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Divergent Roles for CD4$^+$ T Cells in the Priming and Effector/Memory Phases of Adoptive Immunotherapy[1]

Hong-Ming Hu,*† Hauke Winter,[2]* Walter J. Urba,*‡ and Bernard A. Fox[3]*†‡§

The requirement for CD4$^+$ Th cells in the cross-priming of antitumor CTL is well accepted in tumor immunology. Here we report that the requirement for T cell help can be replaced by local production of GM-CSF at the vaccine site. Experiments using mice in which CD4$^+$ T cells were eliminated, either by Ab depletion or by gene knockout of the MHC class II β-chain (MHC II KO), revealed that priming of therapeutic CD8$^+$ effector T cells following vaccination with a GM-CSF-transduced B16BL6-D5 tumor cell line occurred independently of CD4$^+$ T cell help. The adoptive transfer of CD8$^+$ effector T cells, but not CD4$^+$ effector T cells, led to complete regression of pulmonary metastases. Regression of pulmonary metastases did not require either host T cells or NK cells. Transfer of CD8$^+$ effector T cells alone could cure wild-type animals of systemic tumor; the majority of tumor-bearing mice survived long term after treatment (>100 days). In contrast, adoptive transfer of CD8$^+$ T cells to tumor-bearing MHC II KO mice improved survival, but eventually all MHC II KO mice succumbed to metastatic disease. WT mice cured by adoptive transfer of CD8$^+$ T cells were resistant to tumor challenge. Resistance was mediated by CD8$^+$ T cells in mice at 50 days, while both CD4$^+$ and CD8$^+$ T cells were important for protection in mice challenged 150 days following adoptive transfer. Thus, in this tumor model CD4$^+$ Th cells are not required for the priming phase of CD8$^+$ effector T cells; however, they are critical for both the complete elimination of tumor and the maintenance of a long term protective antitumor memory response in vivo. ***The Journal of Immunology,*** **2000, 165: 4246–4253.**

CD4$^+$ T cells are required for the generation and maintenance of cytolytic CD8$^+$ T cells (1) and are generally believed to be essential for the generation of both a cellular and a humoral antitumor immune response (2–6). However, the contribution of CD4$^+$ T cells to the development, expression, and maintenance of antitumor immunity is still the subject of intense investigation. These investigations generally employ either active-specific or adoptive immunotherapy approaches. Active-specific immunotherapy strategies vaccinate the host to prime and expand therapeutic effector and memory T cells (7). In this model one cannot readily separate the contribution of CD4$^+$ or CD8$^+$ T cells to the priming, expansion, effector, or memory phases of the antitumor immune response. Using active-specific immunotherapy, several studies have demonstrated that CD4$^+$ T cells play a critical role in the development of a therapeutic antitumor immune response (2, 5, 8, 9). Alternatively, adoptive immunotherapy strategies exploit in vitro conditions to activate and expand primed T cells, generally with IL-2, for infusion to the tumor-bearing host (10–12). Since one animal is vaccinated and used as the source of primed T cells, and another animal, with systemic tumor, is the recipient of in vitro expanded effector T cells, the contribution of CD4$^+$ and CD8$^+$ T cells to distinct phases of T cell differentiation can be readily determined. In this system, effector T cells generated from tumor-infiltrating lymphocytes or tumor vaccine draining lymph nodes (TVDLN)[4] of weakly immunogenic tumors are generally CD8$^+$ T cells (12, 13). However, effective elimination of systemic tumor requires the coadministration of either tumor-sensitized CD4$^+$ T cells or systemic IL-2 (14, 15). This suggests that the principal role of CD4$^+$ T cells at the effector stage is to provide IL-2 to support or expand CD8$^+$ effector T cells. However, CD4$^+$ T cell help is required during the in vitro activation and expansion of effector T cells from LN draining a non-gene-modified weakly immunogenic tumor vaccine (15, 16). This requirement for CD4$^+$ T cells could not be replaced by addition of IL-2 in the culture medium. Recent studies have demonstrated that tumor cells genetically modified to express certain cytokines were more effective as vaccines (17, 18). In most studies GM-CSF-secreting tumor vaccines were among the most effective vaccine strategies (17, 19, 20). Since GM-CSF can activate and cause differentiation of dendritic cells (21), we examined whether one reason for its effectiveness as a vaccine adjuvant might be its ability to bypass the requirement for CD4$^+$ T cell help. Our studies employed a tumor vaccine genetically engineered to secrete GM-CSF and investigated the requirements for CD4$^+$ T cells during priming, in vitro activation and expansion, therapeutic efficacy following adoptive transfer, and ability to support/maintain a protective antitumor memory response.

*Laboratory of Molecular and Tumor Immunology, Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR 97213; †Department of Biochemistry and Molecular Biology, Oregon Graduate Institute, Portland, OR 97291; and ‡Oregon Cancer Center and §Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, OR 97201

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[2] Current address: Department of Surgery, Ludwig Maximilians Universität München, Klinikum Grosshadern, Marchioninistrasse 15, 81377 Munich, Germany.

[3] Address correspondence and reprint requests to Dr. Bernard A. Fox, Laboratory of Molecular and Tumor Immunology, Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR 97213. E-mail address: foxb@ohsu.edu

[4] Abbreviations used in this paper: TVDLN, tumor vaccine draining lymph node; LN, lymph node; KO, knockout; wt, wild type; LAK, lymphokine-activated killer cells; D5-G6, a GM-CSF-producing clone of B16BL6; CM, complete medium; TRP, tyrosinase-related protein; AIT, adoptive immunotherapy.

