

# Antigen-specific CD4<sup>+</sup> T-Cell Help Is Required to Activate a Memory CD8<sup>+</sup> T Cell to a Fully Functional Tumor Killer Cell<sup>1</sup>

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## Abstract

Although the importance of CD4<sup>+</sup> T-cell help for generation of an effective CD8<sup>+</sup> effector cytotoxic T cell (CTL) response is well established, the role of T-cell help in the activation of memory T cells to become fully functional tumor killer cells is undefined. Using synthetic peptide immunizations corresponding to the major CTLs and T-helper epitopes of ovalbumin, adoptive transfers of ovalbumin-specific memory CTLs (mCTLs), and ovalbumin as the tumor-specific antigen in a mouse tumor model, we have determined that T help is essential for the activation of mCTLs to kill tumors. Our data show that T-helper cells specific for the tumor-associated antigen are required for the reactivation of mCTLs by antigen presented indirectly from tumor. In contrast, effector CTLs do not need T help to kill tumors. These results have implications for induction of tumor immunotherapy by immunization.

## Introduction

CD8<sup>+</sup> mCTLs<sup>3</sup> play a major role in the elimination of viruses and tumors (1–3). Before mCTLs can eliminate viruses and tumors, these cells are required to proliferate and acquire effector function. The role CD4<sup>+</sup> T-helper cells play in the induction of CD8<sup>+</sup> effector T cells from naïve precursors is known (4–7). However the role of T-helper cells in the activation of mCTLs when the antigen is presented by the tumor is not established. To dissect the role of T-helper cells in the activation of memory T cells, we used adoptive transfer of tumor antigen-specific mCTLs in a tumor protection model in mice where tumor protection is solely dependent on the presence of active antigen-specific CD8<sup>+</sup> T cells. We have shown in this study that tumor protection by memory T cells is observed only if tumor antigen-specific T help is provided. The requirements for the reactivation of memory T cells are important not only for understanding the mechanisms of generation of functional CTLs but also for the design of better vaccines.

## Materials and Methods

**Mice.** Ovalbumin (SIINFEKL) T-cell receptor transgenic OT-1 Rag<sup>-/-</sup> mice on a C57BL/6J background (henceforth referred to as OT-1 mice; Ref. 8) were a kind gift from Dr. William Heath (Walter and Eliza Hall Institute, Melbourne, Australia). Specific pathogen-free C57BL/6J mice (female, 6–8 weeks old) were bought from Animal Resources Centre (Perth, Australia) and were kept at the Princess Alexandra Hospital Biological Research Facility under germ-free conditions.

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<sup>3</sup> The abbreviations used are: mCTL, memory cytotoxic T cell; SIINFEKL, major H-26 CTL epitope in ovalbumin; OVT, ovalbumin T-helper peptide; KLH, keyhole limpet hemocyanin; eCTL, effector CTL; CFA, complete Freund's adjuvant.

**Synthetic Peptides.** Synthetic peptides (Auspep Ltd., Melbourne, Australia) were checked for purity and toxicity as described earlier (9).

**Adoptive Transfer of OT-1 Transgenic T Cells.** mCTLs were generated in T cell reconstituted Rag<sup>-/-</sup> mice as described previously (10). Briefly, a single cell suspension of pooled lymph node cells (axillary, brachial, cervical, inguinal, periaortic, mediastinal, and mesenteric) were prepared from OT-1 mice. CD8<sup>+</sup> T cells from the preparations were shown to be ~90% SIINFEKL tetramer specific by flow cytometry. OT-1 cells (5 × 10<sup>5</sup>) in 0.1 ml of PBS were injected into tail vein of syngeneic Rag<sup>-/-</sup> mice, and these mice were immunized 1 day later with 50 μg of the K<sup>b</sup> CTL epitope peptide of ovalbumin (SIINFEKL) and Complete Freund's adjuvant s.c. on the tail and in the scuff of the neck.

