed CD45.1⁺ target cells at 3, 5, and 7 d postimmunization. Four days posttransfer, ~50% of the site I-pulsed targets were eliminated in mice that received TCR-I cell transfer, whereas no target cell killing was detected at this time point in mice that received immunization alone (Fig. 2A, left panels). Site I target cell elimination corresponded with the appearance of high levels of TCR-I T cells at early time points (Fig. 2A, right panels). The presence of TCR-I T cells did not significantly alter 16-h in vivo cytotoxicity against site IV-pulsed target cells at any of the time points tested, but it dramatically reduced the percentage of endogenous site IV-specific CD8⁺ T cells that accumulated in the spleens of immunized mice (Fig. 2A, lower panels). Consistent with this decrease in site IV-specific T cell accumulation, cytotoxicity was reduced to 52% on day 7 if the assay was harvested after only 4 h (Fig. 2B). These results indicate that increasing the naive CD8⁺ T cell precursor frequency accelerates the kinetics of the functional site I-specific response, enhances total accumulation...
of site I-specific CD8+ T cells within the lymphoid organs, and limits accumulation of the endogenous site IV-specific CD8+ T cell response, effectively reversing the immunodominance hierarchy.

The above experiment revealed that the presence of excess site I-specific precursors inhibited accumulation of the endogenous site IV-specific CD8+ T cells, but it did not completely block their initial activation. Thus, we determined the dose of TCR-I T cells required to inhibit accumulation of the endogenous site IV-specific CD8+ T cells. C57BL/6 mice received various doses of CD8-enriched GFP+ TCR-I T cells, followed by immunization with B6/WT-19 cells. Doses of $10^5$ and $10^6$ TCR-I T cells blocked accumulation of normal levels of endogenous site IV-specific CD8+ T cells, whereas normal levels of site IV-specific CD8+ T cells accumulated when only $10^5$ TCR-I cells were transferred (Fig. 2C). This result corresponded with a decreased accumulation of site I-specific T cells (Fig. 2C) and, at the lowest dose, it also allowed the emergence of endogenous GFP+ site I-specific T cells (Fig. 2D). These results suggest that a large excess of site I-specific precursors is required to reverse the SV40 TAg immunodominance hierarchy. Additionally, excess TCR-I cells block accumulation of the endogenous site I-specific CD8+ T cells more efficiently than the accumulation of endogenous site IV-specific CD8+ T cells.

Elimination of competing T Ag epitopes minimally alters accumulation of site I- and IV-specific CD8+ T cells

Because the above experiments demonstrated that skewing the initial frequency of epitope-specific naive CD8+ T cells reverses the immunodominance hierarchy, we investigated the potential role of competition among the physiologically low endogenous T Ag-specific CD8+ T cells in regulating T cell accumulation. C57BL/6 mice were immunized with B6/K-TagI cells that express a T Ag variant in which sites II/III, IV, and V have been inactivated or B6/Tag-IV–only cells that express a T Ag variant in which sites I, II/III, and V have been eliminated. Control mice were immunized with B6/WT-19 cells expressing wild type T Ag. The frequency of T Ag epitope-specific CD8+ T cells was determined by MHC tetramer staining of splenocytes 10 d postimmunization. Samples were gated on CD8+ cells, and the percentage of tetramer+ CD8+ cells is indicated. D. The percentage of GFP+ and GFP+ (host) site I-specific CD8+ T cells within the samples shown in C was determined. Error bars indicate SE. Imm only, mice receiving only immunization; TCR-I+Imm, mice receiving TCR-I cells and immunization.