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Materials and Methods

Mice and tumor cell lines

Female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC II gene knockout (KO) mice (C57BL/6 Tac-[KO] A^{β} N5) and MHC I and II double KO mice were purchased from Taconic Farms (Germantown, NY). Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996), and all animal protocols were approved by the Earle A. Chiles Research Institute animal care and use committee. D5 is a poorly immunogenic subclone of the spontaneously arising B16BL6 tumor (22). D5-G6 is a stable murine GM-CSF-transduced D5 clone, which secretes GM-CSF at 400 ng/ml/10^6 cells/24 h (22). Both were provided by Dr. Suyu Shu, Cleveland Clinic Foundation (Cleveland, OH). MCA-101 is a chemically induced fibrosarcoma (gift from Dr. Nicholas Restifo, National Cancer Institute, National Institutes of Health) (23). Tumor cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml of gentamicin sulfate. This was further supplemented with 50 μM 2-ME (Aldrich, Milwaukee, WI), and 10% FBS (Life Technologies, Grand Island, NY). Tumor cells were harvested two or three times per week by brief trypsinization and were maintained in T-75 or T-150 culture flasks.

Adoptive immunotherapy

Vaccination was performed by injecting 1×10^6 D5-G6 tumor cells s.c. into both the fore and hind flanks of mice. Eight days later, inguinal and axillary LNs (D5-G6 TVDLN) were collected, and single-cell suspensions were prepared and cultured at 2×10^6 cells/ml of CM in 24-well plates with 50 μl of a 1/40 dilution of 2C11 ascites (anti-CD3). This dilution was determined previously to be optimal for T cell activation. After 2 days of activation, the T cells were harvested and subsequently expanded at 0.1×10^6 cells/ml in CM containing 60 IU/ml IL-2 (gift from Dr. Marty Giedlin, Chiron, Emeryville, CA) in Lifecell tissue culture flasks (Nexell Therapeutics, Irvine, CA) for 3 additional days. Effector T cells were then harvested and washed twice in HBSS, and graded numbers of effector T cells were injected i.v. into B6 mice with 3-day experimentally established D5 pulmonary metastases (five mice per group unless indicated otherwise). Pulmonary metastases were generated by tail vein injection of $0.1–0.2 \times 10^6$ tumor cells. Starting on the day of T cell infusion, mice received 90,000 IU of IL-2 i.p. once per day for 2 or 4 days. Animals were sacrificed 11–13 days following tumor inoculation by CO_2 narcosis. Lungs were resected and fixed in Feketes solution. The number of pulmonary metastases was counted in a blinded fashion. Metastases that were too numerous to count accurately were assigned a value of 250. In some experiments effector T cells were transferred into mice depleted of T or NK cells either by sublethal irradiation (500 rad) 1 day before T cell transfer or by injection of anti-NK1.1 mAb (PK136). In some experiments T cells were transferred into tumor-bearing MHC II KO mice or MHC I and II double KO mice.

Monoclonal Ab-mediated depletion of NK cells or T cell subsets

In vivo depletion of $CD4^+$ and $CD8^+$ T cells was accomplished by treatment with mAbs produced by the GK1.5 (anti-CD4, American Type Culture Collection, Manassas, VA; TIB 207), 2.43 (anti-CD8, American Type Culture Collection, TIB 210), or PK136 (anti-NK1.1, American Type Culture Collection, HB-191) hybridomas, respectively. Vaccination was performed on day 0, and mice were given 50 μl of ascites (anti-CD4 or CD8) i.p. in 0.5 ml of HBSS 1 day before and 2 and 5 days after vaccination. This regimen effectively depleted either $CD4^+$ or $CD8^+$ T cells (data not shown) as determined by flow cytometric analysis with labeled anti-CD4 or CD8 mAb (PharMingen, San Diego, CA). Purified rat Ig (500 μg; I-4131, Sigma, St. Louis, MO) was used as control Ab. NK cell depletion was achieved by administration of 100 μl of PK136 hybridoma ascites i.p. 1 day before T cell transfer.

Flow cytometric analysis

Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA), and data were analyzed with CellQuest software (Becton Dickinson). FITC- or PE-conjugated anti-CD4, CD8, NK1.1, I-Aβ, and CD11c were purchased from PharMingen. FITC-conjugated OX-40 and Ly6-C Abs were prepared in our laboratory with purified Ly6.2-C Ab (from culture supernatant of hybridoma 143-4-2, American Type Culture Collection, CRL-1970) and OX86 Ab (gift from Dr. Andrew W. Weinberg). Purified anti-CD16/CD32 mAb was prepared from culture supernatant of the 2.4G2 hybridoma (American Type Culture Collection) and used to block nonspecific binding to Fc receptors.

Cytotoxicity assays

Six-hour ^{51}Cr release assays were used to detect tumor-specific cytotoxicity of effector T cells. Tumor targets, including D5, D5 treated with IFN-γ, and MCA-101, were labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA), washed, and incubated with effector T cells as previously described (24). D5 tumor cells were incubated overnight with 5 ng/ml recombinant IFN-γ to up-regulate MHC class I expression. Standard 3-day lymphokine-activated killer (LAK) cells, generated by culturing splenocytes with 6000 IU/ml IL-2 for 3 days, were used as a positive control for tumor cell lysis in all experiments.

Survival and tumor challenge experiments

Mice bearing 3-day pulmonary metastases were treated with effector T cells generated as described above, but instead of being sacrificed to count pulmonary metastases, they were observed for overall survival. Mice were sacrificed when they became cachectic or moribund. To determine whether surviving mice were immune, they were rechallenged s.c. with 2×10^4 D5 tumor cells ($10 \times TD_{100}$) 50 or 150 days after adoptive immunotherapy. In some experiments mice were depleted of either CD4 or CD8 subsets by injection of 50 μl of ascites (anti-CD4 or CD8) i.p. in 0.5 ml of HBSS 1 day before and 2 and 5 days after rechallenge with D5 tumor cells. The incidence of tumor formation and tumor growth were assessed every other day. Age-matched naive mice served as controls. The significance of differences in survival time between the two experimental groups was determined by Kaplan-Meier analyses and log rank tests.

