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Development of Antigen-Specific CD8⁺ CTL in MHC Class I-Deficient Mice through CD4 to CD8 Conversion¹

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CD8⁺ CTL are the predominant tumoricidal effector cells. We find, however, that MHC class I-deficient mice depleted of CD8⁺ T cells are able to mount an effective antitumor immunity after immunization with fused dendritic/tumor cells. Such immunity appears to be mediated by the generation of phenotypic and functional CD8⁺ CTL through CD4⁺ to CD8⁺ conversion, which we have demonstrated at the single cell level. CD4⁺ to CD8⁺ conversion depends on effective in vivo activation and is promoted by CD4⁺ T cell proliferation. The effectiveness of this process is shown by the generation of antitumor immunity through adoptive transfer of primed CD4⁺ T cells to provide protection against tumor cell challenge and to eliminate established pulmonary metastases. The Journal of Immunology, 2004, 172: 7848–7858.

The ability of CD4⁺ and CD8⁺ T cells to recognize tumor-specific or associated Ags is the foundation of antitumor immunity. Much research has been devoted to the study of CD8⁺ T cells, given the fact that most nonhemopoietic tumors express only MHC class I molecules and that predominant tumoricidal effector cells are CD8⁺ CTL. Therefore, as most tumors do not express MHC class II, CD4⁺ T cells have been assumed to provide help to B cells and CD8⁺ T cells. CD4⁺ T cells are known to be required for priming, generation, and maintenance of CTL (1–6). In the priming phase, CD4⁺ T cells activate APCs so that the latter acquire the capacity to stimulate CD8⁺ T cells (7–9). CD4⁺ T cells also function to maintain the numbers and cytotoxic capacity of CD8⁺ T cells and promote the infiltration of CD8⁺ T cells in tumor (5, 6). There is increasing evidence, however, that CD4⁺ T cells play a more direct role, beyond delivery of assistance in the generation of antitumor immunity (10). During the effector phase, CD4⁺ T cells mediate tumor rejection by a number of mechanisms, including: 1) activation of eosinophils and macrophages to produce both tumoricidal superoxide and NO (1); and 2) mediation of tumor rejection by indirect effects of IFN-γ (11, 12). Hung et al. (1) have also shown that immunization of CD4⁺/⁻ mice with B16 melanoma cells transplanted with GM-CSF is ineffective in priming a systemic immune response capable of rejecting the tumor challenge. Unexpectedly, a significant fraction of similarly immunized CD8⁺/⁻ mice mounted a successful tumor rejection, suggesting a more crucial role for CD8⁺ T cells in antitumor immunity. Rather than simply providing help for CD8⁺ T cells, CD4⁺ T cells express both Th1 and Th2 cytokines and recruit other antitumor effector cells in addition to CD8⁺ T cells. These properties, however, are still limited to the assistance delivered or killing mediated by CD4⁺ T cells. Several questions still remain unanswered, including that of why abrogation of CD4⁺, but not CD8⁺, T cells compromises antitumor immunity (1–6). Furthermore, it is not clear how the depletion of CD4⁺ T cells affects the maintenance of CD8⁺ T cells in the tumor. To address these issues, we have embarked on an investigation to dissect the role of CD4⁺ and CD8⁺ T cells in antitumor immunity by using MHC class I and/or class II knockout mice. We show that CD4⁺ T cells primed by fusions of dendritic cells (DC) and tumor cells in MHC class I knockout mice proliferate and convert into phenotypic and functional CTL, thus linking CD4⁺ T cells directly to CTL. Moreover, we were able to demonstrate that adoptive transfer of primed CD4⁺ T cells in vivo can confer sufficient antitumor immunity to reject tumor challenge and to treat established pulmonary metastases.

Materials and Methods

Mice
Six- to 8-wk-old C57BL/6 wild-type (WT), MHC class I (β₂m⁻/⁻), class II (Abh⁻/⁻), and class I and II (β₂m⁻/⁻/Abh⁻/⁻) knockout mice were obtained from Taconic Farms (Germantown, NY). The β₂m⁻/⁻ mice with a homozygous mutation in the gene for β₂m lack MHC class I expression and are, thus, devoid of CD8⁺ T cells (13). The Abh⁻/⁻ mice with a homozygous mutation in the gene for abh abrogate MHC class II expression and are, thus, devoid of CD4⁺ T cells (14). Accordingly, β₂m⁻/⁻/Abh⁻/⁻ mice lack both MHC class I and II and are, thus, devoid of CD4⁺ and CD8⁺ T cells (15). β₂m⁻/⁻ and Abh⁻/⁻ mice were developed on C57BL/6 background. The MUC1 transgenic mice (MUC1.Tg, a kind gift from Dr. S. J. Gendler, Mayo Clinic, Scottsdale, AZ) expressing MUC1 at a level similar to that found in humans (16) were congenic on the C57BL/6 background at n > 10. The mice were maintained in microisolation cages under specific pathogen-free conditions, and PCR was performed routinely to identify MUC1.Tg-positive mice in the colony. Age- and sex-matched mice were used for all experiments.

Cell preparation
Murine MC38 colon adenocarcinoma cells were stably transfected with a MUC1 cDNA (MC38/MUC1) (17, 18) and murine B16 melanoma cells

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4 Abbreviations used in this paper: DC, dendritic cell; β₂m, β₂-microglobulin; DP, double positive; LCMV, lymphocytic choriomeningitis virus; LNC, lymph node cell; Tg, transgenic; WT, wild type.
with a MUC1 cDNA (B16/MUC1) (19). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. DC were obtained from bone marrow culture of WT C57BL/6 mice and were cultured in 20 ng/ml murine rGM-CSF medium (Sigma-Aldrich, St. Louis, MO) for 5 days. The purified T-LNC were added to MC38/MUC1 cells in the presence of 50% polyethylene glycol (Sigma-Aldrich), as described previously (3). The fused cells (FC/MUC1) were used for immunization.

Flow cytometry

Mice were immunized twice s.c. on days 0 and 7 with FC/MUC1 cells (5 × 10⁶). Control mice were injected with PBS. On day 14, the draining lymph node cells (LNC) were isolated and cultured with 5 U/ml purified MUC1 Ag (19). The supernatants were added to the cell culture. At the indicated time, the T-LNC were collected by passing LNC through nylon wool and dual stained with FITC-conjugated anti-CD4 (clone H129.19) mAb and PE-conjugated anti-CD8 (clone 53-6.7) mAb (BD PharMingen, San Diego, CA) for 30 min on ice. The cells were washed, fixed, and analyzed by FACSscan (BD Biosciences, Bedford, MA) with CellQuest analysis software.