**Flow Cytometric Analysis.** SIINFEKL-specific tetramers labeled with phycoerythrin were provided by the National Institute of Allergy and Infectious Disease Tetramer Core Facility (Atlanta, GA). Allophycocyanin conjugate CD8, CD44-conjugated biotin, and streptavidin-conjugated fluorescein isothiocyanate were purchased from PharMingen (San Diego, CA). Mice were sacrificed at the time points indicated, and single cell suspensions were prepared from individual spleens and lymph nodes. Cells were stained at 4°C for 30 min in PBS containing 0.1% BSA and 0.1% NaN<sub>3</sub> and analyzed by flow cytometry, acquiring 10,000–50,000 live cells/sample. The data were acquired using a Becton Dickinson FACSCaliber Flow cytometer and were analyzed by using Cellquest (Becton Dickinson) and FLOWJO (TreeStar, San Carlos, CA) programs.

**Tumor Challenge Experiments.** Tumor challenge experiments were performed as described previously (11). Briefly, mice received s.c. injections in the neck scuff with the ovalbumin gene-transfected tumor cell line EG7.Ova (12, 13) or the parent EL4 tumor cell line, and 10–15 days after the tumor challenge, mice were killed and tumor weights recorded.

**IFN-γ ELISPOT Assays.** The assays were performed using a previously published method (3). Briefly, to quantify ovalbumin-specific CD8<sup>+</sup> T cells, CTL epitope peptide (SIINFEKL) was added to plates at a concentration of 1 μg/ml, and the plates were incubated for 16 h. To quantify ovalbumin-specific CD4<sup>+</sup> T-helper cells, the ovalbumin I-A<sup>b</sup>-restricted T-helper epitope peptide (OVT amino acid sequence = ISQAVHAAHAEINEAGR; Ref. 14) was added to plates at a concentration of 8 μg/ml and incubated for 40 h. Spot numbers/10<sup>6</sup> cells were reported.

## Results

**Generation of Ovalbumin-specific Memory CD8<sup>+</sup> T Cells.** To study the role of T-helper cells in the activation of mCTLs, control of growth of a murine tumor-expressing ovalbumin as a tumor-specific antigen was used as a model. Tumor control in this model was previously shown to be dependent on functional CD8<sup>+</sup> tumor antigen-specific T cells, and CD4<sup>+</sup> T cells have no effect on the growth of tumors (11). Therefore this method can be used to determine the presence of active CD8<sup>+</sup> T cells *in vivo*. Ovalbumin is a well-studied protein, and its CTLs and T-helper epitopes are known. To generate sufficient mCTLs to be detected by flow cytometric analysis, adoptive transfer of naïve T cells from ovalbumin-specific T-cell receptor transgenic mice (OT-1; Ref. 8) into Rag<sup>-/-</sup> mice, which were subsequently immunized with ovalbumin, was undertaken. Of the naïve lymph node and spleen CD8<sup>+</sup> T cells from donor OT-1 mice, 89 and 62%, respectively, are SIINFEKL specific, as measured using

SIINFEKL MHC class I tetramers (data not shown), in agreement with the published data measured using antibodies against V $\alpha$ 2 chain (8). These OT-1 lymph node and spleen cells were adoptively transferred to syngeneic Rag<sup>-/-</sup> mice. Recipients were immunized 1 day after transfer with SIINFEKL/CFA to generate large numbers of SIINFEKL-specific activated CTL. To determine the time course of T-cell activation and the development of mCTLs, T lymphocytes from mice sacrificed at days 7, 14, or 21 after transfer were examined for the expression of CD8 and the memory T-cell marker CD44 (15). After 21 days, a high percentage of CD8<sup>+</sup> cells are CD44 positive (Fig. 1), indicating that the majority of surviving CD8<sup>+</sup> T cells have a memory phenotype.