Results and Discussion

Increased number of dendritic cells in D5-G6 TVDLN, but not D5 TVDLN

It is now well accepted that antitumor immunity is initiated by the acquisition of tumor-associated Ags by APCs and their subsequent presentation to immunocompetent T cells. Dendritic cells are thought to be the key APC responsible for cross-priming the immune response to many different Ags (25, 26). CTL priming by a GM-CSF gene-modified tumor vaccine also occurs via a cross-priming pathway (27, 28). To examine whether local secretion of GM-CSF increased the number of dendritic cells available for cross-priming an antitumor immune responses in TVDLN, we stained the cells from lymph nodes draining either unmodified D5 tumor or GM-CSF-producing D5-G6 tumor with anti-CD11c and anti-MHC II I-Aβ, two markers of dendritic cells. Naive LN and TVDLN of D5-vaccinated mice contained similar numbers of dendritic cells (1.4 and 1.6%), whereas TVDLN of D5-G6-vaccinated mice exhibited a 3-fold increase in the number of double-positive DCs (4.8%; Fig. 1).

Increased number of T cells with memory phenotype in D5-G6 TVDLN

The activation status of freshly isolated TVDLN T cells 8 days following vaccination was assessed by flow cytometry. Expressions of OX40 and Ly6-C were used as markers for the number of activated CD4 and CD8 T cells, respectively (29, 30). Vaccination with D5-G6 increased the percentage of $OX40^+CD4^+$ T cells to 11.2% compared with 4.4% for D5 TVDLN or 3.9% for naive LN $CD4^+$ T cells (Fig. 2). A similar pattern was seen for Ly6-C^+CD8^+ expression. In naive LNC 28.6% of $CD8^+$ T cells expressed Ly6-C^+. D5-G6 TVDLN had a 16.4% increase (from 28.6 to 45.0%) in Ly6-C^+CD8^+ T cells compared with a 6% increase (from 28.6 to 34.6%) for $CD8^+$ T cells from D5 TVDLN (Fig. 2). Thus, there is an increased number of activated $CD4^+$ and $CD8^+$ T cells in D5-G6 TVDLN, consistent with the hypothesis that the increased number of DCs are priming more T cells to tumor-associated Ags.

D5-G6 primes therapeutic T cells in MHC class II KO mice

A major contribution of $CD4^+$ T cells to the cross-priming of $CD8^+$ CTL occurs via the activation of dendritic cells by CD40/

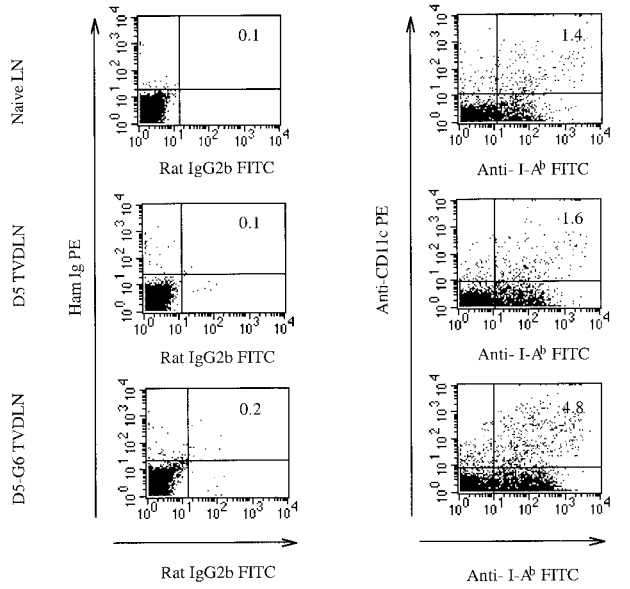

FIGURE 1. D5-G6 vaccination increased the percentage of dendritic cells in D5-G6 TVDLN. Single-cell suspensions of naive LN, D5, and D5-G5 TVDLN were stained with FITC-labeled control rat IgG2b and PE-labeled control hamster (Ham) IgG, FITC-labeled I-A^b (IgG2a), and PE-labeled CD11c mAb (hamster IgG). Samples were analyzed by CellQuest with FACScan (Becton Dickinson). Ten thousand events were collected and analyzed gated on live cells. The number in the *top right* quadrant is the percentage of double-positive cells.

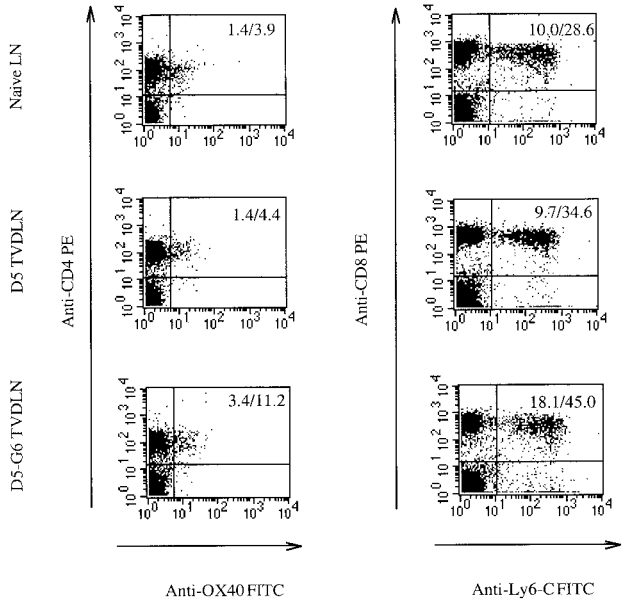

FIGURE 2. Both CD4 and CD8 T cells are primed in D5-G6 TVDLN. Naive, D5, and D5-G6 TVDLN were stained with either PE-conjugated anti-CD4 Ab and FITC-labeled OX-40 Ab or PE-conjugated anti-CD8 Ab and FITC-labeled anti-Ly6-C Ab. The first number presented in the *upper right* quadrant is the percentage of double-positive cells in the total population. The second number is the percentage of double-positive CD4 (*right* three dot plots) or CD8 (*left* three dot plots) T cells.

