

# Development of Antitumor Immune Responses in Reconstituted Lymphopenic Hosts<sup>1</sup>

Hong-Ming Hu,<sup>2</sup> Christian H. Poehlein, Walter J. Urba, and Bernard A. Fox

Robert W. Franz, Cancer Research Center, Earle A. Chiles Research Institute, Providence Portland Medical Center [H.-M. H., C. H. P., W. J. U., B. A. F.], Portland, Oregon 97213, and Departments of Biochemistry and Molecular Biology [H.-M. H., B. A. F.] and Molecular Microbiology and Immunology [B. A. F.], Oregon Health and Science University, Portland, Oregon 97201

## Abstract

When naïve T cells reconstitute lymphopenic hosts, they transiently proliferate and differentiate into memory-like T cells. Here we report that tumor-specific T cells preferentially expand in tumor vaccine-draining lymph nodes after a melanoma vaccine given to RAG1 mice reconstituted with naïve T cells from normal mice. The percentage of tumor-specific Tc1 T cells detected by intracellular cytokine staining was increased ~4-fold in reconstituted lymphopenic hosts compared with normal hosts. Concomitantly, vaccination-induced Th1 T cells were also dramatically increased in vaccinated, reconstituted RAG1 hosts. T cells derived from reconstituted RAG1 hosts exhibited a higher level of melanoma-specific cytotoxicity *in vitro*. These cells were significantly more potent at mediating tumor regression *in vivo* after adoptive transfer into mice bearing established pulmonary metastases. Vaccination is best performed concomitantly with reconstitution; delayed vaccination resulted in T cells with less therapeutic activity.

## Introduction

Naïve T cells, which normally do not proliferate, or do so at a very slow rate, are driven to proliferate vigorously when they are transferred into lymphopenic hosts [either irradiated or RAG1 knock-out (RAG1) mice; Ref. 1]. The self-peptides bound by host MHC class I and class II molecules not only control T-cell survival (2) but also induce homeostatic proliferation (lymphopenia-driven proliferation) in the periphery of T cell-deficient hosts (1). Homeostatic proliferation occurs in both the CD4 and CD8 T-cell compartments; CD4<sup>+</sup> T cells depend on self-MHC II/peptide ligands (3), whereas CD8<sup>+</sup> T cells depend on self-MHC I/peptide ligands (4). Surprisingly, naïve CD8<sup>+</sup> T cells that undergo homeostasis-driven proliferation acquire many characteristics of memory and effector T cells. These characteristics include high levels of expression of CD44 and Ly6C, increased IFN- $\gamma$  production, and CTL activity (5–9). Recent data also suggested a subset of naïve CD4<sup>+</sup> T cells acquired memory-like function (secretion of IL-3-2 and IFN- $\gamma$ ) after expansion in RAG1 mice (10). Because reconstitution of the T-cell compartment in lymphopenic hosts is regulated by the peptides occupying MHC class I and II molecules at the time of T-cell recovery, there may be an opportunity to skew the T-cell repertoire during T-cell recovery by engaging the available MHC class I and class II molecules with peptides of particular interest. If, as it appears, naïve T cells are more

sensitive to activation by weak self-antigens during reconstitution of lymphopenic hosts, there may be a window during which immune tolerance may be broken. If MHC class I and class II molecules presented self peptides, which had been shown to serve as tumor-associated antigens, during a lymphopenic episode, the host may be repopulated with tumor-reactive T cells that could lead to better tumor control. To test this hypothesis, we monitored tumor-specific CD4 and CD8 T cells in TVDLNs from RAG1 mice vaccinated with melanoma cells after *i.v.* reconstitution with naïve spleen cells. Adoptive immunotherapy was performed using effector T cells generated from the TVDLNs of RLP hosts.