**CD8<sup>+</sup> Memory T Cells Need Antigen-specific CD4<sup>+</sup>T-helper Cells to Achieve Tumor Protection.** Rag<sup>-/-</sup> mice were adoptively transferred with OT-1 cells and immunized with minimal CTL peptide

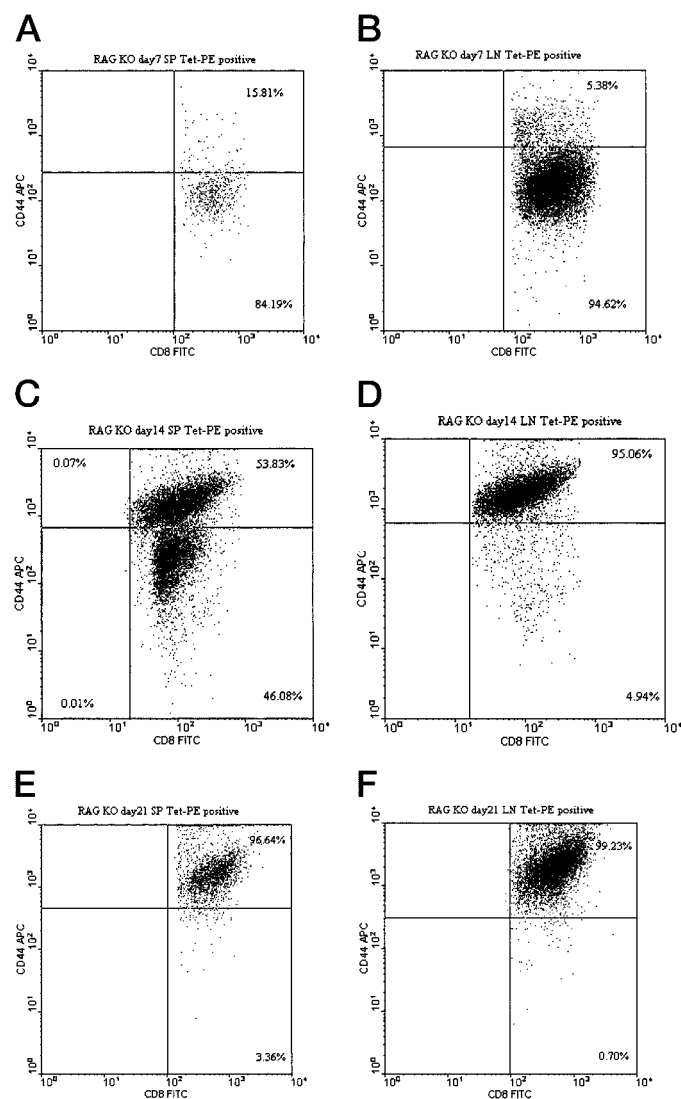


Fig. 1. OT-1 cells transferred to Rag<sup>-/-</sup> mice and immunized with the minimal CTL epitope peptide (SIINFEKL) and CFA adjuvant acquire a CD44<sup>high</sup> memory phenotype with time. OT-1 lymph node cells ( $5 \times 10^5$ ) were transferred to Rag<sup>-/-</sup> mice through tail vein injection, and mice were immunized with 50  $\mu$ g of SIINFEKL peptide in CFA 1 day later. At day 7 (A and B), day 14 (C and D), and day 21 (E and F), mice were sacrificed, and splenocytes (A, C, and E) or lymph node cells (B, D, and F) were isolated and analyzed. The percentage of CD44<sup>high</sup>, SIINFEKL-specific CD8<sup>+</sup> T cells was assessed by three-color flow cytometry staining with SIINFEKL MHC class I tetramer and antibodies to CD8 and CD44. Plots shown are gated on the SIINFEKL tetramer-positive population. Values are mean percentages of CD44<sup>+</sup> cells within the SIINFEKL tetramer-positive population.