FIGURE 2. Increasing the naive precursor frequency of site I-specific CD8+ T cells reverses the immunodominance hierarchy. A, Groups of three mice were immunized with B6/WT-19 cells with and without adoptive transfer of $1 \times 10^6$ TCR-I transgenic CD8+ T cells. Mice received CFSE-labeled CD45.1 target cells at the indicated time points following immunization. After 16 h, splenocytes were harvested and stained for CD45.1, and the percentage of target cell killing was determined (left panels). Tetramer staining with Db/I and Kb/IV was performed on the same cell populations (right panels), with the percentage of CD8+ cells that were MHC tetramer+ indicated. This experiment was repeated twice, with similar results. B, Groups of three mice received B6/WT-19 immunization or TCR-I cells and B6/WT-19 immunization. Seven days following immunization, CFSE-labeled CD45.1+ targets were injected; after 4 h, isolated splenocytes were stained for CD45.1, and the percentage of killing was determined. C, D. Groups of three mice received B6/WT-19 immunization and GFP+ TCR-I cells at the indicated doses or B6/WT-19 immunization alone (No AT). C. The frequency of site I- and IV-specific CD8+ T cells was determined by MHC tetramer staining of splenocytes 10 d postimmunization. Samples were gated on CD8+ cells, and the percentage of tetramer+ CD8+ cells is indicated. D. The percentage of GFP+ and GFP+ (host) site I-specific CD8+ T cells within the samples shown in C was determined. Error bars indicate SE. Imm only, mice receiving only immunization; TCR-I+Imm, mice receiving TCR-I cells and immunization.
NK1.1+ cells. Representative plots from the IgG- and NK cell depletion. The gate represents the percentage of stained with anti-NK1.1 to determine the efficiency of TAP to and 3 d postimmunization with B6/WT-19 (B6) or three mice received control IgG or anti-NK1.1 1 d prior to and 3 d postimmunization with B6/WT-19 (B6) or B6/WT-19 cells. The results demonstrate that the kinetics and magnitude of T cell accumulation was altered in mice immunized with TAP1-/WT-Tag cells relative to mice immunized with B6/WT-19 cells, with the peak of the response occurring at day 7 for the former (Fig. 4A). In contrast, sites I- and site IV-specific CD8+ T cells continued to accumulate in the spleens of mice immunized with B6/WT-19 cells out to day 10. These results indicate that the magnitude and duration of the site I- and site IV-specific CD8+ T cell responses are reduced under conditions in which only cross-presentation of T Ag occurs, although the immunodominance hierarchy remains the same.

One possible explanation for these results is that the B6- and TAP1-/derived cells express different amounts of T Ag, leading to differential T cell triggering and expansion. However, we found that the TAP1-/WT-Tag and B6/WT-19 cells express similar levels of T Ag by Western blot analysis (data not shown), suggesting that the observed differences are explained by a lack of MHC surface expression rather than variation in the amount of Ag used for immunization. We also considered that the difference in T cell magnitude could be attributed to increased susceptibility of TAP1-/WT-Tag cells to NK cells (39), resulting in accelerated removal of TAP1-/WT-Tag cells. To examine this possibility, we depleted B6 mice of NK cells by injection of anti-NK1.1 mAb prior to immunization and quantified the site IV-specific CD8+ T cell response at 7 and 10 d postimmunization. We chose to examine the more robust site IV-specific response in this experiment. Depletion of NK cells following administration of anti-NK1.1 was verified by flow cytometry (Fig. 4C). The results indicate that depletion of NK cells did not enhance the response to site IV following immunization with TAP1-/WT-Tag cells on day 7 or 10 (Fig. 4B). Therefore, the minimal T Ag-specific response generated by TAP1-/WT-Tag immunization cannot be attributed to enhanced NK cell-mediated clearance of TAP1-/ cells.
We next considered that the reduced accumulation of site I- and site IV-specific CD8+ T cells following immunization with TAP1+/−/WT-Tag cells might be explained by an earlier termination of CD8+ T cell proliferation. We used in vivo BrdU incorporation to determine the proportion of proliferating cells at various times following immunization. Because total T Ag-specific T cell numbers are low at early time points postimmunization, we examined the proliferative response of adoptively transferred TCR-I T cells. As outlined in Fig. 5A, B6 mice received TCR-I T cells plus immunization with B6/WT-19 or TAP1+/−/WT-Tag cells the same day. Mice received BrdU injections at 12 and 24 h prior to analysis at the indicated times postimmunization. TCR-I T cells accumulated to similar levels by day 5 following immunization with TAP1+/−- or B6-derived T Ag-transformed cells (Fig. 5A). However, the proportion of cells that incorporated BrdU in the 24-h preceding analysis was 5-fold higher in mice immunized with B6/WT-19 cells. By day 6, TCR-I T cells had declined to 3.5% in TAP1+/−/WT-Tag-immunized mice but continued to expand in B6/WT-19 immunized mice, with 46% still proliferating. To ensure that TCR-I T cells could proliferate in response to immunization with TAP1+/−/WT-Tag cells, some mice were assessed at 3 d postimmunization. At this early time point, a similar proportion of TCR-I T cells incorporated BrdU in response to B6/WT-19 and TAP1+/−/WT-Tag immunization (Fig. 5B). These data indicate that the increased magnitude of T Ag-specific CD8+ T cells induced by immunization with B6/WT-19 cells is associated with a prolonged expansion phase and suggest that the proliferative response to cross-presented T Ag subsides prior to 5 d postimmunization.