## Materials and Methods

**Mice, Tumor Cell Lines, and Monoclonal Antibodies.** Female C57BL/6J (B6) mice and RAG1 KO mice (B6.129S7-*Rag1<sup>tm1Mom</sup>* stock number 002216) were purchased from the Jackson Laboratory (Bar Harbor, ME). 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 and D5-G6 tumor cells lines have been described previously (11). D5 is a subclone of the B16 melanoma cell line B16BL6. D5-G6, which is a clone produced by transducing D5 with MFG-mGM-CSF retroviral vector, produces ~200 ng/ml/10<sup>6</sup> cells/24 h of GM-CSF. MCA 310 is a chemically induced fibrosarcoma from C57BL/6J mice. B16F10 melanoma is from American Type Culture Collection; B16F10-CHITA.28 is a stable clone that expresses the human MHC class II transactivator, which also exhibits high level I-A<sup>b</sup> expression on the cell surface. CL8-1 is a stable clone of B16 melanoma that was transfected with a plasmid that encodes H-2 K<sup>b</sup>. Tumor cells were cultured in 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  $\mu$ g/ml of gentamicin sulfate. This was further supplemented with 50  $\mu$ M 2-mercaptoethanol (Aldrich, Milwaukee, WI) and 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). Tumor cells were harvested two to three times per week by brief trypsinization and were maintained in T-150 or T-225 culture flasks. Hamster antimouse CD3 (clone 2c11) and CD28 (clone PV1) mAbs (American Type Culture Collection) were purified from culture supernatant by ammonium sulfate precipitation and ion exchange column chromatography.

**Reconstitution, Vaccination, and *in Vitro* Sensitization.** RAG1 mice were reconstituted with 20 million naïve spleen cells from wild-type B6 mice and then vaccinated with the D5-G6 tumor vaccine as described previously (11). Briefly, four aliquots of  $1 \times 10^6$  tumor cells each were injected into both the fore and hind flanks of recipient mice. Eight days after vaccination, two enlarged inguinal and two axillary TVDLNs were collected, and single cell suspensions were prepared by pressing lymph nodes between two sterile glass slides. The TVDLN cells were cultured at  $1 \times 10^6$  cells/ml of CM in 24-well plates with 5  $\mu$ g/ml 2c11 antibody (anti-CD3) and 5  $\mu$ g/ml anti-CD28 mAb. After 2 days of activation, the T cells were harvested and subsequently expanded at  $0.1 \times 10^6$  cells/ml in CM containing 60 IU/ml IL-2 (Chiron Co., Emeryville, CA) in Lifecell tissue culture flasks (Nexell Therapeutics, Inc., Irvine, CA) for 3 additional days. These *in vitro* activated and expanded cells are referred to as effector T cells.

**Adoptive Immunotherapy.** Effector T cells were washed twice in HBSS and injected *i.v.* into B6 mice in which pulmonary metastases were established

Received 4/30/02; accepted 6/3/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This study was supported by grants from the Chiles Foundation, the M. J. Murdock Charitable Trust, and NIH Grant RO1 CA 80964 (to B. A. F.).

<sup>2</sup> To whom requests for reprints should be addressed, at Providence Portland Medical Center, 4805 NE Glisan Street, Portland, OR 97213. Phone: (503) 215-6531; Fax: (503) 215-6841; E-mail: hhu@providence.org.

<sup>3</sup> The abbreviations used are: IL, interleukin; RLP, reconstituted lymphopenic; TVDLN, tumor vaccine draining lymph node; GM-CSF, granulocyte/macrophage-colony stimulating factor; CM, complete medium; mAb, monoclonal antibody; TNF, tumor necrosis factor.

3 days earlier by tail vein injection of  $0.2 \times 10^6$  D5 tumor cells. Starting on the day of T-cell infusion, mice received 90,000 IU IL-2 i.p. once per day for 3 days. Animals were sacrificed at 13 days after tumor inoculation by CO<sub>2</sub> narcosis. Lungs were resected and fixed in Fekete's solution. The number of macroscopic pulmonary metastases was counted, and metastases that were too numerous to count accurately were assigned a value of 250.

**Intracellular Cytokine Staining and Fluorescence-activated Cell Sorter Analysis.** For intracellular IFN- $\gamma$  release assays, four million effector T cells were either not stimulated or stimulated with  $2 \times 10^5$  D5, MCA 310, F10, F10-CIITA.28 tumor cells, or anti-CD3 antibody in 2 ml of CM in 24-well plates. Brefeldin A was added, and the cells were incubated at 37°C for 14–16 h. Cells were harvested and stained with FITC-labeled anti-CD8 antibody, Cy-chrome labeled anti-CD3 antibody, and PE-labeled anti-IFN- $\gamma$  antibody after fixing and permeabilization (Cytofix/Cytoperm kit; PharMingen, San Diego, CA). Fifty thousand gated events based on forward and light scatter were collected and analyzed, and all of the analysis was gated on CD3<sup>+</sup> cells.