Cell sorting

The β₃m⁻/⁻ mice were immunized twice s.c. on days 0 and 7 with FC/MUC1 cells (5 × 10⁶). Control mice were injected with PBS. On day 14, the draining LNC were collected and passed through nylon wool to deplete B cells and APCs. The single T-LNC were dual stained with FITC anti-CD4 mAb and PE anti-CD8 mAb for 30 min on ice. The stained T-LNC subsets were sorted into separate tubes by MoFlo (Cytomation, Fort Collins, CO) with Summit v3.0 analysis software. The CD4⁺ T cells were sorted again, and the purity was assessed by real-time RT-PCR and/or FACS analysis. The repeatedly sorted CD4⁺ T-LNC were used for analysis of CD8 mRNA expression with real-time or single cell RT-PCR. In the kinetic studies of CD4⁺ conversion, the resorted CD4⁺ T cells (1 × 10⁶) were cultured in complete RPMI 1640 medium with 5 U/ml purified MUC1 Ag and syngeneic DC (1 × 10⁶). On day 5, 5L-2 (20 U/ml; BD Labware, Bedford, MA) was added to the cell culture. On days 0, 2, 4, 6, 8, 10, and 12, the CD4⁺ T-LNC, after being passed through nylon wool to remove DC, were stained with FITC anti-CD4 and PE anti-CD8 for 30 min on ice. The stained T-LNC were sorted into separate tubes by MoFlo (Cytomation, Fort Collins, CO) with Summit v3.0 analysis software. The CD4⁺ T cells were sorted again, and the purity was assessed by real-time RT-PCR and/or FACS analysis.

Real-time RT-PCR

One-step reverse transcription, amplification, and data collection were performed by the Cepheid Smart Cycler system (Cepheid, Sunnyvale, CA). Reverse transcription was conducted at 50°C for 20 min. The standard amplification protocol consists of an initial activation of hot start Taq DNA polymerase at 95°C for 15 min, followed by melting step at 94°C for 15 s, annealing at 72°C for 10–30 s, and extension at 72°C for 10–30 s. Temperature ramp was constant at 20°C/s. Measurements of SYBR Green fluorescence were taken at the end of the extension phase at 72°C. Species were amplified with following oligonucleotide pairs: murine CD4 primer (5'-TCC TTC CCA CTC AAC-3' and 5'-AAG CGA GAC CTG GGT TAG CT-3', 200 bp) or CD8 primer (5'-GCT CAG TCA TCA GCA ACT CG-3' and 5'-ATC ACA GCA GGA GTC CAA TC-3', 200 bp). PCR-amplified products were analyzed on 1.5% agarose gel.

Single cell RT-PCR

Lymph nodes were collected from FC/MUC1-immunized mice and teased into single cells. T-LNC were purified through nylon wool, and CD4⁺ and CD8⁺ T cells were selected by single cell sorting with anti-CD4 FITC and CD8 PE staining. The cells were kept cool during isolation. A single CD4⁺ T cell, after repeated cell sorting, was deposited into 96-well V-bottom plates with RT-PCR buffer containing RNase inhibitor. Single cell RT-PCR was conducted using the Qiagen OneStep RT-PCR kit (Qiagen). Briefly, the single cell was thawed and transferred into microfuge tubes with single cell RT-PCR master mix containing 10 µl of 5× Qiagen One-step RT-PCR buffer, HPLC-purified primer, 2 µl of dNTP mix (containing 10 nM each dNTP), 2 µl of Qiagen OneStep RT-PCR enzyme mix, 5–10 U of RNase inhibitor, and RNase-free water. Reverse transcription was conducted at 50°C for 30 min. The protocol for PCR amplification consisted of an initial activation of hot start Taq polymerase at 95°C for 15 min, followed by melting at 94°C for 30 s, annealing at 60°C for 35 s, and extension at 72°C for 30 s. The primers were used for single PCR amplification: murine CD4 primer (5'-TCC TTC CCA CTC AAC TTT GC-3' and 5'-AAG CGA GAC CTG GGT TAG CT-3', 200 bp) or CD8 primer (5'-GCT CAG TCA TCA GCA ACT CG-3' and 5'-ATC ACA GCA GGA GTC CAA TC-3', 200 bp). For dual amplification, the following murine CD8 primer was used: 5'-TCT GTC GTG CCA GCA TTT CTT C-3' and 5'-CCT-TCC-TGT-CTG-ATC-AGG-3', 292 bp (Life Technologies, Rockville, MD). PCR-amplified products were analyzed on 1.5% agarose gel.

31 Cr cytotoxicity assay

Murine MC38, MC38/MUC1, B16, B16/MUC1 tumor cells, and human MCF-7 breast cancer cells (2 × 10⁵ cells/well) were prelabeled with 31Cr for 60 min at 37°C. The T cells were distributed in 96-well V-bottom plates at the indicated target concentrations and coincubated with targets for 5 h at 37°C. The Ab-blocking assays, the target cells were incubated with anti-MHC class I mAb (M1/42.3.9.8) for 30 min at 4°C before addition of the effector cells. For anti-CD4 or anti-CD8 blocking, the effector cells were preincubated with anti-CD4 (OKT 1.5) or anti-CD8 (2.43) mAb. The supernatants were assayed for 31Cr release in a gamma counter, and CTL activity was determined at various E:T ratios. Spontaneous release of 31Cr was assayed by incubation of targets in the absence of effector. Maximum and total release of 31Cr were determined by incubation of targets with 10% Triton X-100. Percentage of specific 31Cr release was determined by the following equation: percentage specific release = (experimental − spontaneous)/(maximum − spontaneous) × 100.

CFSE labeling

CD4⁺ T-LNC from β₃m⁻/⁻ mice, immunized with FC/MUC1 cells or PBS, were isolated and selected by cell sorting. The single CD4⁺ T-LNC were resuspended in serum-free medium at a concentration of 1 × 10⁶ cells/ml and incubated in a shaking bath for 10 min at 37°C with the vital fluorescent dye CFSE (final concentration 4 µM; Molecular Probes, Eugene, OR). The CFSE-labeled T cells were washed twice and resuspended in medium (1 × 10⁵ cells/well) with purified MUC1 Ag (5 U/ml) and DC (1 × 10⁵ cells/well) for in vitro culture. On days 1, 2, 3, 4, 5, 6, 8, and 10, the CD4⁺ T-LNC populations were passed through nylon wool to remove nonadherent cells and then stained with PE anti-CD8 mAb to determine coreceptor expression during cell division. For analysis of CFSE-labeled cells, 5,000–10,000 events were collected by two-color FACSscan analysis and/or using Modfit LT cell cycle analysis software (Verity Software House, Topsham, ME).