We next investigated whether our results could be explained by differences in Ag duration in vivo following immunization with TAP1+/−/WT-Tag cells versus B6/WT-19 cells. To examine this possibility, B6 mice were immunized with TAP1+/−/WT-Tag or B6/WT-19 cells; 7 d later, they received CD8-enriched CFSE-labeled naïve TCR-I T cells. Analysis of splenocytes isolated after 3 d showed that TCR-I T cells proliferated to a similar extent in both groups of mice (Fig. 5C). These results indicate that cross-presentation of T Ag site I persists up to 7 d postimmunization with TAP-deficient or TAP-sufficient T Ag-transformed cells. Because this length of time is beyond the point when TCR-I T cells showed decreased proliferation in response to TAP1+/−/WT-Tag immunization, Ag duration is likely not a major limiting factor.

**CD11c+ cells are not required at late times postimmunization to promote T Ag-specific CD8+ T cell accumulation**

CD11c+ dendritic cells, particularly the CD8α subset, are important for cross-priming CD8+ T cells (40). To evaluate whether CD11c+ cells are needed to sustain T cell proliferation following immunization with TAP-sufficient B6/WT-19 cells, we determined whether elimination of CD11c+ cells following initial T cell expansion altered accumulation of TCR-I T cells at day 7. In this approach, CD11c-DTR mice, which express the high-affinity simian DT receptor from the CD11c promoter (22), received 1 × 106 CD8-enriched TCR-I T cells and immunization with B6/WT-19 cells. CD11c+ cells were depleted on day 4 postimmunization by injection of DT, and the frequency of site I-specific CD8+ T cells was determined on day 7 (Fig. 6B). Injection of 2 ng/g DT into CD11c-DTR mice resulted in the depletion of ~90% of CD11c+ cells (Fig. 6A). We note that similar to previous reports (22), GFP expression on CD11c+ cells was low in CD11c-DTR mice. Despite elimination of CD11c+ cells at day 4, site I-specific CD8+ T cells accumulated to equivalent levels in CD11c-depleted versus control-treated mice (Fig. 6B).