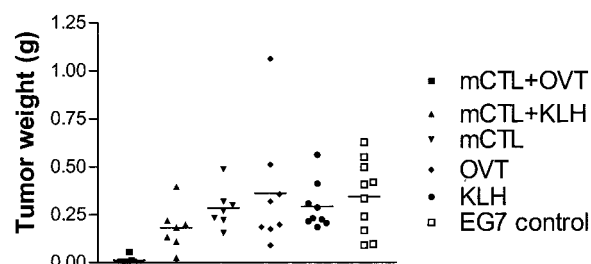


Fig. 2. CD8<sup>+</sup> memory T cells need antigen-specific CD4<sup>+</sup> T helper cells to achieve tumor protection. Ovalbumin CTL epitope (SIINFEKL)-specific T cells were parked in Rag<sup>-/-</sup> mice for 42 days to generate mCTL and then adoptively transferred ( $1 \times 10^6$  cells) to C57BL/6 mice (indicated as  $\blacksquare$ ,  $\blacktriangle$ , and  $\nabla$ ). Two groups of recipient C57BL/6J mice were immunized 8 days prior with 50  $\mu$ g of OVT in incomplete Freund's adjuvant (indicated as  $\blacksquare$  and  $\blacklozenge$ ) or 20  $\mu$ g keyhole limpet hemocyanin (KLH) protein as control in incomplete Freund's adjuvant (indicated as  $\blacktriangle$  and  $\bullet$ ). Mice were challenged with  $3 \times 10^6$  ovalbumin-expressing tumor cells (EG7) in the scruff of the neck 1 day after adoptive transfer. Normal C57BL/6 control mice (indicated as EG7 control,  $\square$ ) were challenged with EG7 without any treatment. Significance of differences was measured using Student's *t* test. mCTL+OVT ( $\blacksquare$ ) versus EG7 control ( $\square$ ):  $P = 0.0004$ . mCTL+OVT ( $\blacksquare$ ) versus mCTL+KLH ( $\blacktriangle$ ):  $P = 0.0080$ . mCTL+OVT ( $\blacksquare$ ) versus OVT ( $\blacklozenge$ ):  $P = 0.0113$ . mCTL+OVT ( $\blacksquare$ ) versus mCTL ( $\nabla$ ):  $P = 0.0006$ . mCTL+OVT ( $\blacksquare$ ) versus KLH ( $\bullet$ ):  $P = 0.0010$ . mCTL+KLH ( $\blacktriangle$ ) versus KLH ( $\bullet$ ):  $P = 0.0039$ .