CD40L cross-linking (8, 31–33). $CD4^+$ T cell help can also be replaced by injecting mice with an anti-CD40 mAb that engages CD40 on dendritic cells, a signal that is normally provided by CD40L on activated $CD4^+$ T cells (32, 33). However, some CTL responses to viral Ags develop independently of $CD4^+$ T cell help (34). In this case, direct activation of CTL is mediated by dendritic cells, which, secondary to viral infection, have acquired costimulatory properties in the absence of the cross-linking of CD40 (31). To address directly the requirement for $CD4^+$ T cell help in the priming of tumor-specific $CD8^+$ T cells, we attempted to generate effector T cells from MHC II KO mice. These mice lack MHC class II-restricted $CD4^+$ T cells and fail to prime CTL responses efficiently (32, 33). As shown in Table I, the adoptive transfer of effector T cells generated from MHC II KO mice vaccinated with D5-G6 mediated regression of pulmonary metastases at both high (70×10^6 cells) and low doses (35×10^6 cells) of T cells transferred. This shows that both the priming and subsequent generation of therapeutic $CD8^+$ T cells could be accomplished in the absence of MHC class II-restricted $CD4^+$ Th cells by vaccination with the GM-CSF-secreting tumor, D5-G6. While MHC class II-restricted $CD4^+$ T cells are eliminated in MHC II KO mice, a minor population of $CD4^+$ T cells, many of them restricted by CD1 molecules, have been found in these mice (35). Recent studies have shown that these CD1-restricted $CD4^+$ T cells can provide help for CTL against *Toxoplasma gondii* or for Ab responses to GPI-linked proteins of *Plasmodium* and *Trypanosoma* (36). To determine whether this subpopulation of T cells may provide T cell help in the generation of an antitumor immune response in MHC II KO mice, MHC II KO mice were depleted of $CD4^+$ T cells by Ab administration before and after D5-G6 vaccination. Flow cytometric analysis confirmed the depletion (from 4.0 to 0.2%) of this minor population (data not shown). Effector T cells generated from CD4-depleted mice were highly therapeutic; complete regression of pulmonary metastases was observed with depletion of minor population of $CD4^+$ T cells (Table I).

$CD4^+$ T cell-independent priming of CTL

To generate both $CD4^+$ and $CD8^+$ effector T cells independently, C57BL/6 wild-type (wt) mice were depleted of $CD4^+$ or $CD8^+$ T cells by Ab administration before and following D5-G6 vaccination. Flow cytometric analysis of TVDLN obtained from anti-CD4 or anti-CD8-treated mice confirmed the depletion of these subsets (data not shown). Effector T cells generated from nondepleted, CD4-depleted, and CD8-depleted wt mice were then adoptively transferred into mice with established pulmonary metastases. To generate effector T cells from D5-G6 TVDLN, we routinely activated LN cells with anti-CD3 Ab for 48 h and then expanded the activated T cells in CM with low dose IL-2 (60 IU/ml). Previously we have shown that in vitro activated effector T cells generated from D5-G6-vaccinated mice are tumor specific, as evidenced by tumor-specific IFN-γ release and tumor-specific cytotoxicity (37). To determine the cytolytic activity of effector T cells generated from CD4-depleted mice, short term ^{51}Cr release assays were performed. Target cells included D5 (very low level of MHC I expression), D5 treated in vitro with recombinant IFN-γ to up-regulate MHC I expression (D5/IFN-γ), and a syngeneic fibrosarcoma, MCA-101. LAK cells were used to prove that targets could be lysed. Both D5 and MCA 101 tumor cells were lysed equally by LAK cells; however, LAK-mediated lysis of IFN-γ-treated D5 was reduced (Fig. 3*A*). We frequently observe this pattern of reduced susceptibility to LAK killing when MHC class I has been up-regulated by IFN-γ. The effector T cells generated from TVDLN in D5-G6-vaccinated mice exhibited a low level D5-specific lysis, which became more detectable if D5 cells were pretreated with IFN-γ (Fig. 3*B*). As expected, $CD8^+$ effector T cells (Fig. 3*C*), but not $CD4^+$ effector T cells (Fig. 3*D*), were highly cytolytic.

Effector T cells generated from CD4-depleted mice were highly therapeutic; complete regression of pulmonary metastases was observed at both the high (70×10^6) and low (35×10^6) doses of T cells transferred (Table II). In contrast, effector T cells from CD8-depleted mice were ineffective at both the high and low doses of T

Table I. *Therapeutic T cells can be generated from MHC II KO mice*

Donor	Immunotherapy[a]			Mean No. of Lung Metastases		
	Vaccine	T cells $\times 10^6$	IL-2	Expt. 1	Expt. 2	Expt. 3
None			+	250	250	250
MHC II KO	D5-G6	70	+	0[c]	0[c]	0[c]
MHC II KO	D5-G6	35	+	0[c]	0[c]	0[c]
CD4 depleted[b]	D5-G6	70	+	0[c]	0[c]	ND

[a] Eight days after s.c. vaccination of 1×10^6 D5-G6 tumor cells, TVDLNs were harvested from MHC II KO mice, activated with anti-CD3 for 48 h, and then expanded for 3 days in CM with 60 IU/ml IL-2 to generate effector T cells. wt recipient mice were injected with 2×10^5 D5 tumor cells i.v. 3 days before adoptive transfer of effector T cells. IL-2 (90,000 IU in 0.5 ml HBSS) was injected i.p. once per day for 4 days after cell transfer.

[b] CD4 depletion of MHC II KO mice was performed by the injection of 50 μl anti-CD4 ascites (GK1.5) 1 day before and 2 and 5 days after vaccination with D5-G6.

[c] $p < 0.05$ compared with group receiving IL-2 alone.

cells transferred (Table II). Consistent with our findings that class II-restricted $CD4^+$ T cells were not required to generate therapeutic T cells in the MHC II KO mice, depletion of $CD4^+$ T cells by mAb treatment did not eliminate the priming of tumor-specific CTL or therapeutic $CD8^+$ effector T cells. This suggests that neither the class II-restricted $CD4^+$ T cells nor the non-MHC II-restricted $CD4^+$ NKT cells are required for efficient priming of $CD8^+$ T cells.