**Cytotoxicity Assay.** *In vitro* cytotoxicity of effector T cells was determined as described previously (11). Tumor cells were incubated with 100 mCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (DuPont NEN, Boston, MA) for 90 min, washed twice, and plated into round-bottomed, 96-well plates with  $1 \times 10^4$  target cells/well in triplicate. The target cells were incubated with effector T cells at the indicated E:T ratios in a total volume of 200  $\mu$ l of CM at 37°C in a CO<sub>2</sub> incubator. The supernatant was harvested and counted, and the percentage of specific lysis was calculated as described previously (11). Maximum lysis was determined by incubating target cells with 2% Triton X-100 detergent.

## Results

### RLP RAG1 Hosts Developed Strong Tc1 and Th1 Responses.

To compare the immune responses in vaccinated normal or RLP hosts, we performed intracellular cytokine staining and flow cytometry on TVDLN lymphocytes to determine the frequency of tumor-specific T cells. TVDLNs from normal and RLP RAG1 hosts were stimulated with anti-CD3 antibody and anti-CD28 and expanded in IL-2 to generate effector T cells (11). Effector T cells were restimulated *in vitro* for 14–18 h with D5, MCA 310, F10, F10-CIITA.28, CL8-1, or anti-CD3 antibody in the presence of 5  $\mu$ M brefeldin A. D5, MCA-310, and CL8-1 tumor cells all express class I MHC but do not express MHC class II molecules; F10 and F10-CIITA.28 tumor cells express low and high levels of class II MHC I-A<sup>b</sup> molecules, respectively (Fig. 1). As expected, the control tumor, MCA 310, failed to stimulate effector T cells from either normal or reconstituted RAG1 hosts. However, the background was significantly higher in RLP RAG1 hosts (Fig. 2). In normal hosts, the percentage of TVDLN CD8 T cells that produced IFN- $\gamma$  ranged from 1.12 to 2.61%, depending on the tumor cells used for stimulation (Fig. 2A). The percentage of CD8 T cells stimulated to release IFN- $\gamma$  in response to the different cell lines is as follows: control MCA-310 (0.88%); D5 (1.83%); F10 (1.12%); F10-CIITA.28 (1.79%); and CL8-1 (2.61%; Fig. 2A). There appears to be a direct correlation between MHC class I expression by the stimulator cells and the percentage of IFN- $\gamma$ -producing CD8 T cells. The percentage of IFN- $\gamma$  producing cells at baseline was higher in effector T cells obtained from RLP vaccinated RAG1 mice: 2.24% and 2.5% CD8<sup>+</sup> T cells producing IFN- $\gamma$  without stimulation or stimulated with MCA-310 in RLP hosts, compared with 0.55% with no stimulation and 0.88% with control MCA-310 in normal hosts. There was also a large increase in the percentage of melanoma-specific IFN- $\gamma$ -secreting CD8 T cells after stimulation of TVDLN effector T cells from RLP RAG1 hosts with different cell lines derived from B16 melanoma: D5 (6.64%); F10 (6.91%); F10-CIITA.28 (10.29%); and CL8-1 (8.16%). Although the “background” stimulation by the control tumor MCA-310 was greater in RAG1 mice compared with normal mice, the increase in melanoma-specific T cells also appeared to be greater in RAG1 mice. Interestingly, although <1% of CD4 T cells (CD3<sup>+</sup>CD8<sup>-</sup>) from D5-G6 vaccinated normal hosts responded to F10 or F10-CIITA (expressing low and