Tumor rejection in vivo

C57BL/6 WT, MUC1.Tg. β₃m⁻/⁻, and Abb⁻⁻ mice were immunized twice s.c. with 5 × 10⁶ FC/MUC1 cells. Control mice were injected with irradiated MC38/MUC1 cells or PBS. Fourteen days after immunization, 5 × 10⁵ MC38/MUC1 tumor cells were administered s.c. and tumor growth was monitored. To exclude the effect of residual CD4⁺ and/or CD8⁺ T cells, some mice were treated with anti-CD4 and anti-CD8 Abs before and after FC/MUC1 immunization, and then challenged s.c. with 5 × 10⁵ MC38/MUC1 tumor cells. Mice injected with anti- rat IgG or PBS were used as control.

To study the prevention of MC38/MUC1 tumor growth in Abb⁻⁻ mice with adoptive transferred CD4 T-LNC, the Abb⁻⁻ mice were first immunized s.c. with 5 × 10⁶ FC/MUC1 on days 0 and 7. On days 12 and 16, the mice were injected i.v. with 5 × 10⁶ CD4⁺ T-LNC from FC/MUC1-immunized β₃m⁻/⁻ mice that were cocultured with MUC1 Ag for 3 days. On day 14, the recipient Abb⁻⁻ mice were challenged s.c. with 5 × 10⁵ MC38/MUC1 tumor cells on either the right or the left side of the flank. Abb⁻⁻ mice injected with PBS or naive CD4 T-LNC from β₃m⁻/⁻ mice were used as controls. Tumor incidence and growth were monitored and recorded up to 40 days.

In the treatment of MC38/MUC1 tumor metastases in Abb⁻⁻ and β₃m⁻/⁻/Abb⁻⁻ mice, the Abb⁻⁻ and β₃m⁻/⁻/Abb⁻⁻ mice were injected i.v. with 10 × 10⁶ MC38/MUC1 tumor cells on day 0. On days 2 and 4, the Abb⁻⁻ mice were treated i.v. with 5 × 10⁶ CD4⁺ T-LNC from β₃m⁻/⁻ mice immunized with FC/MUC1. The β₃m⁻/⁻/Abb⁻⁻ mice received injection on days 2, 4, and 15. Mice injected with PBS or CD4 T-LNC from naive β₃m⁻/⁻ mice stimulated by anti-CD3 mAb (0.1 µg/ml)
for 3 days were used as controls. The mice were sacrificed on day 28, and the lungs were removed and stained with India ink. The number of pulmonary metastatic nodules was counted from each lung.

**Statistical analysis**

Statistical significance was analyzed using χ² and Student’s t tests.

**Results**

**Differential antitumor immunity in MHC class I and II knockout mice induced by DC/tumor fusion cells**

We have shown that DC/tumor fusion cells (FC/MUC1) induce systemic antitumor immunity in both WT and MUC1.Tg mice (3,21). To dissect the roles of CD4⁺ and CD8⁺ T cells in such DC/tumor fusion cell-induced antitumor immunity, we have used MHC class I and MHC class II knockout mice. MHC class I and II molecules are vital in the positive selection of CD8⁺ T cells, respectively, within the thymus. Mice with a disrupted β₂m gene (β₂m⁻/⁻ mouse) are virtually devoid of CD8⁺ T cells (13), whereas mice with a disrupted Aβ (H2β) gene (Abb⁻/⁻ mouse) are depleted of CD4⁺ T cells (14). MHC class I knockout (β₂m⁻/⁻) and MHC class II knockout (Abb⁻/⁻) mice were immunized twice (days 0 and 7) s.c. with FC/MUC1 cells, with WT and MUC1.Tg mice used for controls. One week after the second immunization, the mice were challenged with murine MC38 adenocarcinoma cells stably transfected with a MUC1 cDNA (MC38/MUC1). Immunization with FC/MUC1 fusion cells provided 100% protection against challenge with MC38/MUC1 tumor cells in WT and MUC1.Tg mice (Fig. 1A). Unexpectedly, FC/MUC1 vaccination induced sufficient antitumor immunity to reject the tumor challenge in all β₂m⁻/⁻ mice (Fig. 1A). In contrast, impaired antitumor immunity was observed in Abb⁻/⁻ mice immunized

![Graph](https://example.com/graph.png)

**Figure 1.** Effect of CD4⁺ and/or CD8⁺ T cells in cell-mediated antitumor immunity. A, C57BL/6 WT (●), MUC1.Tg (○), β₂m⁻/⁻ (△), and Abb⁻/⁻ (▲) mice (n = 11/group) were immunized s.c. twice with 5 × 10⁶ FC/MUC1 cells on days 0 and 7. MUC1.Tg mice immunized with 5 × 10⁶ irradiated MC38/MUC1 carcinoma cells (○) or PBS (□) were used as control. On day 14, mice were challenged with 5 × 10⁶ MC38/MUC1 tumor cells. They were followed for 40 days, and tumor incidence was determined. B and C, β₂m⁻/⁻ mice (B, n = 7/group) immunized twice with FC/MUC1 cells were treated four times with anti-CD4 mAb (●) and anti-CD8 mAb (▲) i.p. before and after immunization. Abb⁻/⁻ mice (C, n = 7/group) immunized twice with FC/MUC1 cells were treated four times with anti-CD4 mAb (●) and anti-CD8 mAb (▲) i.p. before and after immunization. The mice in control groups without immunization were treated with anti-rat IgG (●, n = 8) or PBS (□, n = 5). D, β₂m⁻/⁻ mice (n = 6/group) immunized twice with FC/MUC1 cells were chronically treated i.p. with anti-CD8 mAb (▲), anti-CD4 mAb (●), and anti-rat IgG (●) before and after immunization up to 24 days. Nonimmunized mice were injected with PBS (□, n = 5) as control. On day 14, mice were challenged with 5 × 10⁶ MC38/MUC1 tumor cells. Tumor growth was monitored for up to 35 days, and tumor incidence was determined. The results were obtained from two independent experiments. E and F, Kinetics of phenotypic change in activated T-LNC from mice immunized with FC/MUC1 and cocultured with MUC1 Ag in vitro. Inguinal LNC isolated from WT, MUC1.Tg, β₂m⁻/⁻, and Abb⁻/⁻ mice treated with PBS (E) or from WT, MUC1.Tg, β₂m⁻/⁻, and Abb⁻/⁻ mice immunized with FC/MUC1 cells (F) were cocultured with 5 U/ml purified MUC1 Ag. IL-2 (20 U/ml) was added to cultures on day 5 to maintain LNC. At the indicated time, T-LNC obtained by passing LNC through nylon wool were stained with FITC anti-CD4 and PE anti-CD8 mAbs and then subjected to two-color flow cytometry. Similar results were obtained in three independent experiments.
with FC/MUC1, suggesting a key role for CD4+ T cells in immunity. Six of 11 immunized mice developed tumors, although tumor appearance was delayed compared with nonvaccinated mice (Fig. 1A). The difference in tumor incidence between immunized βm−/− and immunized Abb−/− mice is statistically significant (p < 0.01).