To ensure that cross-presentation by CD11c+ cells was reduced in DT-treated mice, we determined the efficiency of cross-priming of naïve TCR-I T cells following transfer into freshly treated CD11c-DTR mice. CD11c-DTR mice received CFSE-labeled TCR-I T cells followed by injection of DT. Mice were then immunized with B6/WT-19 cells 12 h following depletion; 3 d later, the percentage of proliferating TCR-I T cells was determined. In the absence of CD11c+ cells, TCR-I T cells failed to proliferate by 3 d postimmunization, whereas proliferating cells represented 93% of TCR-I T cells in nondepleted mice (Fig. 6C). Results similar to those presented in Fig. 6B were obtained when CD11c+ cells were depleted from TCR-I donor cells prior to CD8 enrichment and adoptive transfer into CD11c-DTR mice (Supplemental Fig. 2). This result indicates that cotransfer of low numbers of contaminating CD11c+CD8α+ cells, known to efficiently cross-prime naïve T cells in vivo (40, 41), is unlikely to play a role in our results.
to contribute to the late proliferative response following immunization with B6/WT-19 cells. Taken together, the results indicate that CD11c+ cells are necessary during the initial priming of TCR-I T cells following immunization with B6/WT-19 cells, but they are not required later in the primary response to promote maximum accumulation. This finding is consistent with the hypothesis that cross-presentation drives the early proliferative response, but direct presentation by T Ag-transformed cells is required to promote extended CD8+ T cell proliferation.

**Discussion**

The results of this study demonstrate that the CD8+ T cell response to cell-associated Ag is dramatically enhanced by increasing the naive precursor frequency and the presence of nonhemopoietic cells that can directly present the relevant determinants. Although the former is regulated by development of the immune system (42), the latter can be manipulated during immunization to optimize the number of responder cells. Our results indicate that maximal T cell accumulation is achieved by prolonging T cell proliferation during the primary response in the presence of direct presentation. However, functional immunodominance is established early after immunization, even when Ag is only cross-presented.

Our finding that the in vivo kinetics of CD8+ T cell-mediated killing is staggered for T cells targeting two unique determinants within the same protein Ag is best explained by differences in the initial precursor frequency. Several studies have shown a correlation between the naive precursor frequency and the relative magnitude of the T cell response to a given determinant (8–10). Using a similar approach, we acquired preliminary data indicating that the ratio of site IV/site I T cell dominance is maintained from the naive state to the peak of the response (A. Tatum and T. Schell, unpublished observations). This observation is consistent with results from Obar et al. (10), demonstrating that the hierarchy of Tag cells. The frequency of TCR-I T cells was assessed 4 d following the second immunization (day 9 postprimary immunization). Control mice received only a single immunization at day 0 or 5. Mice that received immunization only at day 0 with TAP1-/-/WT-Tag cells achieved only 6% TCR-I T cells at 9 d postimmunization compared with 42% in mice immunized with B6/WT-19 cells (Fig. 7). Mice that received a second dose of TAP1-/-/WT-Tag cells showed no additional expansion of TCR-I T cells. In contrast, TAP1-/-/WT-Tag-immunized mice that received B6/WT-19 cells for the second dose at day 5 had ~40% site I-specific CD8+ T cells by day nine. Importantly, day-5 immunization with B6/WT-19 cells alone yielded <10% site I-specific CD8+ cells. This result demonstrates that the addition of cells directly presenting T Ag site I at a time coincident with the initiation of T cell contraction fully restores T cell accumulation. Importantly, this prolonged response was not induced following immunization with cells that can only donate Ag for cross-presentation, indicating that Ag load is not a limiting factor in determining the magnitude of the T Ag-specific CD8+ T cell response.

**FIGURE 6.** CD11c+ cells are not required late in the primary response to promote T cell accumulation. A, CD11c-DTR mice (three/group) received 2 ng/g DT, and splenocytes were harvested after 16 h and stained for CD11c. The figure shows a representative mouse from the group. The percentage of CD11c+ cells of total splenocytes is indicated. B, Groups of three CD11c-DTR mice received 1 × 106 TCR-I CD8+ T cells and immunization with 5 × 107 B6/WT-19 cells. As indicated in the timeline, mice received 2 ng/g DT i.p. on day 4, and splenocytes were harvested on day 7. Splenocytes were stained with Db/I tetramer and anti-CD8 to determine the frequency of Db/I+CD8+ T cells. C, Groups of three CD11c-DTR mice received 1 × 106 CFSE-labeled TCR-I CD8+ T cells. The next day, mice were injected with 2 ng/g DT and were immunized with 5 × 107 B6/WT-19 cells 12 h later. Proliferation of CFSE-labeled TCR-I CD8+ T cells was determined after 3 d by co-staining with anti-CD8 and Db/I tetramer. Dot plots show the frequency of CD8+ T cells specific for site I, and histograms show the percentage of CD8+ Db/I tetramer+ cells that divided. The data are representative of mice from each group.