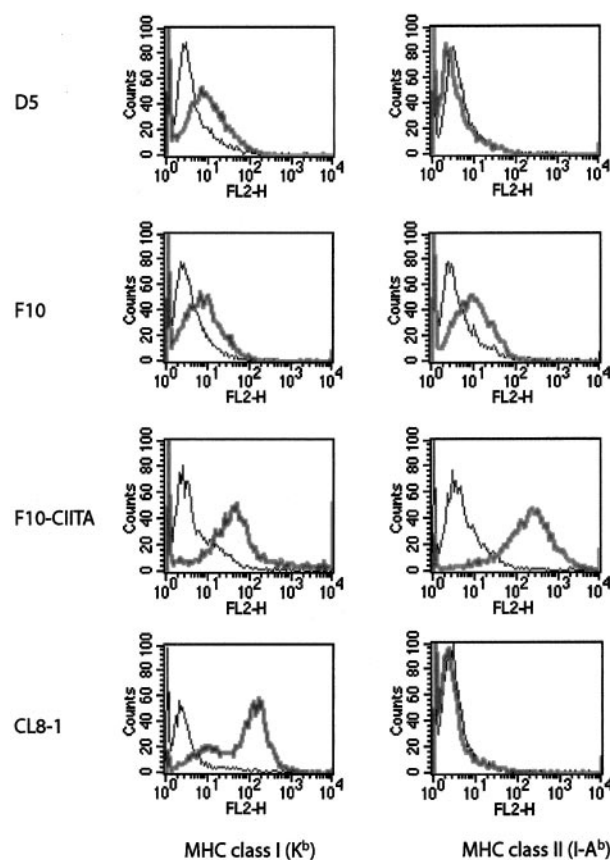


Fig. 1. Expression of MHC class I and class II molecules on melanoma cell lines. D5 (B16BL6-D5), F10 (B16BL6-F10), F10-CIITA (clone 28 of F10 cells transfected with human *CIITA* gene), and CL8-1 (clone 8-1 of B16BL6 cells transfected with H-2 *K<sup>b</sup>* gene) are different subclones derived from B16 melanoma. For class I expression, tumor cells were stained with biotin-labeled anti-*K<sup>b</sup>*, biotin-labeled isotype mAbs (PharMingen), and then with phycoerythrin-conjugated streptavidin (Jackson Immune Research Laboratory). For class II expression, tumor cells were directly labeled with phycoerythrin-conjugated anti-I-A<sup>b</sup> or isotype mAbs. Thin line, background staining by isotype control antibody; thick line, staining of the class I or class II antibodies.

high level of I-A<sup>b</sup> molecules, respectively), a much larger proportion of CD4 T cells from vaccinated RLP RAG1 mice were stimulated to release IFN- $\gamma$  by F10 (3.62%) and F10-CIITA.28 (13.13%; Fig. 2B). A summary of these data are also presented as a bar graph for easy comparison (Fig. 2, E and G).

The production of TNF- $\alpha$  by CD4 and CD8 tumor-specific T cells in vaccinated normal and RLP RAG1 hosts was also examined. Results similar to those for IFN- $\gamma$  production were obtained. CD8 T cells from normal hosts exhibited an increased TNF- $\alpha$  response after stimulation with each of the melanoma cell lines but not the MCA-310 cell line (Fig. 2C). Although baseline or nonspecific TNF- $\alpha$  production was higher, the largest increase in melanoma-specific TNF- $\alpha$ -producing CD4 T cells was found in RAG1 RLP compared with normal hosts (Fig. 2, C and D). A summary of these data is also presented as a bar graph for easy comparison (Fig. 2, F and H). The significantly higher number of melanoma-specific T cells observed in RLP RAG1 mice supports our hypothesis that antigen-specific T cells can be expanded preferentially in reconstituted lymphopenic hosts if appropriate antigen is presented during immune reconstitution.

To determine whether effector T cells generated from RLP RAG1 mice exhibited tumor-specific cytotoxicity, a 6-h <sup>51</sup>Cr-release assay was performed. Although effector T cells from both normal and RLP RAG1 mice failed to kill control tumor MCA 310, effector T cells generated from RLP RAG1 mice, but not normal mice, were highly cytolytic to D5 tumor cells *in vitro* (Fig. 3). These data demonstrate