Although βm−/− and Abb−/− mice are virtually depleted of CD8+ and CD4+ T cells, respectively (13, 14), they retain ~2% of residual CD8+ or CD4+ T cells. We, therefore, next attempted to exclude a role for such residual CD8+ or CD4+ T cells in antitumor immunity. The βm−/− and Abb−/− mice were treated with anti-CD8 and anti-CD4 Abs (mAb), respectively. When the residual CD8+ T cells in βm−/− mice and the CD4+ T cells in Abb−/− mice were depleted before and after the first and second immunizations, tumor incidence increased only slightly over the anti-rat IgG control, whereas cross-depletion of T cells by treating βm−/−-deficient mice with anti-CD4 Ab and Abb−/− mice with anti-CD8 Ab resulted in tumor growth similar to that in nonvaccinated mice (Fig. 1, B and C). These findings suggest that the residual CD4+ and CD8+ T cells in MHC knockout mice play only a marginal role in antitumor immunity. Prolonged depletion of CD8+ T cells with anti-CD8 mAb in βm−/−-deficient mice increased tumor incidence significantly, suggesting that CD8+ T cells appeared in the late phase of immune response (Fig. 1D). There is a significant difference in tumor incidence in βm−/− mice treated with anti-CD8 mAb for short and long duration (p < 0.025).

It is currently thought that CD8+ T cells are essential in the killing of MHC class I-positive tumor cells because most tumor cells express only MHC class I molecules. However, this fails to explain our in vivo findings that mice depleted of CD8+ T cells are still protected against tumor challenge after FC/MUC1 immunization. To address this question, we next investigated the mechanism of the cell-mediated immune response. We analyzed the phenotype of T-LNC isolated from mice immunized with FC/MUC1 and compared them with those from nonvaccinated mice. LNC were isolated from WT, MUC1.Tg, βm−/−, and Abb−/− mice and cocultured with MUC1 Ag. The T-LNC, obtained after passing LNC through nylon wool at multiple time points, were stained with anti-CD4 and CD8 mAb and subjected to two-color flow cytometry analysis. In the naive T cells from nonvaccinated WT, MUC1.Tg, βm−/−, and Abb−/− mice, the CD8+ T cells decreased during the 10 days of culture with MUC1 Ag (Fig. 1E). However, in T-LNC from FC/MUC1-immunized mice, we observed a shift from CD4+ to CD8+ populations (Fig. 1F). On day 0, there were distinct CD4+ and CD8+ T cell subsets in WT and MUC1.Tg mice. Surprisingly, a T cell population double positive (DP) for CD4 and CD8 and CD8 was observed in FC/MUC1-immunized WT, MUC1.Tg, or βm−/− mice compared with those in their naive counterparts (Fig. 1, E and F). The DP-T cells increased on day 2 of culture. Interestingly, we observed increased CD8+ T cells accompanied by decreased CD4+ T cells during 10 days of culture (Fig. 1F). This shift from CD4+ to CD8+ was more apparent in βm−/− mice. On day 0, there were ~2% CD8+ T cells in FC/MUC1-immunized βm−/− mice. From day 4 on, CD8+ T cells increased in numbers significantly. The shift from CD4+ to DP and then to CD8+ T cells was obvious, and the trend was still ongoing at the end of the experiment. Unlike the T cells from WT and MUC1.Tg mice, substantial numbers of CD4+ T cells still remained on day 10 of culture in βm−/− mice (Fig. 1F). In contrast, in FC/MUC1-immunized Abb−/− mice, minimal CD4+ T cells were shown over 10 days of culture, and there was no evidence of the shift from CD8+ to CD4+ (Fig. 1F).

Because there is no shortage of CD8+ T cells in βm−/− mice, these experiments suggest as an explanation for our in vivo findings that βm−/− mice maintain normal cellular immunity. The generation of CD8+ T cells in βm−/− mice is as exuberant as that in WT and MUC1.Tg mice, if not more so. Furthermore, the appearance of DP-T cells in the T-LNC indicates an alternative source of CD8+ T cells in βm−/− mice.

**Source of CD8+ T cells in βm−/− mice**

The finding that immunized βm−/− mice develop CD8+ T cells is of particular interest because these mice are constitutively depleted of peripheral CD8+ T cells. However, CD8+ CTL have been found previously to be induced in βm−/− mice by a variety of stimulators, and the residual CD8+ T cells have been postulated as the source of CTL. One possible explanation for the findings is that CTL are derived from the expansion of the small residual population of CD8+ T cells that persists in these mice. Our findings in Fig. 1F suggest an alternative source of CTL: the progressive generation of CD8+ T cells through CD4+ to CD4+CD8+ DP-T cells, then to CD8+ T cells. The emergence of DP-T cells is of particular significance for this mechanism because it is unlikely that we should observe DP-T cells in the previously proposed mechanism of expansion by the residual CD8+ T cells. Based on our observation, we therefore hypothesize that the CD8+ T cells observed after immune stimulation in the present study are generated by CD4+ to CD8+ conversion with DP-T cells as potential intermediates in the process.

To test this hypothesis, we next studied CD8 expression in purified CD4+ T cells. Because the purity of such CD4+ T cell populations is critical in avoiding false-positive results, we used a variety of methods to purify the CD4+ T cells. We found that repeated CD4+ T cell sorting is a highly sensitive and reproducible method to purify CD4+ T cells. Fig. 2A shows the progressive purification of our CD4+ T cells by repeated cell sorting. The doublets were gated out in each cell sorting. To assess the purity of sorted CD4+ T cells, we spiked naive CD4+ T cells with increasing numbers of CD8+ T cells and checked the gene expression with real-time RT-PCR. Resorted naive CD4+ T cells from βm−/− mice were collected into each well (5000 cells/well with purity of 99.89%). CD8+ T cells were spiked at 10-fold increments, 106 to 107, into 5000 naive CD4+ T cells. Total RNA was extracted from the cells, and real-time RT-PCR was performed using CD4- or CD8-specific primers. CD4, but not CD8, expression was detected in 5000 naive CD4+ T cells by real-time RT-PCR (Fig. 2B). In contrast, expression of CD8 was detected when 106 to 107 CD8+ T cells were spiked into 5000 naive CD4+ T cells with graded increase of signal (Fig. 2B). Our experiments indicate that we can detect the presence of as few as 1 CD8+ T cell in the population. We were thus able to show that repeated CD4+ T cell sorting eliminates CD8+ T cell contamination and leads to a pure population of CD4+ T cells, at least at the 5000-cell level.

