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Cognate Memory CD4⁺ T Cells Generated with Dendritic Cell Priming Influence the Expansion, Trafficking, and Differentiation of Secondary CD8⁺ T Cells and Enhance Tumor Control

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CD4⁺ T cells are known to provide support for the activation and expansion of primary CD8⁺ T cells, their subsequent differentiation, and ultimately their survival as memory cells. However, the importance of cognate memory CD4⁺ T cells in the expansion of memory CD8⁺ T cells after re-exposure to Ag has been not been examined in detail. Using bone marrow-derived dendritic cells pulsed with cognate or noncognate MHC class I- and class II-restricted peptides, we examined whether the presence of memory CD4⁺ T cells with the same Ag specificity as memory CD8⁺ T cells influenced the quantity and quality of the secondary CD8⁺ T cell response. After recombinant vaccinia virus-mediated challenge, we demonstrate that, although cognate memory CD4⁺ T cells are not required for activation of secondary CD8⁺ T cells, their presence enhances the expansion of cognate memory CD8⁺ T cells. Cognate CD4⁺ T cell help results in an approximate 2-fold increase in the frequency of secondary CD8⁺ T cells in secondary lymphoid tissues, and can be accounted for by enhanced proliferation in the secondary CD8⁺ T cell population. In addition, cognate memory CD4⁺ T cells further selectively enhance secondary CD8⁺ T cell infiltration of tumor-associated peripheral tissue, and this is accompanied by increased differentiation into effector phenotype within the secondary CD8⁺ T cell population. The consequence of these improvements to the magnitude and phenotype of the secondary CD8⁺ T cell response is substantial increase in control of tumor outgrowth. The Journal of Immunology, 2007, 179: 5829–5838.

The potential for immunological control of tumor outgrowth is well established. However, although extensive efforts are currently being made to determine vaccine formulation that will most effectively elicit immune responses against tumors, little clinical benefit after vaccination against tumor has been demonstrated (1). One potential explanation for this deficit is that to date efforts in tumor vaccine development have been predominantly focused on eliciting CD8⁺ T cell responses, with secondary regard for CD4⁺ T cells.

Although it is generally acknowledged that a variety of pathogens or peptides with high MHC-binding affinity (2, 3) can elicit primary CD8⁺ T cell responses in the absence of CD4⁺ T cells, the varied contribution of CD4⁺ T cells to the orchestration of effective CD8⁺ T cell responses has recently become appreciated. In the first instance, the expression of CD40L (CD154) on activated CD4⁺ T cells has been shown to play an essential role in the activation of CD8⁺ T cells. CD40L-mediated stimulation of dendritic cells (DC)³ induces the expression of cytokines (4, 5) and costimulatory molecules (6), licensing the DC to initiate the productive expansion of CD8⁺ T cells (7–9). Alternatively, direct stimulation of CD8⁺ T cells via CD40 ligand expressed on activated CD4⁺ T cells has been implicated in CD8⁺ T cell activation (10). Subsequent to activation, the secretion of cytokines such as IL-2 by activated CD4⁺ T cells supports the expansion of effector CD8⁺ T cells (11, 12). Finally, CD4⁺ T cells have been demonstrated to provide pivotal support in the development of CD8⁺ T cells that are destined to form memory CD8⁺ T cells, and their subsequent capacity to reactivate and expand after secondary exposure to Ag (13–17). Thus, in the majority of instances CD4⁺ T cells contribute to the activation, expansion, and survival of primary CD8⁺ T cells and provide signals that support the establishment and maintenance of memory CD8⁺ T cells.

Less information is available about the role of CD4⁺ T cells in the expansion of memory CD8⁺ T cells. Several studies of pathogenic infections have argued that the activation and expansion of secondary CD8⁺ T cells from memory CD8⁺ T cells is independent of CD4⁺ T cells (14, 15, 18). In contrast, other studies have indicated deficiencies in secondary responses in the absence of CD4⁺ T cells (19–21), and it has been suggested that the discrepancy in CD8⁺ T cell dependence may be a consequence of the pathogen studied. It is of particular importance to note that the majority of these conclusions have been made after physical or genetic ablation of CD4⁺ T cell populations, thus asking whether CD4⁺ T cells are required for secondary CD8⁺ T cell expansion, not whether cognate (shared Ag specificity) memory CD4⁺ T cells can enhance secondary CD8⁺ T cell expansion. In the setting of immunotherapeutic vaccination against tumor, the benefit of concomitantly generating cognate memory CD4⁺ T cells along with tumor-reactive CD8⁺ T cells, thus justifying an extensive effort to identify MHC class II-restricted epitopes within tumor Ags, is a critical question. Evidence supporting a role for memory CD4⁺ T cells in enhancing tumor-specific responses includes the capacity

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of adoptively transferred cognate CD4+ T cells to facilitate cognate CD8" T cell infiltration of tumors (22); the ability of cognate CD4+ T cells to rescue vaccine-induced dysfunctional CD8" T cells (23); and, primarily, the observation that mice primed with MHC class I and class II-restricted peptides derived from tumor Ags are more resistant to outgrowth of tumor cells (24, 25). Although the potential efficacy of targeting the generation of tumor-specific memory CD4" T cells is apparent, the mechanisms by which cognate memory CD4" T cells support secondary CD8" T cells have not been established. To further explore this question, we have asked whether bone marrow-derived DC (BMDC) that have been pulsed with MHC class II-restricted epitopes can elicit specific CD4+ T cells and assessed whether and how the presence of cognate memory CD4" T cells generated in this manner enhance the capacity of memory CD8" T cells to respond to Ag rechallenge. We find that the presence of cognate memory CD4" T cells increases the capacity of memory CD8" T cells to proliferate after Ag rechallenge, migrate into tumor-associated tissue and differentiate into effector phenotype cells, resulting in substantially enhanced control of tumor outgrowth.