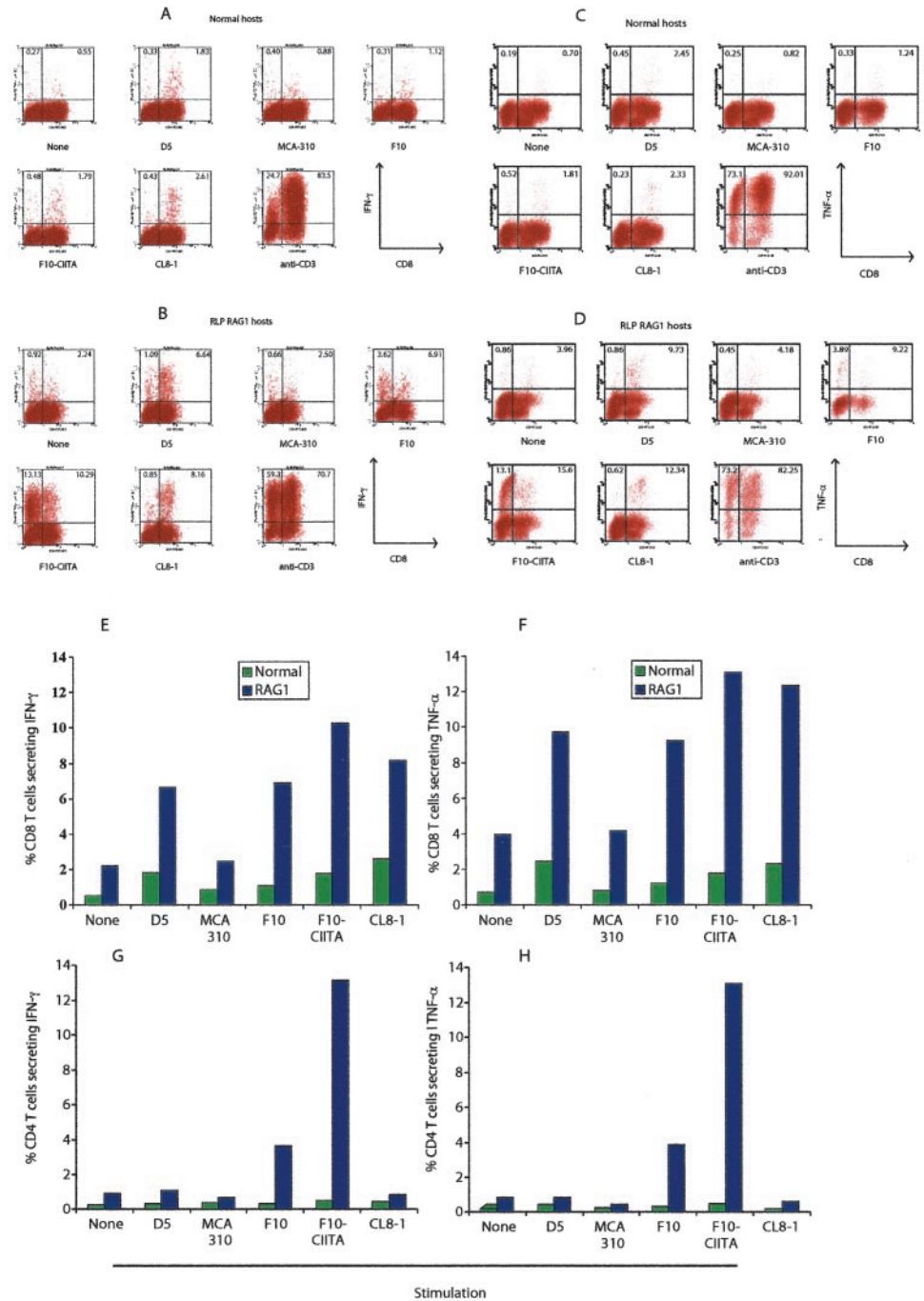


Fig. 2. Selective expansion of IFN- $\gamma$  producing (A and B) and TNF- $\alpha$  producing (C and D) CD4<sup>+</sup> and CD8<sup>+</sup> T cells in vaccinated normal and RLP RAG1 mice. TVDLN effector T cells were derived from D5-G6 vaccinated normal or RLP RAG1 hosts by *in vitro* stimulation with anti-CD3 and CD28 and expansion in IL-2. T cells were then not stimulated or restimulated *in vitro* with D5, MCA 310, F10, F10-CIITA.28, CL8-1, or anti-CD3 antibody in the presence of brefeldin A. T cells were harvested and stained with FITC-labeled anti-CD8 antibody and Cy-chrome labeled anti-CD3 antibody. T cells were fixed and permeabilized before being stained with either phycoerythrin-labeled isotype control antibodies or anti-IFN- $\gamma$  or TNF- $\alpha$  antibodies. Flow cytometry analysis of labeled cells was performed with a B-D FACScalibur. Fifty thousand “live” cell gated events were collected. All of the dot plots represent CD3<sup>+</sup> live cell gated events. The number represents the percentage of IFN- $\gamma$ <sup>+</sup> in either CD8<sup>+</sup>CD3<sup>+</sup> (top right quarter) or CD8<sup>-</sup>CD3<sup>+</sup> (top left quarter) T cells. A and C present the intracellular IFN- $\gamma$  and TNF- $\alpha$  staining results in normal hosts, whereas B and D present the results from RLP RAG1 hosts. Similar results were obtained from two other independent experiments. Data are presented as bar graphs for easier comparison (E-H).

that vaccination during reconstitution of a lymphopenic host can result in a strong type 1 (both Th1 and Tc1) immune response.

**Effector T Cells Obtained from Vaccinated RLP RAG1 Mice Are Highly Therapeutic.** To determine whether the presence of a higher frequency of Th1 and Tc1 cells in the TVDLNs from RLP RAG1 mice would confer superior therapeutic efficacy *in vivo*, we adoptively transferred effector T cells into mice bearing established 3-day pulmonary metastases. Each mouse also received 90,000 IU IL-2 by i.p. injection for 3 days