We next studied the effect of FC/MUC1 immunization of βm−/− mice on coreceptor expression in CD4+ T cells. Seven days after the secondary immunization, the T-LNC were isolated and the CD4+ T cells were selected by immunofluorescence cell sorting. CD4+ T cells were sorted again and the doublets were gated out (Fig. 2C). Real-time RT-PCR was used to assess the coreceptor expression in the freshly sorted CD4+ T cells. Both CD4 and, to a lesser extent, CD8 were detected in the repeatedly sorted CD4+ T cells from FC/MUC1-immunized mice (Fig. 2D). In contrast, only CD4 was detected in repeatedly sorted naive CD4+ T cells. These results indicate that FC/MUC1 priming results in the expression of CD8 in CD4+ T cells and that CD8+ T cells in βm−/−
mice immunized with FC/MUC1 are generated by means of CD4+ to CD8+ conversion.

Detection of CD8 expression in CD4+ T cells after FC/MUC1 immunization by single cell RT-PCR

To confirm the existence of CD8-expressing CD4+ T cells in the FC/MUC1-immunized mice and determine its frequency, we next analyzed the freshly isolated and resorted CD4+ T cells by single cell RT-PCR for CD4 and CD8 expression. Single cell RT-PCR offers the advantages of sensitivity, resolution, and identification of expression of multiple gene products simultaneously. It is particularly useful in determining the frequency of gene expression in particular cell populations and the existence of cells with segregated gene expression patterns in a phenotypically homogeneous population (22–25). Lymph nodes were collected from β2m−/− mice immunized with FC/MUC1 or irradiated MC38/MUC1 tumor cells or treated with PBS and teased into single cells. T-LNC were purified through nylon wool, and CD4+ and CD8+ T cells were selected by single cell sorting with anti-CD4 FITC and anti-CD8 PE staining. CD4+ T cells were then further purified by repeated cell sorting with doublets gated out, as illustrated in Fig. 2. Then single CD4+ T cells were deposited into 96-well V-bottom plates with cyclone unit attached to the MoFlo cell sorter. One-step single cell RT-PCR was conducted using CD4 and/or CD8 primers. The detection of CD8 expression in CD4+ T cells immunized with FC/MUC1 is shown in Fig. 3A, lanes 1 and 9. This finding was further supported by detection of both CD4 and CD8 expression in single CD4+ T cells from FC/MUC1-immunized β2m−/− mice simultaneously using both CD4 and CD8 primer sets (Fig. 3B, lanes 3, 5, and 8). In a parallel study, CD8 transcript was...
detected in a pool of resorted CD4+ T cell from which the single cell was derived (Fig. 3C). In contrast, no CD8 was detected in CD4+/H11545 T cells from /H9252 2 m/H11002 mice immunized with irradiated MC38/MUC1 or treated with PBS (Fig. 3). The frequency of CD8-expressing CD4+/H11545 T cells, determined by single CD8 primer and CD4/CD8 double primer pairs, was 10.9% (Table I). These findings confirm CD8 expression in CD4+/H11545 T cells in FC/MUC1-immunized mice and render the strongest support for intracellular CD4 to CD8 conversion.

**Kinetics of CD8+ T cell generation in β2m−/− mice**

The finding that CD4+ T cells from immunized, but not naive, mice have the potential to undergo CD4 to CD8 conversion indicates the requirement of effective activation and/or proliferation. We next studied the kinetics of CD8+ T cell generation in /H9252 2 m/H11002 mice. CD4+/H11001 T cells were isolated from /H9252 2 m/H11002 mice immunized with FC/MUC1 and further purified by repeated cell sorting with doublets gated out, as illustrated in Fig. 2. They were then cultured, stained with FITC anti-CD4 and PE anti-CD8/H9251 or PE anti-CD8/H9252 mAb, and analyzed by two-color flow cytometry every other day. On days 0 and 10, naive CD4+ T cells maintained the expression of CD4 and αβ TCR (Fig. 4A). In contrast, in cells from immunized mice, we observed progressive conversion of CD4+ T cells to DP-T cells and then to single CD8+ T cells during 12-day culture (Fig. 4B). On day 0, only CD4 molecules were expressed in CD4+ T cells. By day 2, DP-T cells emerged from CD4+ T cells, and these were followed by single

### Table I. Detection of CD8 expression in CD4+ T cells by single cell RT-PCR

<table>
<thead>
<tr>
<th>T Cells</th>
<th>Immunization</th>
<th>Single Primer</th>
<th>Double Primers</th>
<th>Total CD8 Expression</th>
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<td>13/13</td>
<td>0/10</td>
<td>0/31</td>
</tr>
<tr>
<td></td>
<td>MC38/MUC1</td>
<td>8/8</td>
<td>0/10</td>
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<td></td>
<td>FC/MUC1</td>
<td>22/22</td>
<td>2/20</td>
<td>6/53</td>
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*a* CD8-expressing CD4+ T cells from FC/MUC1-immunized β2m−/− mice are statistically significant compared with those from mice immunized with irradiated MC38/MUC1 or treated with PBS (p < 0.05).
FIGURE 4. Phenotypic analysis of CD4⁺ to CD8⁺ conversion. T-LNC from naive or FC/MUC1-immunized β₂m⁻/⁻ mice were isolated, stained with FITC anti-CD4 mAb and PE anti-CD8α mAb, and subjected to repeated single cell sorting (A and B). The resorted CD4⁺ T cells from naive β₂m⁻/⁻ (A) or from β₂m⁻/⁻ mice immunized with FC/MUC1 (B) were cocultured with MUC1 Ag and syngeneic DC. On days 0, 2, 4, 6, 8, 10, and 12, the T cells were collected and purified through nylon wool to remove DC. Cells were stained with FITC anti-CD4, PE anti-CD8β, and CyChrome anti-αβTCR mAb, and analyzed by two- or three-color flow cytometry for CD4, CD8, and/or αβTCR expression. C and D, The association of CD4⁺ to CD8⁺ conversion with T cell proliferation. Sorted CD4⁺ T cells from naive or FC/MUC1-immunized β₂m⁻/⁻ mice were labeled with CFSE and cultured in the presence of purified MUC1 Ag and DC. CD4⁺ T cells from naive β₂m⁻/⁻ mice collected on days 1 and 8 of culture (C) or from β₂m⁻/⁻ mice immunized with FC/MUC1 collected on days 1–10 of culture (D) were purified to remove DC and then stained with PE anti-CD8β mAb and analyzed by two-color flow cytometry using ModFit LT cell cycle analysis software. The arrows in the bottom panels of C and D indicate the number of rounds of cell division in the CFSE-labeled T cells.