Materials and Methods

Animals
C57BL/6 mice were obtained from Taconic. Thy1.1" OT-I (26) and OT-II (27) TCR-transgenic mice (expressing TCR specific for H2-Kb-OVA257 or H1-1a" OVA233 complexes, respectively) were used with permission from Dr. F. Carbone (University of Melbourne, Melbourne, Australia). Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at the University of Virginia.

Cell lines

LB15.13 were maintained in RPMI 1640 containing 5% FBS supplemented with SerXtend (Irvine Scientific). B16f0-OVA was generated by transfecting B16f1 melanoma cells with a plasmid containing the OVA gene deleted of its secretion sequence (provided by Dr. Andrew Lew, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia), and selected initially by flow cytometric sorting after staining with 25.D1.16 Ab that is specific for H2-Kb"OVA257 complexes (provided by Dr. Ron Germain, National Institutes of Health, Bethesda, MD) and subsequently maintained in RPMI 1640 plus 5% FBS containing 10 µg/ml blastocycin (Invitrogen Life Technologies). OVA expression was assessed frequently by flow cytometric analysis.

Peptides

Synthetic peptides presented by H2-Kb" (OVA257: SIINFEKL) or H2-IAb" (OVA323: ISQAVHAAHAEINEAGR and HBC128: TPPAYRPPNAPIL) were made by standard Fmoc chemistry using a model AMS422 peptide synthesizer (Gilson Co.) in the Biomolecular Core Facility at the University of Virginia (Charlottesville, VA). All peptides were purified to 

Viruses

Recombinant vaccinia viruses expressing full-length OVA (OVA-vac) or a minigenic expressing OVA257 (OVA257-vac) were gifts from Dr. Jon Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Dendritic cells

DCs were generated as described (28) with modifications (29). Immature DC were isolated on a StemSep column (StemCell Technologies) after incubation with a mixture of Abs that enrich for DC. To induce maturation, DC were incubated in a 2:1 ratio overnight with CD40L-transfected NIH-3T3 fibroblasts (a gift from Dr. Rejean Lapointe, National Cancer Institute, Bethesda, MD). Peptides (OVA257, 10 µg/ml; OVA233, 100 µg/ml; or HBC128, 100 µg/ml) were included during the maturation process. DCs were characterized by co-staining with anti-CD11c-PE and anti-CD86-PE or anti-CD70-PE. All Abs were purchased from eBioscience.

Immunization, Abs, and T cell preparation

Initial priming was via tail vein injection of 1 × 10⁶ peptide-pulsed BMDC. CD4" T cell depletion was achieved by i.p. injections of 100 µg of GK1.5 (rat IgG2b; American Type Culture Collection) for 4 days. Depletion and re-establishment was confirmed by flow cytometric analysis of blood lymphocytes obtained from tail vein samined from specific H2-Kb" mice. Secondary CD8" T cells responses were generated by challenge of primed mice at least 3 wk after priming with 5 × 10⁶ PFU of either OVA-vac or OVA257-vac diluted in PBS containing 0.1% BSA. Primary and secondary CD8" T cell responses were assessed 7 and 5 days after immunization, respectively. Quiescent memory CD8" T cells were enumerated after CD8" T cell enrichment. Briefly, splenocytes were depleted of RBC (RBC lysis buffer; Sigma-Aldrich) and then incubated with Abs to enrich for CD8" cells after passage over a StemSep (StemCell) column. Preparations were consistently 85–95% CD8" as assessed by flow cytometry. To isolate lung lymphocytes the chest cavity was opened and 3–5 ml of 10 U/ml heparin (Sigma-Aldrich) in 1× PBS was injected into the right ventricle of the heart. The lungs were then removed, cut into small pieces, and placed in a 15-ml cone with 5 ml of HBSS (Invitrogen Life Technologies). An enzyme mixture of 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, and 60 U/ml DNase I (all from Sigma-Aldrich) was added to each tube and incubated for 1 h in a 37°C water bath. After incubation, the lung pieces were homogenized and filtered through Nytex mesh. The cells were then spun down at 1200 rpm for 5 min and resuspended in 2.3 ml of RPMI containing 10% FBS. Nycodenz (2.7 ml, 40%; Sigma-Aldrich) in 1× PBS was added to the cells and mixed well. The lung mixture was underlayered under 1.5 ml of medium in a separate 15-ml cone. The cells were spun at 1500 × g at 4°C for 20 min. The lymphocytes were collected at the interface between the two layers and washed with medium.