Regression of established pulmonary metastases following adoptive immunotherapy is independent of host T cells or NK cells

To ensure that the observed antitumor effects were directly mediated by the adoptively transferred T cells, we had to exclude indirect mechanisms mediated via host T or NK cells. First we transferred effector T cells into tumor-bearing mice ablated of lymphocytes by a sublethal dose of irradiation. While all irradiated control mice treated with IL-2 alone developed >250 pulmonary metastases, complete tumor regression was observed in irradiated mice that received both IL-2 and effector T cells (Table III). Complete eradication of pulmonary metastases was also seen in tumor-bearing mice depleted of NK cells by injection of anti-NK1.1 Ab (PK136; Table III). Depletion of NK cells by anti-NK1.1 Ab was confirmed by flow cytometric analysis and by elimination of LAK activity as determined by in vitro assays with cells obtained from treated mice (data not shown). Because irradiation did not completely eliminate all T cells, adoptive immunotherapy was also performed in either CD4-deficient MHC II KO or CD4 and CD8-deficient MHC I and II KO tumor-bearing mice. Again, significant ($p < 0.05$) tumor regression occurred in these mice as well. These results clearly indicate that effector T cells can mediate regression of pulmonary metastases without the participation of host T cells or NK cells.

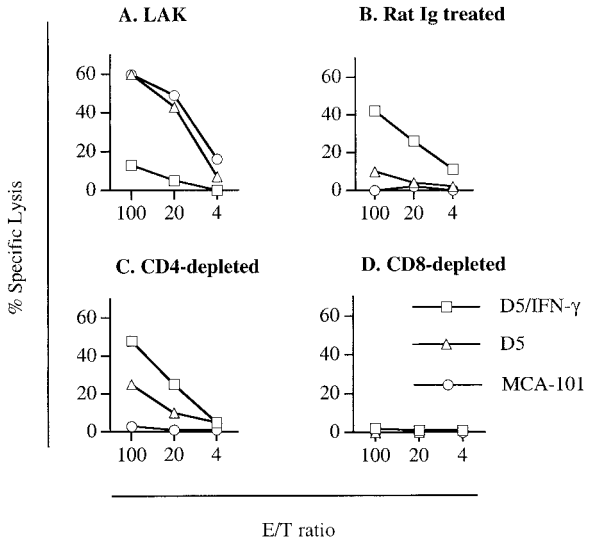

FIGURE 3. CD4-independent priming of CTL in mice vaccinated with D5-G6. Effector T cells generated from TVDLN of D5-G6 vaccinated, rat IgG-treated, or CD4-depleted mice were tested for cytolytic activity against D5, D5 pretreated with IFN-γ, and MCA-101. Using a 6-h ^{51}Cr release assay, cytolytic activity was determined for LAK (*A*), effector T cells generated from D5-G6 vaccinated rat IgG-treated mice (*B*), effector T cells from D5-G6-vaccinated mice depleted of CD4 (*C*), or CD8 (*D*) cells by mAb treatment. The data represent one of three experiments with similar results.

Long term survival after adoptive immunotherapy with $CD8^+$ effector T cells

To determine whether adoptive immunotherapy with $CD8^+$ effector T cells improved survival, mice bearing 3-day established pulmonary metastases received $CD8^+$ T cells and were observed for survival after adoptive T cell therapy (Fig. 4). In experiment 1, 8 of 9 (89%) mice receiving 70×10^6 effector T cells survived for >100 days. The one mouse that died did so 21 days after the death of the last control mouse, which received no T cells. In experiment 2, all mice receiving 35×10^6 effector T cells survived >100 days, while all control mice that received only IL-2 therapy died before day 25. In the third experiment, 30 of 30 mice that received 35×10^6 effector T cells generated from CD4-depleted mice survived at least 50 days. Thus, effector T cells from D5-G6-vaccinated CD4-depleted mice not only decreased the number of pulmonary metastases, but apparently cured the majority of mice treated.

Transfer of $CD8^+$ T cells prolongs survival but does not cure MHC II KO mice

Our results suggest that $CD4^+$ T cells do not play a critical role during either the priming phase or the acute effector phase (as measured by the reduction in pulmonary metastases) in this adoptive immunotherapy model. To examine whether $CD8^+$ effector T cells can cure mice of systemic tumor without $CD4^+$ T cell help, wt $CD8^+$ T cells were adoptively transferred into MHC II KO mice bearing established pulmonary metastases (Fig. 5). Both wt and MHC II KO mice treated with IL-2 alone were dead before day 25. The adoptively transferred $CD8^+$ T cells were highly therapeutic as all wt mice receiving effector T cells survived >80 days. However, while MHC II KO mice treated with wt $CD8^+$ T cells survived longer than MHC II KO mice treated with IL-2 alone ($p < 0.05$), they all died before day 70. These MHC II KO mice

Table II. *$CD8^+$ but not $CD4^+$ effector T cells are therapeutic*

Donor[a]	Immunotherapy[b]			Mean No. of Lung Metastases (SE)		
	Vaccine	T cells $\times 10^6$	IL-2	Expt. 1	Expt. 2	Expt. 3
None			+	250	250	250
Untreated	D5-G6	70	+	0[c]	0[c]	0[c]
Untreated	D5-G6	35	+	12 (6)[c]	1 (2)[c]	50 (15)[c]
CD4 depleted	D5-G6	70	+	0[c]	0[c]	0[c]
CD4 depleted	D5-G6	35	+	0[c]	0[c]	0[c]
CD8 depleted	D5-G6	70	+	250	250	250
CD8 depleted	D5-G6	35	+	250	250	250

[a] CD4 and CD8 depletion was done by injection of 50 μl anti-CD4 ascites (GK1.5) and anti-CD8 (2.43) 1 day before and 2 and 5 days after vaccination with D5-G6.

[b] Eight days after s.c. vaccination of 1×10^6 D5-G6 tumor cells, TVDLNs were harvested and activated with anti-CD3 for 48 h and then expanded for 3 days in CM with 60 IU/ml IL-2 to generate effector T cells. Recipient mice were injected with 2×10^5 D5 tumor cells i.v. 3 days before adoptive transfer of effector T cells. IL-2 (90,000 IU in 0.5 ml HBSS) was injected i.p. once per day for 4 days after cell transfer.