CD8⁺ T cells as early as day 6 (Fig. 4B). Such progressive conversion from CD4⁺ T cells to DP-T cells and then to single CD8⁺ T cells was obvious even at the end of the experiment. Interestingly, comparable numbers of DP and CD8⁺ T cells were stained with anti-CD8α or anti-CD8β mAb, suggesting that these cells express heterodimers of CD8 molecules (Fig. 4B). In a parallel study, cultured CD4⁺ T cells were stained with Cy-αβTCR, FITC CD4, and PE CD8α mAb, and analyzed by three-color flow cytometry every other day. Whereas CD4⁺ T cells were converted to CD8⁺ T cells, the expression of αβTCR on these cells was maintained (Fig. 4B). These results indicate that CD8⁺ T cells generated from CD4⁺ T cells in β₂m⁻/⁻ mice are phenotypically conventional CD8⁺ T cells.

To visualize the process of CD4 to CD8 conversion, CD4⁺ T cells were isolated from β₂m⁻/⁻ mice, purified by cell sorting, labeled with the fluorescent dye CFSE, and then cocultured with syngeneic DC and MUC1 Ag. Because CFSE is partitioned equally during cell division (26, 27), this method can monitor cell division and determine the relationship between cell division and differentiation. At the indicated times, the cells were collected, purified through nylon wool, stained with PE-conjugated anti-CD8 mAb, and subjected to two-color flow cytometry analysis. On days 1 and 8, naive CD4⁺ T cells from β₂m⁻/⁻ mice showed minimal division with no evidence of CD4 to CD8 conversion (Fig. 4C). In contrast, on days 1–10, CD4⁺ T cells from FC/MUC1-immunized β₂m⁻/⁻ mice showed vigorous cell division in the presence of DC and MUC1 Ag. Beginning on day 2, CD4⁺ T cells underwent division, and the cells undergoing cell division increased in number to 81% on day 10, with 65% of the cells having divided more than six times (Fig. 4D). Moreover, cell division was accompanied by CD4⁺ to CD8⁺ conversion, as demonstrated in a parallel study. The population of CD8-expressing T cells increased from day 1 and reached 37% on day 10 (Fig. 4D). These experiments demonstrate directly that the CD4⁺ T cells from β₂m⁻/⁻ mice converted to CD8⁺ T cells in association with cell division. Taken together, the results indicate that the process of CD4⁺ to CD8⁺ conversion depends on effective in vivo activation and is promoted by CD4⁺ T cell proliferation.

MHC class I-restricted CTL

We then analyzed CTL activity during the process of CD4⁺ to CD8⁺ conversion. The sorted CD4⁺ T cells from β₂m⁻/⁻ mice immunized with FC/MUC1 were cocultured with DC and MUC1 Ag. The CTL activity was determined by the standard 5¹Cr release assay on days 1, 6, and 12. On day 1, there was no CTL activity...
against $^{51}$Cr-labeled MC38/MUC1 (relevant, MHC class I and MUC1 positive), B16/MUC1 (MHC class I and MUC1 positive), B16 (nonrelevant, MHC class I positive and MUC1 negative) targets (Fig. 5A). The CTL activity increased linearly with time, reaching 63 and 40% against MUC1 and MHC class I-positive MC38/MUC1 and B16/MUC1 tumor cells, respectively, on day 12 (Fig. 5A). In contrast, there is minimal CTL activity against B16 or MC38 tumor cells (Fig. 5, A and B), suggesting the Ag specificity of these CTL. The MHC class I-restricted lysis of MUC1-positive targets was further demonstrated in mAb-blocking assays. As shown in Fig. 5B, anti-MHC class I mAb inhibited the killing of MUC1 and MHC class I-positive targets. Moreover, using anti-CD4 or anti-CD8 mAb preincubation of effectors, anti-CD4 mAb significantly reduced the lysis of MC38/MUC1 and B16/MUC1, whereas anti-CD4 mAb had little effect on lysis of targets (Fig. 5C). These results suggest that the CD8 $^+$ T cells derived from CD4 $^+$ T cells in $\beta^+_m{m}^{\text{m}}/\text{m}$ mice immunized with FC/MUC1 are functional CD8 $^+$ T cells, and that they are Ag-specific and MHC class I-restricted CTLs.

**Therapeutic value of primed CD4 T cells**

We have shown that immunization with FC/MUC1 provided <50% protection against tumor challenge in MHC class II knockout mice (Fig. 1A). The data presented by others and ourselves support the notion that CD4 $^+$ T cells are essential for antitumor immunity. Previously, the finding that mice depleted of CD4 $^+$ T cells failed to provide protection against tumor challenge was interpreted as lack of help from CD4 $^+$ T cells to CD8 $^+$ T cells. Our results, however, provide evidence that CD4 $^+$ T cells from immunized mice give rise to CD8 $^+$ T cells. Thus, the CD4 $^+$ T cells have direct therapeutic value. To determine whether adoptive transfer of CD4 T cells primed in vivo by FC/MUC1 immunization can enhance antitumor immunity, CD4 T cells from $\beta^+_m{m}^{\text{m}}/\text{m}$ mice were injected i.v. into $\text{Abb}^+/\text{m}$ mice that were twice vaccinated with FC/MUC1. The tumor incidence was determined up to day 40. $\text{Abb}^+/\text{m}$ mice twice immunized with FC/MUC1 that received CD4 T-LNC from naive $\beta^+_m{m}^{\text{m}}/\text{m}$ mice have only 40% protection against tumor challenge (Fig. 6A). This result is consistent with the findings in Fig. 1A. In contrast, the immunized $\text{Abb}^+/\text{m}$ mice that received primed CD4 T cells from $\beta^+_m{m}^{\text{m}}/\text{m}$ mice immunized with FC/MUC1 had 100% protection against tumor challenge (Fig. 6A). The tumor incidence in $\text{Abb}^+/\text{m}$ mice receiving immunized CD4 T-LNC compared with that of the control group receiving naive CD4 T-LNC is statistically significant ($p < 0.01$). The enhanced antitumor immunity was accompanied by increased CTL activity in the lysis of MUC1-positive targets and, at a lesser extent, in the lysis of MC38 tumor cells, suggesting the induction of polyclonal CTL (Fig. 6, B and C). In contrast, there was minimal CTL activity against unrelated MUC1-negative, but MHC class I-positive B16 or human MCF-7 breast tumor targets (Fig. 6C). These data indicate that primed CD4 T cells can enhance antitumor immunity in an Ag-specific manner.