Adaptive transfer

Naive OT-II CD4" T cells were obtained by density-mediated centrifugation of single-cell suspensions from spleen and lymph nodes of OT-II-transgenic mice on Lympholyte-M. OT-II cells were labeled with 4 µM CFSE (Molecular Probes) according to the manufacturer’s directions, and 5 × 10⁶ OT-II cells were transferred into recipient mice by i.v. injection. Memory OT-II cells were generated by subsequent immunization of recipient mice and resting for 28 days. Memory OT-I cells were generated by i.v. injection of 2 × 10⁶ µg of OVA protein, 3 wk apart. Four weeks after immunization, cells were harvested from lymphoid organs; column enriched for CD8" T cells (StemSep); stained with Abs specific for CD69, CD44, and OVA257-tetramer; and sorted for CD69lowCD44dimexpressing cells using a FACSVantage SE sorter (BD Biosciences) running the DIVA option. Sorted cells were labeled with CFSE (4 µM), and 5 × 10⁶ were transferred into recipient mice via tail vein injection. Tumor challenge with 4 × 10⁶ B16f0-OVA cells was performed 24 h after transfer of memory OT-I cells. Lung lymphocytes from immunized and tumor-challenged mice were isolated and stained with OVA257-tetramer, anti-wt-CD8, and anti-CD28 before sorting (as above) into CD27high and CD27low populations, with subsequent assessment of IFN-γ expression by intracellular cytokine staining (see below).

Proliferation assays

CD4" T cells were magnetically enriched by negative selection (StemSep) from the spleens and lymph nodes of immunized mice. Enriched CD4" T cells were cocultured with LB15.13 cells that had been pulsed overnight with 100 µg/ml OVA233 or HBC128 or left unpulsed. After 3 days of culture, medium was supplemented with 1 µCi of [3H]thymidine (MP Biomedicals) and incorporation of radioactivity was determined by scintillation counting.

Intracellular cytokine staining

Cytokine expression was examined either ex vivo in freshly isolated spleen or in short-term cultures maintained in vitro. Splenocytes from immunized mice were depleted of RBC (RBC lysis buffer; Sigma-Aldrich). To measure the production of intracellular cytokines, stimulator cells were incubated overnight in the presence of 100 µg/ml peptide, washed, and incubated with splenocytes for 5 h at a ratio of 1:1 in medium supplemented with 50 U/ml IL-2 (Chiron) and 10 µg/ml brefeldin A (Calbiochem). For endogenous CD8" T cell responses, stimulated cells were counterstained with a 1/500 dilution of PerCP-conjugated anti-CD8 (eBioscience), washed, then fixed and permeabilized in PermWashFix (BD Pharmingen), followed by staining with a 1/50 dilution of allophycocyanin-conjugated anti-IFN-γ. To assess cytokine production by OT-II cells, CD4" T cells were magnetically enriched (StemCell) from spleen and
lymph nodes of primed mice, incubated with OVA323-pulsed stimulator cells, surface stained with anti-CD4-PeCy5.5 and anti-Thy1.1 PeCy7, and subsequently permeabilized and stained with allophycocyanin-conjugated anti-IFN-γ/H9253, anti-IL-2, or anti-IL-4. Values obtained from unpulsed stimulators were subtracted. For OT-II responses, additional Abs against Thy1.2, V/H9251, or V/H9252 were included. Flow cytometry was conducted on a five-color FACSCalibur using CellQuest software (BD Biosciences) and analyzed with FloJo software (Treestar).

Tetramer staining
MHC tetramers that had been folded around OVA257 were kindly provided by Dr. Vic Engelhard (University of Virginia). T cells were coincubated for 45 min at room temperature with the 1/1000 dilution of tetramer-allophycocyanin and a 1/1000 dilution of anti-CD8-PerCP, washed twice, and fixed in 1% paraformaldehyde. Data were acquired on a FACSCalibur using CellQuest software (BD Biosciences) and analyzed with FloJo software (Treestar).

Tumor outgrowth
B16cOVA cells were harvested and washed twice with HBSS. Immunized mice were challenged with either \( 1 \times 10^5 \) or \( 1 \times 10^4 \) cells i.v. or s.c. for lung or skin tumor growth studies, respectively. For lung growth, challenged mice were euthanized 20 days after tumor challenge, lungs were dissected, and tumor nodules (pigmented) were observed by dissection microscope. For skin growth, challenged mice were observed on a daily basis for nodule formation, and tumor size was charted by cross-sectional measurements using calipers. Mice were euthanized if necrosis was observed in the tumor, or if the tumor reached maximal size permitted by experimental guidelines (225 mm²).

Results

**Immunization with MHC class II-restricted peptide pulsed**

**BMDC elicits peptide-specific CD4⁺ T cells**

To establish the capacity of peptide-pulsed BMDC to elicit CD4⁺ T cell responses in vivo, mice were adoptively transferred with CFSE-labeled OT-II T cells (that express TCR specific for the OVA323 peptide presented by H2-IAb). Twenty-four hours after transfer, recipient mice were challenged with either CD40L-activated, OVA323-pulsed BMDC or OVA-vac. Seven days after challenge, the mice were sacrificed, and spleens were analyzed for OT-II expansion and accumulation and cytokine secretion. In comparison, control mice challenged with unpulsed BMDC in which immunized mice were euthanized 20 days after tumor challenge, lungs were dissected, and tumor nodules (pigmented) were observed by dissection microscope. For skin growth, challenged mice were observed on a daily basis for nodule formation, and tumor size was charted by cross-sectional measurements using calipers. Mice were euthanized if necrosis was observed in the tumor, or if the tumor reached maximal size permitted by experimental guidelines (225 mm²).
no CFSE dilution was observed, in mice that were immunized with OVA-vac OT-II cells exhibited complete CFSE dilution. OT-II cells in mice primed with OVA323-pulsed BMDC also diluted CFSE and accumulated in frequency, although not to the same extent as OVA-vac primed mice (Fig. 1A).