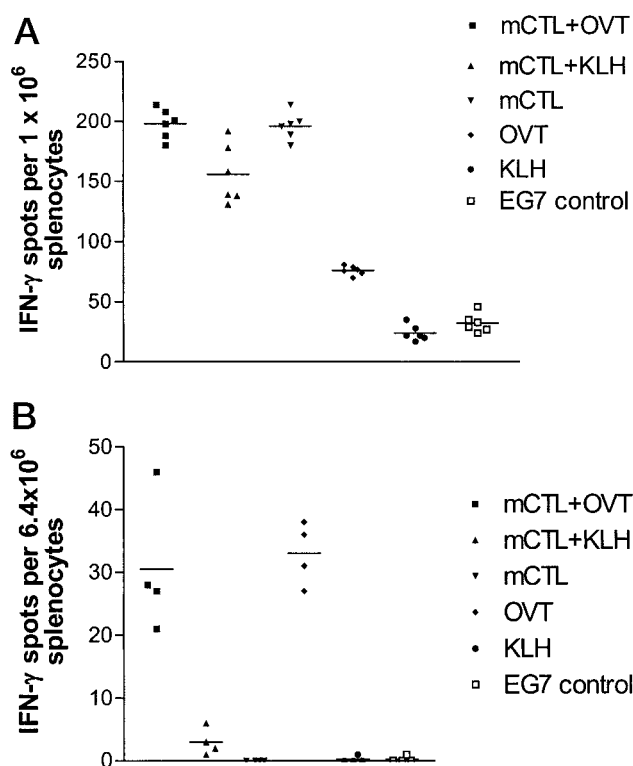


Fig. 3. Absence of tumor protection in mice without antigen-specific T-helper cells is not because of lower levels of tumor antigen (SIINFEKL)-specific CD8<sup>+</sup> T cells. mCTLs ( $1 \times 10^6$ ) were transferred to groups of C57BL/6 mice ( $\blacksquare$ ,  $\blacktriangle$ , and  $\nabla$ ) with or without immunization 8 days prior with 50  $\mu$ g of OVT ( $\blacksquare$  and  $\blacklozenge$ ) or with 20  $\mu$ g of KLH protein control ( $\blacktriangle$  and  $\bullet$ ). C57BL/6 control group (indicated as EG7 control,  $\square$ ) was given no treatment. IFN- $\gamma$  Elispot assays were performed using the splenocytes of these mice, which were challenged *in vitro* with (A) 1  $\mu$ g/ml SIINFEKL peptide or (B) 8  $\mu$ g/ml OVA T-helper peptide to determine the presence of ovalbumin-specific CD8<sup>+</sup> CTLs and CD4<sup>+</sup> T-helper cells, respectively. Significance of differences was measured using Student's *t* test. A, mCTL+OVT ( $\blacksquare$ ) versus EG7 control ( $\square$ ):  $P < 0.0001$ . mCTL+OVT ( $\blacksquare$ ) versus KLH ( $\bullet$ ):  $P < 0.0001$ . mCTL+OVT ( $\blacksquare$ ) versus OVT ( $\blacklozenge$ ):  $P < 0.0001$ . mCTL+OVT ( $\blacksquare$ ) versus mCTL+KLH ( $\blacktriangle$ ):  $P = 0.0037$ . mCTL+KLH ( $\blacktriangle$ ) versus EG7 control ( $\square$ ):  $P = 0.0002$ . mCTL+KLH ( $\blacktriangle$ ) versus KLH ( $\bullet$ ):  $P = 0.0001$ . mCTL+OVT ( $\blacksquare$ ) versus OVT ( $\blacklozenge$ ):  $P = 0.0003$ . mCTL ( $\nabla$ ) versus EG7 control ( $\square$ ):  $P < 0.0001$ . mCTL ( $\nabla$ ) versus KLH ( $\bullet$ ):  $P < 0.0001$ . mCTL ( $\nabla$ ) versus OVT ( $\blacklozenge$ ):  $P < 0.0001$ . OVT ( $\blacklozenge$ ) versus EG7 control ( $\square$ ):  $P = 0.0002$ . OVT ( $\blacklozenge$ ) versus KLH ( $\bullet$ ):  $P < 0.0001$ . B, mCTL+OVT ( $\blacksquare$ ) versus mCTL+KLH ( $\blacktriangle$ ):  $P = 0.0141$ . mCTL+KLH ( $\blacktriangle$ ) versus KLH ( $\bullet$ ):  $P = 0.0121$ . mCTL+OVT ( $\blacksquare$ ) versus EG7 control ( $\square$ ):  $P = 0.0121$ . mCTL+KLH ( $\blacktriangle$ ) versus OVT ( $\blacklozenge$ ):  $P = 0.0010$ . OVT ( $\blacklozenge$ ) versus KLH ( $\bullet$ ):  $P = 0.0008$ . OVT ( $\blacklozenge$ ) versus EG7 control ( $\square$ ):  $P = 0.0008$ .

(SIINFEKL) as described above and were kept for total of 42 days to ensure generation of mCTLs. Memory T cells generated in these mice were then adoptively transferred to syngeneic C57BL/6J mice that had been immunized 8 days previously with OVT to generate ovalbumin-specific T help or with KLH as a source of nonspecific T help. Mice were challenged with EG7 tumor 1 day after the adoptive transfer of mCTLs. Tumor protection was observed only in mice that had received mCTLs and also been immunized with OVT (Fig. 2). No tumor protection was seen in mice that received mCTLs and were unimmunized or immunized with an irrelevant antigen (KLH). Mice immunized with OVT that were not given mCTLs did not demonstrate tumor protection, confirming that CD8<sup>+</sup> T cells specific for ovalbumin are necessary for tumor protection in this assay. Thus, tumor antigen-specific CD4<sup>+</sup> T-helper cells are required for activation of CD8<sup>+</sup> mCTL capable of preventing tumor growth.