**FIGURE 7.** Provision of direct presentation at late times post-T cell activation restores normal T cell accumulation. Groups of three mice received 1 × 106 TCR-I CD8+ T cells and immunization on day 0 with TAP1-/-/WT-Tag (TAP) or B6/WT-19 (B6) cells. Five days after the initial immunization, groups of mice received a second immunization with B6/WT-19 or TAP1-/-/WT-Tag cells. The frequency of TCR-I T cells was assessed 4 d following the second immunization (day 9 postprimary immunization). Control mice received only a single immunization at day 0 or 5. Mice that received immunization only at day 0 with TAP1-/-/WT-Tag cells achieved only 6% TCR-I T cells at 9 d postimmunization compared with 42% in mice immunized with B6/WT-19 cells. Mice that received a second dose of TAP1-/-/WT-Tag cells showed no additional expansion of TCR-I T cells. In contrast, TAP1-/-/WT-Tag-immunized mice that received B6/WT-19 cells for the second dose at day 5 had ~40% site I-specific CD8+ T cells by day nine. Importantly, day-5 immunization with B6/WT-19 cells alone yielded <10% site I-specific CD8+ cells. This result demonstrates that the addition of cells directly presenting T Ag site I at a time coincident with the initiation of T cell contraction fully restores T cell accumulation. Importantly, this prolonged response was not induced following immunization with cells that can only donate Ag for cross-presentation, indicating that Ag load is not a limiting factor in determining the magnitude of the T Ag-specific CD8+ T cell response.
naive CD8\textsuperscript{+} T cells targeting various virus-derived determinants is maintained following virus infection, indicating that T cell expansion is proportional over the course of the primary response. A recent elegant study by van Heijst et al. (43) demonstrated that a high percentage of naive T cells targeting a particular determinant are recruited into the response following virus infection, regardless of the infectious dose, suggesting that the basis for maintenance of the preimmune hierarchy during the primary immune response is the nearly complete recruitment of the available clones. Thus, the early absence of site I versus site IV-specific function observed following immunization is likely due to the subthreshold levels of site I-specific T cells that have accumulated, rather than a lack of T cell priming.

An alternate explanation for the accelerated response of site IV-specific CD8\textsuperscript{+} T cells relative to site I-specific T cells is a potential difference in T cell avidity, allowing more efficient expansion of site IV-specific T cells following immunization. Such a mechanism is supported by recent findings from Zehn et al. (44), in which T cells targeting low-affinity ligands had a shorter duration of proliferation than T cells targeting higher-affinity ligands, leading to lower T cell accumulation and more rapid contraction. Although we showed that detection of a functional site I-specific response is delayed in T Ag-immunized mice, our results indicate that the initiation of endogenous T cell contraction is similar for T cells responding to site I and site IV (Fig. 3). We did not observe differences in the overall avidity of CD8\textsuperscript{+} T cell populations responding to sites I and IV (data not shown), but this may not represent the avidity of the early response to immunization because the population may be skewed toward higher-avidity clones late in the response (44). We note that previous studies failed to define a clear relationship between immunodominance and T cell avidity/TCR affinity. Several studies revealed only a partial correlation (45–47), whereas others found no correlation (48) or an inverse correlation of T cell avidity with immunodominance (49). Overall, these findings suggest that although T cell avidity may be important for prolonging the proliferation of T cell clones within a population, a strict correlation with immunodominance has not been demonstrated.