We next examined the phenotype of primary and secondary CD4\(^+\) T cell effectors induced by BMDC. BMDC are generally CD8\(^-\)/H11001 CD11b\(^+\), which have been associated with the induction of Th2-type CD4\(^+\) T cells (30). In contrast, we have observed strong

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**FIGURE 2.** Coimmunization with MHC class I- and class II-restricted peptides derived from tumor Ag enhances control of tumor outgrowth. Cohorts of mice were immunized i.v. with BMDCs pulsed with the indicated peptides. Mice were challenged by i.v. injection with B16cOVA and sacrificed 20 days later; tumor nodules were then enumerated in excised lungs. Ab-mediated depletions were performed by three sequential injections of 100 \(\mu\)g of either GK1.5 (CD4\(^+\) T cells) or 2.43 (CD8\(^+\) T cells). Statistics were generated with GraphPad Prism using the Student t test. Lines under the \(p\) values link the groups being compared. Data represent one of two similar experiments.

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**FIGURE 3.** Cognate memory CD4\(^+\) T cells increase the magnitude of secondary, not memory, CD8\(^+\) T cell populations. A and B, Cohorts of mice were primed with 1 \(\times\) 10\(^5\) BMDC pulsed with the indicated peptides then rested for 28 days. The frequency of OVA257-specific CD8\(^+\) T cells was assessed 5 days after rechallenge with OVA-vac (33 days after initial BMDC priming) for secondary responses. OVA257-specific CD8\(^+\) T cells were assessed by either MHC-tetramer staining (A) or intracellular staining for the accumulation of IFN-\(\gamma\) (ICS-IFN-\(\gamma\), B), as described in Materials and Methods. Numbers in graphs are the mean frequency of OVA257-specific CD8\(^+\) T cells in the group. Dot plots in B are representative data of a secondary ICS-IFN-\(\gamma\) where CD8\(^+\) T cells from a challenged mouse have been cocultured with OVA257-pulsed (top plot) or unpulsed (bottom plot) stimulator cells. C, Quiescent memory populations were enriched for CD8\(^+\) T cells by magnetic separation 28 days after priming before staining Flow cytometry dot plots in C are representative data of quiescent memory OVA257-tetramer\(^+\) CD8\(^+\) T cells, and are gated on CD44\(^{hi}\)/CD69\(^{lo}\) cells. Numbers in dot plots indicate the percentage of CD8\(^+\) T cells that stain for IFN-\(\gamma\) (B) or OVA-257 tetramer (C). Statistics were generated by a nonparametric Mann-Whitney Student t test using GraphPad Prism software, in which \(p < 0.05\) is considered significant. Data are representative of one of four experiments with similar outcomes.

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**FIGURE 4.** Cognate memory CD4\(^+\) T cells are required for enhanced expansion of secondary CD8\(^+\) T cells. A, Cohorts of mice were primed with BMDC + OVA257/OVA323 or OVA257/HBC128 and rested. One half of each cohort were subsequently depleted of CD4\(^+\) T cells. After a 60-day recovery period, mice were challenged with 5 \(\times\) 10\(^6\) PFU of OVA-vac, and the frequency of secondary CD8\(^+\) T cells specific for OVA257 was determined 5 days after challenge by MHC-tetramer staining. B, Cohorts of mice were primed with BMDC + OVA257/OVA323 or OVA257/HBC128 and rested. Each cohort was challenged with 5 \(\times\) 10\(^6\) PFU of OVA-vac or OVA257-vac, and the frequency of secondary CD8\(^+\) T cells specific for OVA257 was determined 5 days after challenge by MHC-tetramer staining. Data represent one of three similar experiments.
production of IL-12p40 from BMDC after incubation with CD40L-3T3 cells (not shown), which should direct a Th1 phenotype. To assess cytokine secretion by primary effector CD4+ T cells, CFSE-labeled Thy1.1+ OT-II cells were transferred into Thy1.2+ recipients and challenged i.v. with 4 × 10^6 B16cOVA. Mice were sacrificed 3 days (A) or 6 days (B and C) after tumor challenge. Donor cells were identified by staining lymphocytes harvested from spleen, PTLNs, and lung of recipient mice with Abs for CD8, CD27, Thy1.1, and OVA257-tetramers. Activation and proliferation of donor cells were determined by assessing the proportion of cells that had diluted CFSE, and the frequency of cells within each division. B, Total accumulation was assessed 6 days after challenge, and differentiation (C) was determined by staining for CD27 expression. Numbers in dot plots (A and B) refer to the percentage of CD8+ T cells that stain with OVA257-tetramer. Numbers in histograms (B) represent the mean fluorescence index of CFSE fluorescence. Data represent one or two similar experiments.

Enhanced protection against tumor outgrowth is achieved by immunization with BMDC pulsed with both MHC class I- and class II-restricted peptides derived from a tumor Ag