**Absence of Tumor Protection in Mice without Antigen-specific T-helper Cells Is Not because of Lower Levels of Tumor Antigen (SIINFEKL)-specific CD8<sup>+</sup> T Cells.** To determine whether comparable levels of antitumor CD8<sup>+</sup> T cells were present in mice whether or not they were immunized with OVT peptide after passive transfer, IFN- $\gamma$  Elispot was used to assay splenocytes for SIINFEKL-specific CD8<sup>+</sup> T cells. All groups that received mCTLs had comparable levels of SIINFEKL-specific CD8<sup>+</sup> T cells (Fig. 3A). Therefore, inability of mCTLs to eradicate tumor in the absence of tumor-specific T-helper

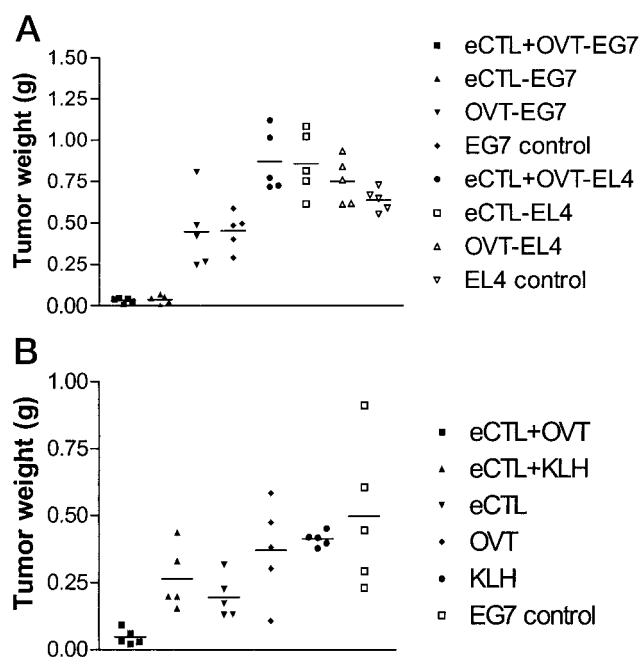


Fig. 4. In contrast to mCTLs, eCTLs do not need T help to kill tumor. eCTLs ( $1 \times 10^6$ ) generated from RAG<sup>-/-</sup> mice were transferred at (A) day 7 or (B) day 14 to C57BL/6 mice that had been immunized 8 days earlier with 50  $\mu$ g of OVT or as control 20  $\mu$ g of KLH. C57BL/6 mice without any treatment were used as controls (EG7 and EL4 controls). Ovalbumin-expressing tumor cells (EG7;  $3 \times 10^6$ ) were injected under scruff of the neck of some groups of mice at (A) day 7 or (B) day 14. The parent tumor cell line EL4 that does not contain ovalbumin gene was injected into other groups of mice at day 7 (A) as nonspecific tumor control. Significance of differences was measured using Student's *t* test. A, eCTL+OVT-EG7 (■) versus OVT-EG7 (▼):  $P = 0.0183$ . eCTL+OVT-EG7 (■) versus EG7 control (◆):  $P = 0.0013$ . eCTL-EG7 (▲) versus OVT-EG7 (▼):  $P = 0.0218$ . eCTL-EG7 (▲) versus EG7 control (◆):  $P = 0.0020$ . eCTL+OVT-EG7 (■) versus eCTL+OVT-EL4 (●):  $P = 0.0006$ . eCTL-EG7 (▲) versus eCTL-EL4 (□):  $P = 0.0010$ . OVT-EG7 (▼) versus OVT-EL4 (△):  $P = 0.0247$ . B, eCTL+OVT (■) versus eCTL (▼):  $P = 0.0203$ . eCTL+OVT (■) versus EG7 control (□):  $P = 0.0227$ . eCTL+KLH (▲) versus KLH (●):  $P = 0.0304$ . eCTL+OVT (■) versus eCTL+KLH (▲):  $P = 0.0171$ . eCTL+OVT (■) versus eCTL (▼):  $P = 0.0203$ . eCTL+OVT (■) versus OVT (◆):  $P = 0.0158$ . eCTL+OVT (■) versus KLH (●):  $P < 0.0001$ . eCTL (▼) versus OVT (◆):  $P = 0.0498$ . eCTL (▼) versus KLH (●):  $P = 0.0066$ . eCTL (▼) versus EG7 control (□):  $P = 0.0465$ .