Whether the staggered functional response toward site I and site IV has any biological significance remains unknown. An intriguing possibility is that naive precursors are present at levels that provide the least interdeterminant interference while allowing optimal accumulation in response to antigenic challenge. For example, our results indicate that increasing the number of naive site I-specific T cells was detrimental to the accumulation of site IV-specific T cells. However, when T cells were present at physiological levels, little to no competition was observed that limited T cell accumulation in response to wild type T Ag versus T Ag variants lacking three of the four determinants (Fig. 3). Similar results were observed in infectious systems using Listeria monocytogenes and LCMV (8, 15). Elimination of the dominant determinants in these pathogens did not change the hierarchy nor enhance the immune response to the subdominant determinants. Interestingly, Kotturi et al. (8) found an overall decrease in the total CD8\textsuperscript{+} T cell response following infection with a LCMV variant lacking the dominant determinants. In this case, CD8\textsuperscript{+} T cell responses to the subdominant determinants could only recover 30\% of the lost dominant response. These results suggest that CD8\textsuperscript{+} T cell accumulation during the primary response is limited by intrinsic factors other than T cell competition, such as the naive precursor frequency. Our results directly support this idea because the addition of naive site I-specific T cells resulted in a dramatically enhanced response to site I.

That the site IV-specific CD8\textsuperscript{+} T cell response may be more biologically important than the response to site I is indicated from studies in SV40 T Ag transgenic mouse models in which T cells targeting the immunodominant site IV determinant can prevent the appearance of new pancreatic tumors (50) and are sufficient to promote the complete regression of established brain tumors (4). This latter result was associated with rapid accumulation of adoptively transferred site IV-specific CD8\textsuperscript{+} T cells within the brain, followed by prolonged T cell persistence at the tumor site. In contrast, site I-specific CD8\textsuperscript{+} T cell entry into the brains of tumor-bearing mice is delayed, despite the presence of a significant population of activated T cells among the donor lymphocytes, and their loss from the brain is rapid relative to site IV-specific CD8\textsuperscript{+} T cells (5). This difference may be partially explained by an increased susceptibility of site I-specific CD8\textsuperscript{+} T cells to tolerance induction (50). The characteristics of the site IV-specific CD8\textsuperscript{+} T cell response that promotes more efficient control of tumors remain to be elucidated.

In the current study, the immunodominance hierarchy of site IV > site I was not altered following immunization with TAP1\textsuperscript{−/−} cells, and a substantial number of T cells accumulated in response to cross-presentation, as found by Chen et al. (23). However, the response to both determinants was increased in mice immunized with TAP-expressing cells as the result of an extended period of T cell proliferation. Thus, when Ag could only be cross-presented, T cell accumulation at later time points was dramatically reduced compared with when Ag was available through cross-presentation and direct presentation. This was despite nearly identical expansion kinetics prior to day 7. A recent study by Thomas et al. (31) showed that during LCMV infection, virus-specific CD8\textsuperscript{+} T cell accumulation is maximized when Ag is presented on nonhemopoietic host cells in addition to pAPCs. However, accumulation of CD8\textsuperscript{+} T cells in response to vesicular stomatitis virus, vaccinia virus, and Listeria monocytogenes infections was not dependent on presentation by nonhemopoietic host cells. The investigators suggested that this difference may be explained by increased survival of nonhemopoietic cells during LCMV infection or by differences in the inflammatory environments. In the present experiments, both forms of presentation likely contributed to the overall T Ag-specific immune response, with Ag presentation by CD11c\textsuperscript{−} APCs driving an extended period of T cell proliferation.