Multiple studies have reported that BMDC pulsed with MHC class I-restricted peptides derived from tumor Ags can elicit CD8+ T cells that protect against tumor challenge. Given that BMDC
pulsed with MHC class II-restricted OVA232 could elicit OVA232-specific CD8+ T cell responses, we next asked whether the inclusion of MHC class II-restricted peptides derived from Ags expressed by the tumor in BMDC vaccines would enhance protection against tumor outgrowth. Mice were primed by either i.v. injection with BMDC pulsed with both OVA257 and OVA232 (OVA257/OVA232; cognate help) or with both OVA257 and HBC128, an irrelevant MHC class II-restricted control (OVA257/HBC128; noncognate help). After 4 wk, primed mice were challenged by i.v. injection with OVA-expressing B16 melanoma cells (B16-cOVA) and examined for tumor metastases 20 days later. As expected, OVA257/HBC128-primed mice exhibited some resistance to tumor nodule formation. Strikingly, OVA257/OVA232-primed contained significantly (p < 0.001) fewer tumor nodules (Fig. 2). Similarly, mice that had been injected s.c. with OVA257/OVA323-pulsed BMDC showed greater resistance to tumor outgrowth after s.c. injection of B16-cOVA than those primed with OVA257/HBC128-pulsed BMDC (data not shown). Antibody-mediated T cell depletion studies of BMDC + OVA257/OVA232-primed mice indicated that the enhanced control of tumor outgrowth was dependent on CD8+ T cells. Further, removal of CD4+ T cells increased with frequency of lung metastases to a level equivalent to that observed in BMDC + OVA257/HBC128-primed mice. Thus, although memory CD8+ T cells could control tumor outgrowth to a certain extent independently of CD4+ T cells, the presence of OVA-specific CD4+ T cells enhanced CD8+ T cell-dependent tumor control.

Enhanced frequency of secondary CD8+ T cells in mice primed with cognate MHC class I and class II peptides results from increased expansion of memory CD8+ T cells

The enhanced CD8+ T cell-dependent protection attained by priming with BMDC pulsed with both class I and class II peptides derived from the tumor Ag suggested that more tumor-specific secondary CD8+ T cells were being produced from this immunization strategy on re-exposure to the tumor Ag. To confirm this, we compared the frequency of OVA257-specific secondary CD8+ T in OVA257/OVA232- and OVA257/HBC128-primed mice after challenge with OVA-vac. At the peak of the secondary response (day 5 after OVA-vac challenge), OVA257-specific CD8+ T cells were found to be twice as frequent in OVA257/OVA232-primed mice as those in OVA257/OVA323-primed mice (p = 0.01; Fig. 3, A and B). We next asked whether the increase in secondary CD8+ T cells was a function of increased frequency of memory CD8+ T cells. Therefore, 28 days after initial priming, after quiescent memory was established, the frequency of OVA257-specific memory CD8+ T cells was examined and found to be equivalent between mice primed with either OVA257/HBC128 or OVA257/OVA323 (Fig. 3C, p = 0.08). Thus, the enhanced frequency of secondary CD8+ T cells generated by cotargeting the generation of cognate memory CD4+ T cells is due to an increased expansion of memory CD8+ T cells, not to an increased frequency of memory CD8+ T cells.

Cognate memory CD4+ T cells are required for enhanced memory CD8+ T cell responses

To confirm that enhanced CD8+ T cell secondary responses required the presence and activity of cognate memory CD4+ T cells, as suggested by the tumor outgrowth data (Fig. 2), we first asked whether the enhanced response would remain after the removal of cognate memory CD4+ T cells. To achieve this, mice were initially primed with OVA257/OVA323- or OVA257/HBC128-pulsed BMDC and then rested for 28 days. One cohort of OVA257/OVA323 primed mice was then depleted of CD4+ T cells (>95% depletion, data not shown), whereas the second cohort received equivalent injections of control Ab. Mice were rested for 60 days to allow the CD4+ T cell population to re-establish (confirmed by flow cytometric analysis of PBL; not shown). After CD4+ T cell re-establishment, all cohorts were challenged with OVA-vac, and the magnitude of the secondary CD8+ T cell response was determined. We found that removal of the OVA232-specific memory CD4+ T cell population resulted in a reduction in the magnitude of the OVA257-specific secondary CD8+ T cell response to a level that was equivalent to that attained in mice primed with OVA257/HBC128-pulsed BMDC (Fig. 4A). For an alternative test, we primed mice with BMDC pulsed with either OVA257/OVA323 or OVA257/HBC128 and subsequently challenged the different cohorts of mice with either OVA-vac or OVA257-vac (which encodes and expresses the OVA257 minigene and thus would not be expected to reactivate OVA232-specific memory CD4+ T cells). Five days after challenge, the magnitude of the OVA257-specific secondary CD8+ T cell response...
CD27 staining. Numbers in histograms refer to the MFI of OVA257/OVA323 or OVA257/HBC128. ramer. CFSE dilution was assessed as a measure of proliferation, enumerated by costaining for CD8, Thy1.1, and OVA257-tetramer. CFSE dilution was assessed as a measure of proliferation, enumerated by costaining for CD8, Thy1.1, and OVA257-tetramer. Cognate memory CD4T cells increase the frequency and number of effector CD8 T cells in PTLN and tumor involved lungs. A. Lymphocytes were isolated from lung tissue and PTLN of BMDC + OVA257/OVA323 or OVA257/HBC128 primed and rested (28 days) mice, 18 days after challenge by i.v. injection with B16-OVA. Lymphocytes were stained for the presence of OVA257-tetramer CD8 T cells. Two representative lung lymphocyte populations are shown. Numbers in histograms refer to the MFI of CD27 staining. D, ICS-IFN-γ staining of OVA257-tetramer CD8 T cells sorted into CD27lo or CD27hi populations from lungs of BMDC + OVA323-primed mice 21 days after i.v. tumor challenge. Data in A, C, and D are representative of one or three similar experiments. Data in B are compiled from four mice in one experiment and are representative of two similar experiments.

was determined by OVA257-tetramer staining. The frequency of OVA257-specific CD8 T cells was similar in the mice that had been primed with OVA257/HBC128-pulsed BMDC, no matter which vaccinia recombinant had been used to challenge the mice (Fig. 4B). In contrast, in mice primed with OVA257/OVA323-pulsed BMDC and challenged with OVA-vac, OVA257-specific CD8 T cells were ~50% more frequent when compared with mice that had been challenged with OVA257-vac. Together, these separate experiments indicate that the reactivation of cognate memory CD4 T cells is required for enhanced expansion of cognate memory CD8 T cells.