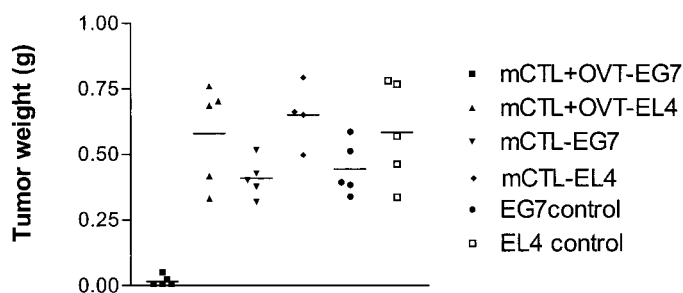


Fig. 5. Study of the long-lived memory CD8<sup>+</sup> T cells generated in C57BL/6J mice. Ovalbumin CTL epitope (SIINFEKL)-specific OT-1 cells were adoptively transferred ( $5 \times 10^5$  cells/mouse) to syngeneic C57BL/6 mice (groups ■, ▲, ▼, and ◆); mice were immunized with SIINFEKL/CFA to activate ovalbumin-specific CD8<sup>+</sup> T cells, and the mice were kept for 100 days to ensure that all transferred OT-1 are of long-lived memory phenotype before challenging with tumor. Eight days before tumor challenge, two groups of mice (■, ▲) were immunized with 50  $\mu$ g of OVT in incomplete Freund's adjuvant as the adjuvant. Untreated C57BL/6 mice were also challenged with tumor as negative control (● and □). Mice were challenged under scruff of the neck with  $3 \times 10^6$  ovalbumin-expressing tumor cell line (EG7) or with the parent tumor line (EL4) as control. Significance of differences was measured using Student's *t* test. mCTL+OVT-EG7 (■) versus mCTL-EG7 (▼):  $P = 0.0004$ . mCTL+OVT-EG7 (■) versus EG7 control (●):  $P = 0.0007$ . mCTL+OVT-EG7 (■) versus mCTL+OVT-EL4 (▲):  $P = 0.0021$ .

cells is not attributable to a difference in mCTL numbers. In mice not transferred with SIINFEKL-specific CTL, low CTL activity is nevertheless observed, possibly attributable to specific CTLs being induced by the tumor cell challenge. Any CTLs induced, however, failed to stop tumor growth. As expected, ovalbumin-specific CD4<sup>+</sup> T-helper cells were demonstrated to be induced by OVT immunization as measured in an IFN- $\gamma$  Elispot assay using the T-helper epitope peptide as the *in vitro* stimulant (Fig. 3B).

**In Contrast to mCTLs, eCTLs Do Not Need T Help to Kill Tumor.** To confirm that cognate T-helper cells are only required for tumor protection when mCTLs rather than eCTLs are present, C57BL/6J mice receiving OT-1 cells were immunized with OVT peptide and challenged with EG7 tumor cells 7 or 14 days after immunization. No antigen-specific T-helper cells were required for tumor killing 7 days after immunization (Fig. 4A). Growth of EL4, the ovalbumin-negative parent tumor cell line of EG7, was not influenced by immunization, confirming that the tumor protection observed was ovalbumin specific. However, when tumor challenged at 14 days after immunization, at which point of time most ovalbumin-specific eCTLs may have died, ovalbumin-specific T-helper cells were needed for tumor protection (Fig. 4B).