after T-cell transfer to support the survival and antitumor activity of the T cells. Effector T cells generated from reconstituted RAG1 KO mice exhibited greater potency on a per cell basis compared with effector T cells from normal hosts (Table 1). Results from two independent experiments demonstrated that  $15 \times 10^6$  effector T cells generated from reconstituted RAG1 KO

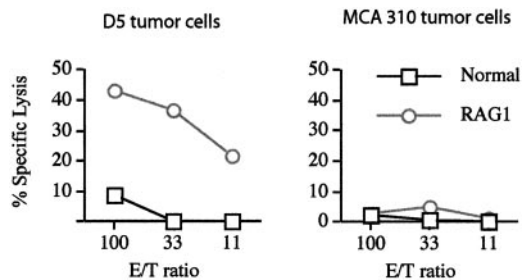


Fig. 3. TVDLN T cells derived from vaccinated RLP RAG1 mice were highly cytolytic. D5 and control MCA 310 tumor cells were labeled with <sup>51</sup>Cr and then incubated with TVDLN effector T cells derived from either normal or RAG1 hosts for 6 h. Effector T cells derived from RAG1 hosts, but not normal hosts, were highly cytotoxic to D5 tumor cells. Effector T cells from both normal and RAG1 mice failed to kill control MCA 310 tumor cells.



Table 1 Concomitant vaccination and reconstitution in RAG1 mice results in highly therapeutic T cells

Group	Immunotherapy <sup>a</sup> at day 3		Mean no. of pulmonary metastases <sup>b</sup> at day 13		
	T-cell donor	T cells × 10 <sup>6</sup>	Exp. 1	Exp. 2	Exp. 3
		None	>250	>250	>250
1	Normal	35	0 <sup>c</sup>	46 (30) <sup>c</sup>	44 (7) <sup>c</sup>
2	Normal	15	19 (29) <sup>c</sup>	189 (49) <sup>c</sup>	>250
3	Normal	5	198 (77)	>250	ND
4	RAG1/RLP	35	ND	ND	ND
5	RAG1/RLP	15	0 <sup>c,d</sup>	0 <sup>c,d</sup>	60 (16) <sup>c,d</sup>
6	RAG1/RLP	5	0 <sup>c,e</sup>	78 (22) <sup>c,e</sup>	172 (55) <sup>c,e</sup>

<sup>a</sup> RAG1 KO mice were reconstituted with 20 × 10<sup>6</sup> naïve spleen cells from wt C57BL6 mice. Immediately after reconstitution, both normal and reconstituted RAG1 mice were vaccinated with D5-G6 cells s.c. TVDLNs were harvested 8 days later and stimulated with anti-CD3/CD28 for 48 h. T cells were expanded in complete medium with 60 IU/ml IL-2 for 3 days. The indicated number of *in vitro* activated T cells (effector T cells) was adoptively transferred into tumor-bearing mice. After T-cell transfer, mice received 90,000 IU IL-2 daily for 3 days.