Cognate memory CD4 T cells enhance the expansion, migration, and differentiation of adoptively transferred memory CD8 T cells in response to tumor

The preceding results indicated that the activation of cognate memory CD4 T cells enhances the expansion of the memory CD8 T cells after re-encounter with Ag in the form of recombinant vaccinia virus. To examine whether this holds true for tumor-derived Ags, we compared the ability of mice that have been primed with OVA323 to support the expansion of memory CD8 T cells specific for OVA257 after challenge with tumor. Memory (CD44hiCD69hi) OT-I cells were sorted, labeled with CFSE, and transferred into Thy1.1 mice that had previously been primed with BMDC + OVA323 or BMDC + HBC128. Twenty-four hours after transfer, recipient mice were challenged by i.v. injection of B16-cOVA. Three and six days after tumor challenge, recipient mice were euthanized, and their lungs, PTLNs, spleens, and inguinal lymph nodes were harvested. Transferred OT-I cells were enumerated by costaining for CD8, Thy1.1, and OVA257-tetramer. CFSE dilution was assessed as a measure of proliferation, and CD27 expression was assessed as a measure of effector phenotype (31). Three days after tumor challenge, we consistently found that BMDC + OVA323-primed mice supported a greater frequency (~2-fold) of secondary CD8 T cells than those found in BMDC + HBC128-primed mice (Fig. 5A). OT-I cells in BMDC + OVA323-primed mice exhibited a ~2-fold lower CFSE staining levels in both the PTLNs and the spleen compared with those found in BMDC + HBC128-primed mice (Fig. 5A), consistent with an additional division. By 6 days after tumor challenge, secondary OT-I cells in BMDC + OVA323-primed mice were still found at greater frequency in lymphoid tissue (PTLNs, spleen) than in BMDC + HBC128-primed mice (Fig. 5B), although the overall frequency of OVA257-specific CD8 T cells was now substantially reduced in the spleen at this time point. Secondary OT-I cells were found to have preferentially accumulated (~5-fold) in lung tissue in OVA323-primed mice compared with HBC128-primed mice, and these lung-resident cells also exhibited a down-regulation of CD27 expression (Fig. 5C). Therefore, we conclude that the presence of cognate memory CD4 T cells not only enhances the proliferation of memory CD8 T cells but also enhances their infiltration of lung tissue and induces effector differentiation. Both of these outcomes could plausibly contribute to enhanced control of tumor.

Cognate memory CD4 T cells enhance the recruitment of endogenous secondary CD8 T cells into lung tissue and the formation of fully differentiated effector cells

We next asked whether cognate memory CD4 T cells influence the differentiation and trafficking of endogenous secondary CD8 T cells in a similar manner to memory OT-I. Therefore we examined the frequency and phenotype of secondary CD8 T cells in
the lungs of mice primed with peptide-pulsed BMDC and challenged with OVA-vac. In contrast to the 2-fold increase in frequency of secondary CD8+ T cells found in the PTLNs and spleen of mice containing cognate memory OVA323-specific CD4+ T cells after OVA-vac challenge (Fig. 3), in the lungs the difference in the frequency of OVA257-tetramer+ CD8+ T cells increased to 4-fold (Fig. 6A). Furthermore, with the secondary OT-I cells, there was an enrichment of CD27low cells within the OVA257-tetramer+ CD8+ T cell population in the lungs of mice primed with BMDC + OVA257/OVA323 (Fig. 6B), indicating that the endogenous OVA257-specific CD8+ T cells in the lung have a more differentiated effector phenotype. Indeed, when examined for their capacity to perform effector functions, a greater proportion of the CD8+ T cells in BMDC + OVA257/OVA323-primed mice secreted IFN-γ, and the vast majority of the IFN-γ secreting CD8+ T cells were of the CD27low phenotype (Fig. 6C), compared with those found in BMDC + OVA257/HBC128-primed mice.

To ascertain whether these recruitment and phenotypic differences in endogenous secondary CD8+ T cell responses to OVA-vac challenge were consistent with those found in tumor-associated lymphocytes, we examined the frequency and phenotype of the OVA257-specific CD8+ T cells in lung tissue of B16cOVA-challenged mice. Strikingly, we found that BMDC + OVA257/OVA323-primed mice containing cognate memory CD4+ T cells had ~8-fold more OVA257-specific CD8+ T cells in their lung tissue compared with BMDC + OVA257/HBC128-primed mice (Fig. 7, A and B). Additionally, in contrast to OVA257-specific CD8+ T cells in the PTLNs of BMDC + OVA257/OVA323 or BMDC + OVA257/HBC128-primed mice, the OVA257-specific CD8+ T cells in lung had a considerably more differentiated phenotype as judged by CD27 down-regulation (Fig. 7C). Further, after flow cytometric sorting of OVA257-tetramer+ CD8+ T cells from lung tissue into CD27high and CD27low populations, ~3-fold greater proportion of the lymphocytes that had down-regulated CD27 produced IFN-γ upon in vitro stimulation than did those that maintained high CD27 expression (Fig. 7D). Thus, in combination these data indicate that the presence of cognate memory CD4+ T cells facilitates the expansion, infiltration of tumor-associated lung tissue, and effector differentiation of endogenous secondary CD8+ T cells in response to both OVA-vac and B16cOVA challenge.