Previous studies showed that cross-presentation of Ag can be biased toward or against particular determinants (27, 51–53), raising the possibility that differences in the magnitude of the sites I and IV-specific CD8\textsuperscript{+} T cell responses could be explained by differences in the efficiency of cross-presentation of these two determinants. In particular, Wolkers et al. (51) showed that determinants located within a functional signal peptide were inefficiently cross-presented in vivo, whereas relocation of the determinant within the same protein or inactivation of the signal peptide promoted efficient cross-priming of epitope-specific CD8\textsuperscript{+} T cells. Likewise Ma et al. (53) found that the efficiency of cross-presentation of the SIINFEKL determinant is influenced by the identity of the flanking amino acids. Previous studies with SV40 T Ag revealed that the immunorecessive site V determinant is weakly cross-presented in vivo relative to the immunodominant site I determinant (27); however, the exchange of site V and site I within T Ag did not enhance the immunogenicity of site V or decrease the immunogenicity of site I (36), suggesting that protein context alone does not dramatically alter the immunogenicity of the T Ag determinants. Although a similar study has not been performed for sites I and IV, immunization with recombinant vaccinia viruses expressing the individual determinants as cytosolic or endoplasmic reticulum-targeted minigenes induces the same hierarchical CD8\textsuperscript{+} T cell response (site IV > site I) observed following immunization with full-length T Ag (33). These results indicate that the observed immunodominance of CD8\textsuperscript{+} T cells responding to site IV versus
site I is independent of the protein context. In addition, the current study reveals that the magnitude of the CD8⁺ T cell response to both sites I and IV is diminished when T Ag is only cross-presented, suggesting that cross-presentation is not biased in this instance. Previous in vitro studies showed that T cells undergo a programmed number of cell divisions following initial Ag encounter (54, 55), whereas in vivo studies revealed that the magnitude of CD8⁺ T cell responses is proportional to the duration of Ag presentation in vivo (18, 43, 56–58). However, this correlation was limited to APCs (54, 55), whereas in vivo studies revealed that the magnitude of immunization, our results do not rule out the possibility of Ag presentation at early time points induces a proliferative program associated with long-term control of SV40 T antigen-induced brain tumors. J. Immunol. 181: 4406–4417.


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References


3. Wang, L. X., S. Shu, and G. E. Plautz. 2005. Host lymphodepletion augments Ag-specific CD8⁺ T cell accumulation to maximal levels following infection/inmunization on T cell accumulation, because changes in Ag levels beyond the first few days had little impact on T cell accumulation. One exception was found using flank infections with HSV, in which only a period of in vivo Ag presentation >4 d promoted optimal HSV-specific CD8⁺ T cell accumulation (58). However, this correlation was limited to APCs that could prime naive T cells, leaving in question any role of non-pAPCs in driving T cell accumulation. In the noninfectious model in the present study, we found that in vivo Ag presentation capable of priming naive T cells persisted at similar levels for up to 7 d, regardless of the cells used for immunization. In addition, CD11c⁺ cells were not required late in the primary response for T Ag-specific CD8⁺ T cells to accumulate to maximal levels following immunization with B6-derived cells. Taken together, these results suggest that after initial T cell activation through cross-priming, interactions with non-CD11c⁺ APCs drive an extended period of T cell proliferation. Although this source of Ag could involve direct presentation by the T Ag-transformed cells used for immunization, our results do not rule out the possibility of Ag transfer to a non-CD11c⁺ cell population.

The question of when direct presentation contributes to extended T cell proliferation remains unknown. Our evidence demonstrates that directly presented Ag can be provided late in the primary response to allow recovery of maximum T cell accumulation. However, we cannot rule out that encounter with directly presented Ag at early time points induces a proliferative program of increased duration. The nature of Ag presentation by non-pAPCs, particularly at earlier time points, may lead to enhanced T cell proliferation due to the absence of negative regulatory signals, such as CTLA-4–B7 interactions (59). These questions will be important to address for optimal design of vaccination approaches targeting CD8⁺ T cells and to better understand the mechanisms that regulate the CD8⁺ T cell response following cell-based vaccination approaches.

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Disclosures

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