[c] $p < 0.05$ compared with group receiving IL-2 or IL-2 plus effector T cells from CD8-depleted D5-G6-vaccinated mice.

succumbed to solitary metastases that developed at various anatomic sites (lung, face, hip, and tail). Thus, when only $CD8^+$ effector T cells are adoptively transferred, host $CD4^+$ T cells appear to be critical for either maintaining effector function of $CD8^+$ T cells or for activation of other host effector mechanisms that are not required for acute tumor regression in the lung, but are important for complete eradication of residual tumor.

Memory responses in mice cured by adoptive immunotherapy

To test whether immunological memory developed in the wt mice cured of systemic tumor by the adoptive transfer of CD4-depleted effector T cells, mice surviving >120 days were subsequently rechallenged with D5 tumor cells. While all naive mice rapidly developed tumor nodules and had to be sacrificed, all eight mice in experiment 1 and three of five mice in experiment 2 were resistant to a subsequent tumor challenge. The two mice in experiment 2 that developed tumor did so much later than the control naive mice that were injected with the same number of tumor cells (Fig. 6). Thus, the adoptive transfer of effector T cells from CD4-depleted mice cured the majority of mice and led to the development of long term protective immunity in 11 of 13 mice.

Requirement for $CD4^+$ Th cells in maintaining the protective memory response

Our data clearly show that when vaccination is performed with this GM-CSF-secreting tumor vaccine, neither priming nor the acute effector function of $CD8^+$ T cells requires $CD4^+$ T cell help. However, the requirement for host $CD4^+$ T cells to cure and develop and/or maintain a protective antitumor $CD8^+$ memory response and the mechanism responsible for this effect need further characterization. Others have reported divergent requirements for $CD4^+$ T cell help depending on the Ag studied and the experimental design. The long term H-Y-specific CTL memory response is independent of $CD4^+$ T cells (38), although it is generally accepted that $CD4^+$ T cells are important for maintaining a long term CTL response against viral Ags and for the clearance of chronic viral infections (39, 40). While our results clearly demonstrated that $CD4^+$ T cells are not critical for priming or effector function immediately following adoptive transfer, we considered it likely that they would be required to maintain a protective antitumor memory response given our results with the MHC II KO mice. To address the role of $CD4^+$ T cells in maintaining the antitumor memory response, we depleted either $CD4^+$ or $CD8^+$ T cells from mice cured by the adoptive transfer of therapeutic $CD8^+$ effector T cells. These mice had survived either 50 days (short term memory) or >150 days (long term memory) and appeared to be tumor free at the time of mAb treatment. These two groups of mice were then challenged with D5 tumor cells and monitored for tumor growth. Mice that had survived 50 days (short term memory) when they were treated with rat IgG or anti-CD4 were resistant to the tumor challenge, but all mice depleted of $CD8^+$ T cells succumbed to

Table III. *Regression of established pulmonary metastases after adoptive transfer dose does not require recipient NK, $CD4^+$, or $CD8^+$ T cells*

Immunotherapy[a]			Recipient[b]	Mean No. of Lung Metastases (SE)	
Vaccine	T cells $\times 10^6$	IL-2	Mice	Expt. 1	Expt. 2
None	None	+	wt	250	250
D5-G6	70	+	wt	10 (4)[c]	0
None	None	+	MHC II KO	250	ND
D5-G6	70	+	MHC II KO	9 (7)[c]	ND
None	None	+	MHC I/II KO	146 (36)	ND
D5-G6	70	+	MHC I/II KO	0[c]	ND
None	None	+	Anti-NK1.1	250	250
D5-G6	70	+	Anti-NK1.1	7 (4)[c]	1 (1)[c]

[a] Eight days after s.c. vaccination of 1×10^6 D5-G6 tumor cells, TVDLNs were harvested from wt mice, activated with anti-CD3 for 48 h, and expanded for 3 days in CM with 60 IU/ml human recombinant IL-2 to generate effector T cells. Recipient mice were injected with 2×10^5 D5 tumor cells i.v. 3 days before adoptive transfer of effector T cells. IL-2 (90,000 IU in 0.5 ml HBSS) was injected i.p. once per day for 2 days after cell transfer.

[b] MHC II KO and MHC I and MHC II double KO mice were injected with 2×10^5 D5 tumor cells to create tumor-bearing mice deficient in either CD4 or both CD4 and CD8 T cells. Purified anti-NK1.1 mAb (PK136) (500 μg) was injected i.v. into wt tumor-bearing mice 1 day before the adoptive transfer of effector T cells.

[c] $p < 0.05$ compared with mice without T cell transfer.