**Discussion**

We examined the capacity of cognate memory CD4+ T cells to enhance the expansion of memory CD8+ T cells. We found that mice containing a memory CD4+ T cell population with the same Ag specificity as memory CD8+ T cells can significantly increase the frequency of secondary CD8+ T cells elicited after re-encountering the Ag. The increase observed is due to an increase in the proliferation of activated CD8+ T cells. Accumulation of secondary CD8+ T cells in tumor-associated lung tissue was also augmented in the presence of cognate memory CD4+ T cells, as was their differentiation state and effector activity. These enhancements coincided with an increased control of tumor outgrowth.

To assess the importance of cognate memory CD4+ T cells in the generation or expansion of memory CD8+ T cells, we utilized peptide-pulsed BMDC for immunization. Although protein-pulsed DCs have been well characterized for their ability to elicit CD4+ T cell responses (30, 32), data pertaining to the capacity of peptide-pulsed BMDC to expand CD4+ T cells are limited. The major issues of concern associated with using peptide-pulsed BMDC to elicit CD4+ T cells included the fact that MHC class II-restricted peptides generally exhibit lower MHC-binding affinity than MHC class I-restricted peptides and therefore may not form stable complexes (33); peptides can bind MHC class II molecules in different binding cassettes compared with peptides derived from naturally processed Ags, potentially affecting the specificity of responding CD4+ T cells (34); and that BMDC grown in culture would predominantly present peptides from components of the culture medium (e.g., FBS proteins) (35). The studies presented here indicate that peptide-pulsed BMDC can effectively induce the activation and expansion of exogenous and endogenous CD4+ T cells specific for the peptide pulsed onto the immunizing BMDC. The resulting effector CD4+ T cell populations are skewed to a Th1 phenotype, secreting IL-2 and IFN-γ, which contrasts with the reports for CD4+ T cells that respond to protein-pulsed ex vivo-derived CD8α+ DC (30, 32) but is consistent with the fact that CD40L-stimulated BMDC secrete IL-12p40 (Ref. 36 and our unpublished data). Importantly, the enhanced secondary CD8+ T cell responses induced after challenge with full-length OVA (expressed either by recombinant vaccinia or transfected tumor cells) generated in mice primed with OVA323-pulsed BMDC strongly suggest that at least a portion of the OVA323-specific CD4+ T cells can also recognize OVA323 that is naturally processed and presented by endogenous DCs.

The data presented in this study indicate an underappreciated role for cognate memory CD4+ T cells in the expansion of a secondary CD8+ T cell response. Previous work has indicated that immunization with both class I- and class II-restricted peptides derived from tumor Ag generated more profound immunity to tumor compared with immunization with class I-restricted epitopes alone (24, 25). However, others have argued that coimmunization with MHC class II-restricted peptides derived from irrelevant Ags raises CD4+ T cell responses that are equivalent in their support to those that are specific for tumor Ags (37). Our results are clearly consistent with the former position, in that greater protection against tumor outgrowth was observed after immunization with BMDC pulsed with class II-restricted peptides derived from the tumor Ag. We have expanded beyond the preceding observation in several ways. Coimmunization with OVA257 and OVA323 had little impact on the frequency of OVA257-specific memory CD8+ T cells generated. In contrast, the increased OVA257-specific CD8+ T cell response seen in OVA257/OVA323-primed mice, as compared with mice primed with OVA257/HBC128, after rechallenge with OVA-vac is attributable to enhanced expansion of memory CD8+ T cells. Although unlikely, it was possible that the enhanced CD8+ T cell secondary responses observed from OVA323-primed mice were a function of a more potent CD4+ T cell response during the priming phase that generated memory CD8+ T cells with enhanced capacity for expansion. Two observations argue against this. First, the deletion of the CD4+ T cell population before challenge with OVA-vac returned the magnitude of the CD8+ T cell response to a level similar to that observed in HBC128-primed mice. Second, the magnitude of the secondary CD8+ T cell response attained by challenge with OVA257-vac, which would not activate cognate memory CD4+ T cells, is similar to that obtained in HBC128-primed mice. Together, these data argue that the presence of cognate memory CD4+ T cells is required for enhanced secondary CD8+ T cell responses.

CD4+ T cell-mediated help is thought to act at two major points in CD8+ T cell responses: during activation by activating DC, and the provision of cytokines that support the proliferation and survival of expanding T cells. The analysis of transferred CFSE-labeled memory CD8+ T cells 3 days after rechallenge with Ag indicated that the majority of transferred memory OT-I cells had been activated. Further, the 2-fold difference in the extent of CFSE dilution (number of divisions) observed between the transferred memory OT-I populations in OVA323- and HBC128-primed mice was consistent with the 2-fold difference in the overall magnitude
of the secondary OT-I population. Thus, it is most likely that secondary cognate CD4+ T cells augment the magnitude of the secondary CD8+ T cell response by enhancing the rate at which secondary CD8+ T cells can expand, not their initial activation. However, because we investigated only a single early time point in the expansion of the memory CD8+ T cells, we cannot rule out differences in the efficiency of activation contributing to differences in the apparent proliferation capacity of the secondary CD8+ T cells. Also, the frequency of cells found with each division was enhanced in mice that had been previously immunized with OVA323-pulsed BMDC, suggesting that enhanced survival of activated memory CD8+ T cells could also be a functional consequence of the presence of cognate secondary CD4+ T cells in this study.