**Study of the Long-lived CD8<sup>+</sup> Memory T Cells Generated in C57BL/6J Mice.** To determine whether the results obtained using Rag<sup>-/-</sup> mice to generate mCTLs were identical to those where mCTLs were generated directly in C57BL/6J mice, tumor protection was sought as follows. One day after transfer of OT-1 T cells, C57BL/6J mice were immunized with SIINFEKL/CFA to activate ovalbumin-specific CD8<sup>+</sup> T cells. After 100 days, the remaining SIINFEKL-specific cells were taken as long-lasting mCTLs. These mice were then immunized with T helper peptide (OVT) to generate ovalbumin-specific T help and challenged with EG7 or EL4 tumor 1 week after immunization. Mice immunized with OVT peptide were protected against tumor challenge, whereas the control mice are not protected as observed with mCTLs generated in Rag<sup>-/-</sup> mice (Fig. 5). Furthermore, killing was antigen specific because no tumor protection was seen when challenged with the parent tumor EL4, which does not contain ovalbumin. Thus, ovalbumin-specific T help is required for mCTL activation, whether memoryCD8<sup>+</sup> T cells were generated *in situ* or transferred passively

## Discussion

Although the requirement of T-help in the activation of a naïve CD8<sup>+</sup> T cell into an eCTL is established (4–7, 16), the role of T-cell help in the activation of a memory T cell into a fully functional antitumor killer cell is not clear at present. Here, we used a mouse tumor model where antigen-specific CD8<sup>+</sup> T cells were shown to be necessary and sufficient for tumor protection (11) and adoptive transfer of antigen-specific memory T cells to study the requirements for activation of the memory T cells. The results show that CD8<sup>+</sup> antitumor memory T cells need the presence of antigen-specific CD4<sup>+</sup> T-helper cells to be activated to achieve tumor protection. In contrast, CD8<sup>+</sup> effector T cells were shown not to need T help for tumor killing.

Other studies using adoptive transfer of influenza-specific CD8<sup>+</sup> memory T cells mixed with influenza virus into SCID mice have shown that memory cells can be activated into effector cells in the absence of CD4<sup>+</sup> T-helper cells and B cells, although memory T cells without the virus either preincubated with the CTL peptide before adoptive transfer or injection of the peptide after the adoptive transfer failed to generate CTLs *in vivo* as measured in a chromium release assay (17). Furthermore, in another study where memory CD8<sup>+</sup> T cells and different levels of memory CD4<sup>+</sup> T-helper cells from latently murine cytomegalovirus-infected mice were adoptively transferred into irradiated murine cytomegalovirus, infected recipients showed that CD4<sup>+</sup> T cells have no effect on the virus killing capacity of CD8<sup>+</sup> T cells in the recipient mice (18). The CD4<sup>+</sup> T-cell-independent activation of CD8<sup>+</sup> T cells observed in these studies could be because of direct stimulation of dendritic cells by the virus bypassing the activation through CD4<sup>+</sup> T cells or because of the effect of very high numbers of memory CD8<sup>+</sup> T cells transferred.

High memory CTL numbers can be achieved in viral infections but numbers are more limited after immunization with peptides/proteins. Therefore, in vaccinations, inclusion of an antigen-specific T-helper component may be essential. We deliberately avoided using viruses as a way of generating large amounts of mCTL because viruses can perturb the immune system. We have used synthetic peptides that are devoid of DNA to dissect out the mechanisms. Synthetic peptide immunizations do not induce large numbers of memory cells, however, and it makes it harder to track the fate of these cells. Therefore, we have used OT-1 ovalbumin T-cell receptor transgenic mouse T-cell adoptive transfer into Rag<sup>-/-</sup> mice as previously described (19) to generate memory cells. We have also transferred OT-1 cells into normal syngeneic mice to validate the use of Rag<sup>-/-</sup> mice in the generation of large numbers of mCTL. The results show that the mCTL generated in Rag<sup>-/-</sup> mice behaves similar to CTL generated in normal mice.

In summary this study shows that to activate CD8<sup>+</sup> memory T cells to kill tumors, tumor antigen-specific CD4<sup>+</sup> T-helper cells are re-

quired. These results have implications for induction of tumor immunotherapy by immunization.

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