To assess whether the enhanced antitumor immunity is due to help provided by the transferred CD4 T cells or to direct participation in tumor killing by the CD4 T cells through CD4 to CD8 conversion, the primed CD4 T cells were used to treat established pulmonary metastases. Naive $\text{Abb}^+/\text{m}$ mice were inoculated with $1 \times 10^6$ MC38/MUC1 tumor cells. On days 2 and 4 postinoculation, the nonimmunized mice received CD4 T-LNC from $\beta^+_m{m}^{\text{m}}/\text{m}$ mice immunized with FC/MUC1. $\text{Abb}^+/\text{m}$ mice injected with PBS or naive CD4 T-LNC from $\beta^+_m{m}^{\text{m}}/\text{m}$ mice stimulated in vitro by anti-CD3 mAb were used as controls. The mice were sacrificed on day 28. Their lungs were collected, stained with India ink, and counted for metastatic tumor nodules. Whereas $\text{Abb}^+/\text{m}$ mice that received CD3 mAb-activated CD4 T-LNC had $>160 \pm 44$ tumor nodules, similar to control mice treated with PBS, mice that received CD4 T cells primed with FC/MUC1 were free of tumor nodules in the lungs (Fig. 6D). The difference in number of tumor nodules between the two groups of mice is statistically significant ($p < 0.01$).

To rule out the involvement of endogenous CD8 T cells mediated by transferred CD4 T cells through cross-priming, $\beta^+_m{m}^{\text{m}}/\text{m}$ $\text{Abb}^+/\text{m}$ double-knockout mice were used. $\beta^+_m{m}^{\text{m}}/\text{m}$ $\text{Abb}^+/\text{m}$ mice that received CD3 mAb-activated CD4 T-LNC had $>202 \pm 57$ tumor nodules.

### FIGURE 5. MHC class I-restricted CTL

Sorted CD4 $^+$ T cells from $\beta^+_m{m}^{\text{m}}/\text{m}$ mice immunized with FC/MUC1 were cultured with MUC1 Ag and DC. A. On days 1, 6, and 12, T cells (E, effector) were collected and incubated with $^{51}$Cr-labeled MC38/MUC1 (○), B16/MUC1 (■), and B16 (□) targets (T, targets). CTL activities were determined by $^{51}$Cr release assay at indicated E:T ratios. B. In the Ab-blocking assays, the target cells were incubated with anti-MHC class I mAb (■) or IgG (□) for 30 min at 4°C before addition of the effector cells cultured for 10 days. C. For anti-CD4 or anti-CD8 blocking, the effector cells on day 10 culture were preincubated with anti-CD4 (□), anti-CD8 mAb (■), or IgG (□). CTL activities against indicated targets at an E:T ratio of 30:1 were determined by $^{51}$Cr release assay. The results are expressed as the mean ± SD of three replicates. Similar results were obtained in two independent experiments.
tumor nodules. By contrast, all but one mouse that received CD4 T cells primed with FC/MUC1 were free of tumor nodules in the lungs (Fig. 6E). Taken together, these data demonstrate that the primed CD4 T cells can confer potent antitumor immunity to eradicate established tumor in a nonimmunized host and directly participate in CTL activity (Fig. 6F) through the mechanism of CD4 to CD8 conversion. It is unlikely that the elimination of pulmonary metastases is due to endogenous CD8 T cells activated by the transferred CD4 T cells through cross-priming because the host CD4 and CD8 T cells were constitutively depleted. In addition, the host APCs do not express MHC class I and II molecules and are poor stimulators in the MLR (15). Cross-priming would be severely compromised. 

Discussion
The fusion of DC and tumor cells is an effective approach to the design of DC-based tumor vaccines (3, 28–32). The DC/tumor fusion cells derive their potency from their unique ability to process and present multiple tumor Ags, including those known or unidentified in the context of costimulatory signals and MHC class I and II molecules. We have shown that immunization with DC/tumor fusion cells activates both CD4 + and CD8 + T cells, which provide protection against tumor cell challenge and treat established tumors (3, 21). However, deletion of CD4 + T cells severely impaired the antitumor immunity. These observations led to the present studies using MHC class I and/or II knock-out mice. We demonstrate in this study that CD4 + T cells play a critical role in antitumor immunity, not only in the priming phase, but also in the effector phase. CD4 + T cells primed in vivo by immunization of β2m–/– mice with FC/MUC1 have been converted into phenotypic and functional CTL. In addition, the adoptive transfer of primed CD4 T cells provides protection against the challenge of tumor cells and eradicates established pulmonary metastases, indicating the potential therapeutic value of primed CD4 T cells. Collectively, these results indicate a novel pathway of CTL generation and shed new light on the function of CD4 + T cells.

MHC molecules play a vital role in the positive selection of thymocytes. It has been shown that mice with a disrupted β2m
gene express few MHC class I molecules and are virtually devoid of CD8\(^+\) T cells (13), and that mice with a disrupted H2\(^b\) gene lack the expression of class I-A molecules on class II-expressing cells and the development of CD4\(^+\) T cells (14). Theoretically, the T cell-mediated immune responses in β\(_m^{-/-}\) and Abb\(^{-/-}\) mice could be compromised. Surprisingly, β\(_m^{-/-}\) mice can mount a CD8\(^+\) T cell-mediated immune response as potent as that in WT mice (33–35). Despite the virtual depletion of CD8\(^+\) T cells, β\(_m^{-/-}\) mice are able to clear virus from their lungs (36, 37) and to reject skin grafts (38) in a way similar to immunocompetent mice. Tumor rejection, mediated by CD8\(^+\) CTL, has been demonstrated in β\(_m^{-/-}\) mice immunized with allogeneic (34, 39–41) or syngeneic tumor cells (35, 41). These contradictory findings of CD8\(^+\) T cell-mediated potent immune responses in mice devoid of CD8\(^+\) T cells are difficult to reconcile. This dilemma led us to ask, first, what is the origin of CTL in β\(_m^{-/-}\) mice? Speculation on the origin of these CTL includes: 1) the residual CD8\(^+\) T cells are selected in the thymus by low levels of H-2D\(^b\) that can be expressed in the absence of β\(_m\) (34, 39, 42); 2) extrathymic CTL development can occur in the presence of high titers of IL-2 (43, 44); 3) the CD8 T cells in β\(_m^{-/-}\) mice contain MHC class I-restricted CD4\(^+\) CTL, which are selected on self MHC class II, and cross-reactively recognize allogeneic MHC class I (45). A second question is how intact is the TCR repertoire of CD8\(^+\) T cells in β\(_m^{-/-}\) mice? Although there are ~2–3% CD8\(^+\) T cells in β\(_m^{-/-}\) mice, they hardly form an intact TCR repertoire of CD8\(^+\) T cells. Their TCR repertoire is skewed (39). Furthermore, the maintenance of naive CD8\(^+\) T cells in the periphery requires MHC class I molecules. It is reasonable to speculate that the limited TCR repertoire of residual CD8\(^+\) T cells in β\(_m^{-/-}\) mice can only react to limited pathogens or Ags. Contrary to this belief, however, strong CD8\(^+\) T cell-mediated immune responses were induced against viral (36, 37) and bacterial (46, 47) pathogens and allogeneic (34, 39, 40) or syngeneic tumor challenges (35). It seems that β\(_m^{-/-}\) mice possess an intact TCR repertoire of CD8\(^+\) T cells against a variety of pathogens and Ags. Our results, however, have provided an explanation for these previous studies in which mice devoid of CD8\(^+\) T cells were still able to mount effective immune responses to reject tumor cells or to clear virus. Whereas our data are consistent with these findings that MHC class I-restricted CTL can develop in β\(_m^{-/-}\) mice, our observations indicate a new pathway for the generation of such CTL. We provide direct evidence that the CD4\(^+\) T cells in fusion cell-immunized β\(_m^{-/-}\) T cells are derived from CD4\(^+\) T cells through CD4\(^+\) to CD8\(^+\) conversion, and not from residual CD8\(^+\) T cells.