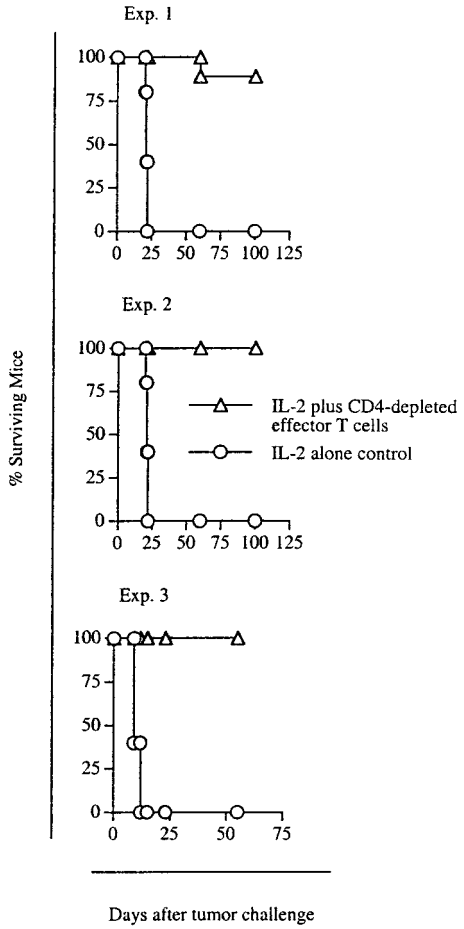

FIGURE 4. Long term survival in mice following adoptive immunotherapy. CD4-depleted effector T cells were generated from TVDLN in CD4-depleted wt mice vaccinated with D5-G6. These cells were adoptively transferred into tumor-bearing wt mice created by i.v. injection of 2×10^5 D5 cells 3 days earlier. All mice also received two injections of 90,000 IU of rIL-2 i.p. daily. In Expt. 1, nine mice were treated with 70×10^6 effector T cells generated from TVDLN of D5-G6-vaccinated CD4-depleted mice and IL-2, and six mice were treated with IL-2 alone (controls). Expt. 2 was the same as Expt. 1, except that 35×10^6 T cells were transferred, and five mice were used in each group. Expt. 3 was the same as Expt. 2, except that 35 mice were used; five of them were used as IL-2 only controls, and 30 mice were treated with CD4-depleted effector T cells.

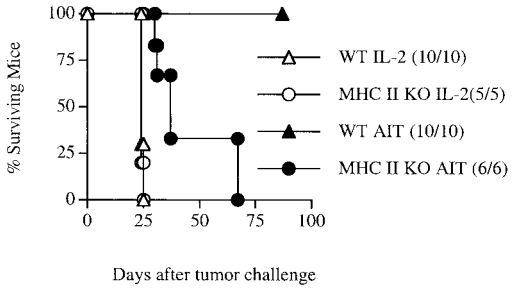

FIGURE 5. Adoptive immunotherapy fails to cure tumor-bearing MHC II KO mice. Adoptive immunotherapy was performed as described in Table I, except that mice were observed for survival. Twenty wt and 11 MHC II KO mice were injected i.v. with D5 tumor cells. Ten wt and six MHC II KO tumor-bearing mice were treated with 70×10^6 effector T cells and IL-2. The remaining mice received IL-2 alone.

tumor growth ($p < 0.05$; Fig. 7*A*). Interestingly, at 150 days following adoptive transfer (long term memory) the majority of CD8-depleted and CD4-depleted mice, but not the control rat Ig-treated mice, developed tumor after the tumor challenge (Fig. 7*B*).

This suggests that $CD8^+$ T cells play the major role in protection against tumor challenge relatively early following adoptive transfer (50 days), but that $CD4^+$ T cells are important in the development and maintenance of long term memory at later time points (150 days). This does not exclude the probability that $CD4^+$ T cells had played a critical role in maintaining the antitumor $CD8^+$ T cells up until the time they were depleted (at 50 days). Future studies will examine whether the requirement for $CD4^+$ T cells at the time of tumor challenge is a function of there being a sufficient number of $CD8^+$ antitumor T cells available to mediate regression in the absence of T cell help at 50 days, but not at 150 days, following adoptive transfer.

Depigmentation in mice that survived a tumor challenge

Five of 14 mice that were cured by adoptive immunotherapy with $CD8^+$ effector T cells and survived a secondary challenge of D5 tumor developed various degrees of depigmentation. This started around day 80 after adoptive immunotherapy and ranged from randomly distributed small white patches to a few large spots that continued to progress over time. One mouse with several large areas of depigmentation is shown in Fig. 8. A naive untreated mouse is shown for comparison.

Our study demonstrates that this GM-CSF-secreting tumor vaccine is able to prime potent antitumor $CD8^+$ effector T cells without $CD4^+$ T cell help. These effector T cells not only cure the majority of mice with pulmonary metastases, but also induce long term immunity against tumor challenge. Similar to the memory responses against cytopathic viruses (40, 41), the cure and maintenance of long-lasting antitumor immunity generated by adoptive transfer of $CD8^+$ T cells is dependent on $CD4^+$ T cell help. In contrast to viral immunity, the role of Ab responses in long term tumor immunity is less clear. An Ab response to the melanosome protein, gp75 tyrosinase-related protein (TRP)-1, was shown to be

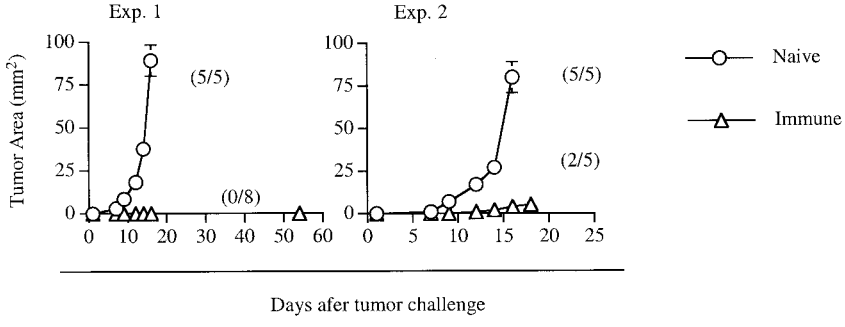

FIGURE 6. Development of antitumor immunity in mice following adoptive immunotherapy. Surviving mice were challenged with 2×10^4 D5 tumor cells s.c. about 120 days after adoptive immunotherapy with CD4-depleted effector T cells. The incidence of tumor formation and tumor size were recorded starting 7 days after challenge. The numbers in parentheses indicate the number of mice that developed tumor of the total number of mice challenged.