The mechanism by which secondary CD4+ T cells could enhance the expansion and survival of secondary CD8+ T cells remains undetermined. It has been recently shown that when activated CD4+ T cells con verge with CD8+ T cells on the same DC, the CD8+ T cells are instructed, in a CD40-dependent manner, to proliferate faster; attain higher levels of IFN-γ production; and up-regulate CD25 earlier (38). Further, productive interactions between naive CD4+ T cells and DCs result in the secretion of CCL3 and CCL4 and an increase in the efficiency of recruiting CCR5-expressing naive CD8+ T cells into CD4+ T cell-CD8+ T cell-DC clusters (39). Thus, in the studies presented here, the increased frequency of OVA323-specific precursors in mice that have previously primed with OVA323 might result in a higher frequency of convolved three-cell clusters and increases in the efficiency of secondary CD8+ T cell activation and proliferation. It is a striking observation, however, that the secondary CD4+ T cells elicited in OVA323-primed mice by OVA-vac challenge provide more support than the large number of primary CD4+ T cells that are rapidly activated (T. N. J. Bullock, unpublished observation) in response to the OVA-vac challenge. This suggests that a qualitative difference between the help provided by secondary CD4+ T cells and that obtained from primary CD4+ T cells, rather than a difference in the frequency of helper CD4+ T cells, likely accounts for the enhanced CD8+ T cells secondary responses. In this regard, the production of IL-2 by activated CD4+ T cells has recently been shown to be of critical importance for the survival and expansion of primary CD8+ T cells (16, 21). However, we found that primary CD4+ T cells activated by peptide-pulsed BMDC effectively produce IL-2 (Fig. 1B), suggesting that there is likely to be little difference in the IL-2 production capacity of primary or secondary CD4+ T cells. IFN-γ secretion by cognate secondary CD4+ T cells has been shown to rescue impaired HSV-specific CD8+ T cell responses after suboptimal priming with heat shock protein complexes (23) and that primary CD4+ T cells examined in this study do not produce as much IFN-γ as BMDC-primed secondary CD4+ T cells. Therefore, it is possible that the enhanced CD8+ T cell secondary responses are a function of superior IFN-γ production by secondary CD4+ T cells, affecting either memory CD8+ T cells directly or a third party, such as the Ag-presenting DC.

We also observed a selective accumulation of OVA257-specific secondary CD8+ T cells in the lungs of tumor-bearing/challenged mice that contained cognate memory CD4+ T cells. Thus, although a 2-fold increase in the frequency of secondary OVA257-specific CD8+ T cells was usually seen in the secondary lymphoid tissues (PTLNs and spleen) of OVA323 compared with HBC128-primed mice, this difference increased to 6- to 10-fold in the lungs. We are currently determining whether this accumulation is due to enhanced proliferation of secondary CD8+ T cells in situ, which may imply a role for lung-resident effector memory CD8+ T cells, or due to trafficking of activated secondary CD8+ T cell into the lung tissue. This result is reminiscent of the enhancement to tumor infiltration by TCR-transgenic CD8+ T cells noted when TCR transgenic CD4+ T cells that were specific for the same tumor Ag were cotransferred into tumor-bearing mice (22). Together, these data suggest that activated CD4+ T cells play a critical role in either the infiltration or retention of CD8+ T cells into tumor-bearing lung. It is currently not clear whether this is due to alterations to the tumor environment (such as an IFN-γ-mediated increase in expression of MHC class I molecules on tumor cells) causing enhanced CD8+ T cell retention at the tumor site or to an alteration in the expression of chemokine/integrins or their receptors leading to enhanced trafficking to the tumor-associated tissue (40–42). Preliminary data obtained with blocking CXCR3-specific Abs suggest that the latter possibility may be true (T. N. J. Bullock, unpublished observation). It would be expected that the increased CD8+ T cell accumulation in the lung tissue plays an important role in the observed increase in tumor control.

The presence of cognate memory CD4+ T cells also appeared to modify the phenotype of the secondary CD8+ T cells. Secondary CD8+ T cells from mice that had been primed with OVA257/OVA323-pulsed BMDC consistently expressed lower levels of CD27 than those that expanded in mice primed with OVA257/HBC128-pulsed BMDC. This was particularly apparent in lung tissues. CD27 down-regulation has been associated with the differentiation of effector CD8+ T cells in both mice and humans (43–46), and we typically found that IFN-γ-secreting secondary OVA257-specific CD8+ T cells had low CD27 expression levels (Figs. 5B and 7C). Engagement of CD70 (the ligand for CD27) is thought to be necessary for CD27 down-regulation, and CD70 expression on DC is tightly controlled by either CD40L- or TLR-mediated stimulation (6, 47, 48), suggesting that secondary CD4+ T cells might enhance the expression of CD70. This may be indicative of enhanced activation of DC in general and provides an additional explanation for the enhanced CD8+ T cell secondary responses evident in mice containing cognate memory CD4+ T cells.

The data presented here clearly indicate that the presence of cognate secondary CD4+ T cells provided a clear advantage in controlling tumor outgrowth. This was evident in both s.c. and metastatic lung disease models. Therefore, this study provides a strong rationale for the inclusion of tumor-Ag-derived, as opposed to generic, MHC class II-restricted peptides in peptide-based vaccines and should provide further impetus for identifying such peptides from the various tumor Ags identified to date (49).

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Disclosures
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References