In fact, there is a line of evidence in the literature implying that CD4\(^+\) T cells are the source of CD8\(^+\) T cells (14). The findings, however, were interpreted as CD8\(^+\) CTL originating from host CD8\(^+\) T cells promoted by transferred CD4\(^+\) T cells. Intracranial infection of normal mice with lymphocytic choriomeningitis virus (LCMV) causes meningitis and death mediated by CD8\(^+\) CTL. In β\(_m^{-/-}\) mice, however, the LCMV meningitis was apparently mediated by CD4\(^+\) T cells (37). Furthermore, the disease could be transferred to recipient β\(_m^{-/-}\) mice by adoptive transfer of immune spleen cells from LCMV-infected β\(_m^{-/-}\) mice (49). Tumor rejection mediated by the CD8\(^+\) CTL in β\(_m^{-/-}\) mice is dependent on CD4\(^+\) T cells, because in vivo depletion of CD4\(^+\) T cells abrogated the antitumor response and resulted in the death of the animals (39).

Because residual CD8\(^+\) T cells have been detected in β\(_m^{-/-}\) mice, it could be argued that the residual CD8\(^+\) T cells contaminate the CD4\(^+\) T cells and that they are the source of CTL. However, this interpretation is not supported by our data. First, the kinetics of CD8\(^+\) T cell generation and the appearance of CD4\(^+\)/CD8\(^+\) T cells (DP-T) preceding CD8\(^+\) T cells argue strongly against the contamination scenario. We postulate that the DP-T cells are intermediates in the process of CD4 to CD8 conversion. In the contamination scenario, we would only observe the expansion of CD8\(^+\) T cells. It is unlikely that we would have observed the DP-T cells if the residual CD8\(^+\) T cells were the source of CTL. Second, single cell RT-PCR detected CD8 expression in CD4\(^+\) T cells. We used freshly isolated and sorted CD4\(^+\) T cell populations devoid of CD8\(^+\) T cells to rule out the possibility that any detection of CD8 expression in CD4\(^+\) T cells resulted from the expansion of contaminating residual CD8\(^+\) T cells. The most convincing evidence, however, is the simultaneous demonstration in single cells of CD4 and CD8 mRNA using CD4/CD8 double primers. The detection of CD8 mRNA indicates the reinitiation of the gene encoding CD8 in some primed CD4\(^+\) T cells. These cells still retain the CD4 molecules on the surface, and thus are positive for CD4. Third, we have purified the CD4\(^+\) population by a variety of techniques including repeated cell sorting, and have confirmed the purity of repeatedly sorted CD4\(^+\) T cells by real-time RT-PCR. Based upon studies adding CD8\(^+\) T cells into purified CD4\(^+\) T cells, we can say that the contamination, if any, would be under 1/5000. These results strongly support the notion that CD8\(^+\) T cells in fusion cell-immunized β\(_m^{-/-}\) mice are derived from CD4\(^+\) T cells through CD4\(^+\) to CD8\(^+\) conversion, and not from residual CD8\(^+\) T cells.

Our studies also indicate that CD4\(^+\) T cells primed in vivo convert into phenotypic and functional CTL, thus linking CD4\(^+\) T cells directly to CTL activity. The CTL in β\(_m^{-/-}\) mice develop in the late phase of immune responses, as evidenced by prolonged anti-CD8 mAb blocking. Delayed CTL development in β\(_m^{-/-}\) mice was reported in the previous study (39). However, these CTL are very potent because mice are fully protected by the generation of such CTL. The finding that adoptive transfer of primed CD4 T cells eliminates established pulmonary metastases in nonimmunized Abb\(^{-/-}\) or β\(_m^{-/-}\)/Abb\(^{-/-}\) mice indicates that the CD4\(^+\) T cells function as potent tumorcidal effectors and participate directly in the killing of tumor cells through CD4\(^+\) to CD8\(^+\) conversion.

Based on the data from single cell RT-PCR, the frequency of CD8-expressing CD4\(^+\) T cells from mice immunized with FC/MUC1 is 10.9%. The frequency is higher than we expected. However, we should remember that single cell RT-PCR was performed in an activated and proliferated population of CD4\(^+\) T cells. The frequency of CD4\(^+\) T cells with potential CD8 expression in naive population is likely to be much lower. The absence of CD8 mRNA in naive CD4\(^+\) T cells probed with real-time RT-PCR or single cell RT-PCR indicates that activation is required for initiation of CD8 expression.

The molecular basis of CD4\(^+\) to CD8\(^+\) conversion is unclear yet due to our in vivo system. Brugnera et al. (50) have demonstrated that thymocytes initially terminated CD8 transcription can be signaled by IL-7 to differentiate into CD8\(^+\) T cells through silencing CD4 transcription and reinitiating CD8 transcription, an event they have referred to as coreceptor reversal. In peripheral T cells, Paiard et al. (51) have reported that activation and culturing of cloned human CD4\(^+\) T cells in medium containing IL-4 result in the acquisition of CD8. The requirement of cytokines and accessory molecules in the CD4\(^+\) to CD8\(^+\) conversion and characterization of the converted CD8\(^+\) T cells are currently under investigation. Finally, whether CD4 to CD8 conversion occurs in WT mice remains to be determined.
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cfying into CD8+ T cells. J. Immunol. 150:3219.


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