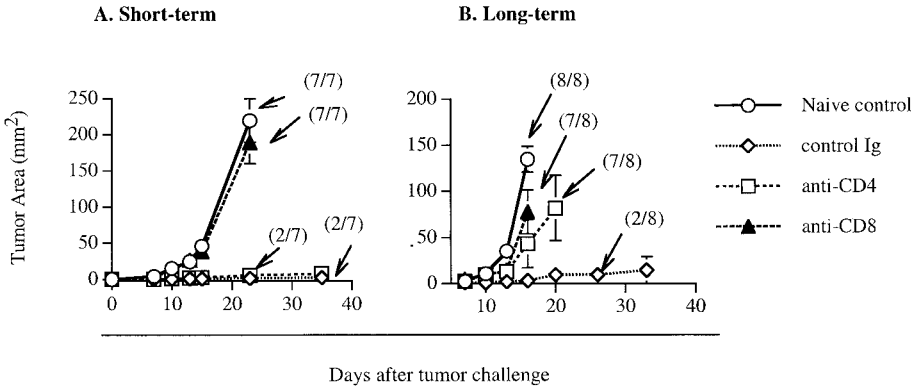

FIGURE 7. $CD8^+$ T cells in immune mice play a major role in protection against a secondary tumor challenge. Fifty days (*A*) or >150 days (*B*) after the adoptive transfer of effector T cells generated from D5-G6 TVDLN, mice were treated with control rat IgG, anti-CD4, or anti-CD8 mAb and then challenged with 2×10^4 D5 tumor cells. Age-matched naive mice were included as a control. The incidence of tumor formation and tumor size were recorded starting 7 days after challenge. The numbers in parentheses indicate the number of mice that developed tumor of the total number of mice challenged.

involved in both the regression of B16 melanoma and autoimmune depigmentation (42, 43).

In the B16 mouse melanoma model, depigmentation can be induced by passive transfer of anti-TRP-1 mAb (42) or active immunization with either insect-derived TRP-1 protein (44), a plasmid DNA containing xenogenic TRP-1 cDNA (45), or a recombinant mouse TRP-2 vaccinia virus (46). Interestingly, mechanisms required for tumor protection induced by TRP-1 DNA vaccination were different from those required for depigmentation (45). Tumor rejection, but not depigmentation, required $CD4^+$ T cells, $NK1.1^+$ cells, and the Fc receptor γ-chain.. Using the same homologous DNA vaccination approach, Bowne et al. were also able to induce tumor immunity by DNA immunization against human TRP-2, but not by immunization with the syngeneic gene (47). Immunization against human TRP-2 induced both autoantibodies and autoreactive cytotoxic T cells. In this case both tumor immunity and depigmentation required $CD8^+$ T cells, but not Abs. Interestingly, only depigmentation required perforin; tumor immunity proceeded in the absence of perforin. Thus, autoimmunity induced against two closely related autoantigens, TRP-1 and TRP-2, involved different mechanisms, i.e., Ab vs a $CD8^+$ T cell response (47). Using vaccinia viruses immunization strategies, Overwijk et al. were able to demonstrate a striking depigmentation and melanocyte destruction in mice vaccinated with murine TRP-1, but not vectors containing other melanocyte differentiation Ags (TRP-2, MART-1, or gp100) (46). These mice also rejected a lethal challenge of B16 melanoma, indicating that the immune response against TRP-1 could destroy both normal and malignant melanocytes. CTL specific for TRP-1 could not be detected in depigmented mice, but high titers of anti-TRP-1 IgG Abs were present, further supporting the hypothesis that different mechanisms (humoral or cellular) can mediate both autoimmunity and antitumor immunity.

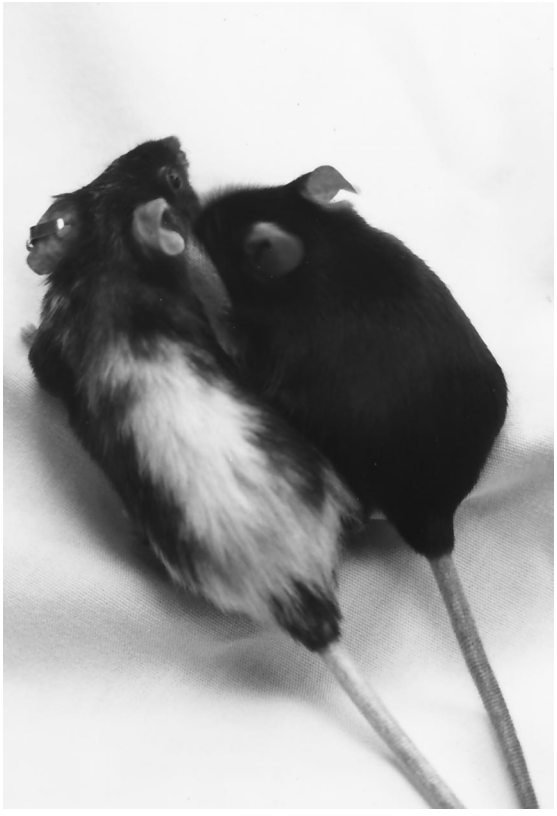

FIGURE 8. Adoptive transfer of effector T cells from TVDLN of D5-G6 vaccinated mice also induced depigmentation in long term surviving mice. Fourteen mice treated with therapeutic T cells generated from CD4-depleted D5-G6-vaccinated mice survived >100 days. Five mice developed depigmentation; one of them is shown here with a normal mouse.

To our knowledge, the results presented in this study are the first direct evidence that adoptive transfer of $CD8^+$ T cells alone could induce depigmentation. In agreement with Bowne et al., (47), we observed that while effector T cells generated from D5-G6-vaccinated wt or perforin knockout mice can cure animals of systemic tumor, only wt effector T cells induce depigmentation (37) (W. Hauke, H.-M. Hu, and B. A. Fox, manuscript in preparation). The $CD8^+$ effector T cells used for adoptive immunotherapy were found to recognize the H-2 K^b-restricted dominant TRP-2 peptide (48) and three mouse gp100 peptides (H.-M. Hu et al., manuscript in preparation), but not the TRP-1 peptide described by Dyall and colleagues (49). Using an ELISA with immobilized proteins from a solubilized melanosome fraction prepared from D5 tumor, we were also able to detect a strong anti-melanosome Ab response in long term surviving mice regardless of whether they developed depigmentation (data not shown). Current studies are investigating the possible contributions of both CD4 Th cells and humoral immunity to the protective antitumor memory response observed in mice cured by adoptive immunotherapy.

Although it remains to be determined whether our observations from the D5 model will extend to other tumor models, elucidation of the mechanism for CD4-independent priming of therapeutic $CD8^+$ T cells will probably yield information critical to the design of therapeutic vaccine and adoptive immunotherapy strategies.

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