<sup>b</sup> Mice were injected i.v. with 2 × 10<sup>5</sup> D5 tumor cells to establish pulmonary metastases. A total of 5 mice were used for each experimental group. ND, not done.

<sup>c</sup> P < 0.05, compared with mice without T-cell transfer.

<sup>d</sup> P < 0.05, group 2 compared with group 5.

<sup>e</sup> P < 0.05, group 3 compared with group 6.

mice mediated complete tumor regression (no metastases), whereas the same number of T cells from normal mice failed to mediate complete tumor regression in three independent experiments. In experiment 1, as few as 5 × 10<sup>6</sup> effector T cells from RAG1 KO mice were able to mediate complete tumor regression, whereas 15 × 10<sup>6</sup> effector T cells from normal mice failed to eradicate tumor. In experiment 2, 15 × 10<sup>6</sup> effector T cells from vaccinated RLP RAG1 hosts mediated complete tumor regression, whereas 35 × 10<sup>6</sup> effector T cells from normal mice were not able to do so. In three of three experiments, 5 × 10<sup>6</sup> T cells from RLP mice mediated significantly

better tumor regression than three times as many, 15 × 10<sup>6</sup>, T cells from normal hosts (P < 0.05). Data from these three independent experiments were combined and represented in a graphic format (Fig. 4A). These results demonstrated that there is direct correlation between *in vivo* therapeutic activity and the percentage of melanoma-specific T cells measured by intracellular cytokine staining. This is consistent with our previous observation that CD8<sup>+</sup> T cells are the effector T cells that mediate the regression of pulmonary metastases in this animal model (11).

**Delayed Vaccination Primes T Cells with Less Therapeutic Activity.** After transfer into lymphopenic hosts, naïve T cells reach their peak of proliferation between days 5 and 7; their number reaches a plateau at day 20, after which the space is “filled,” and the number of T cells in the hosts remains constant (4). To determine whether the improvements in vaccination depended on stimulating T cells during homeostatic proliferation or had something to do with the adoptive transfer of naïve T cells, we vaccinated RAG1 mice 1 week after reconstitution with 20 × 10<sup>6</sup> naïve splenocytes when their proliferation reaches a peak. Normal mice without reconstitution were also vaccinated as the controls. Effector T cells from both groups of mice were generated and adoptively transferred into tumor-bearing mice as described. The results of these experiments are shown in Table 2. Vaccination after adoptively transferred naïve T cells have reached their peak of proliferation is less effective than vaccination at time of T-cell transfer. Although concomitant vaccination experiments were not performed at the same time, T cells from RLP RAG1 mice with delayed vaccination were less therapeutic. In contrast to T cells from RLP RAG1 mice with concomitant vaccination, 5 million effector T cells from mice with delayed vaccination were not more effective than 15 million effector T cells from normal hosts and actually were not

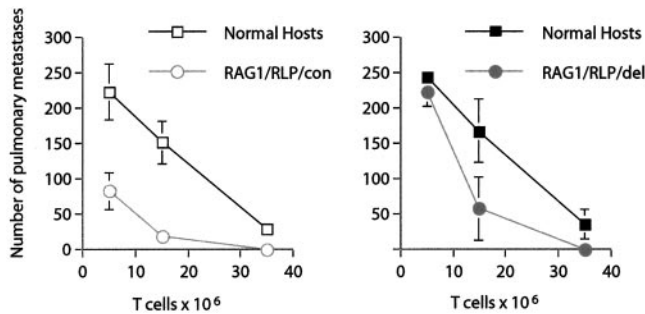


Fig. 4. Concomitant vaccination of reconstituted RAG1 mice generates T cells with greater therapeutic activity than delayed vaccination. Normal or RAG1 mice were reconstituted with 20 × 10<sup>6</sup> naïve spleen cells and vaccinated immediately (A) or delayed for 1 week. TVDLNs were collected 8 days after vaccination and adoptively transfer into mice bearing 3-day D5 lung metastases after activation with anti-CD3 and CD28 mAbs and expanded in IL-2 medium. Five, 15, and 35 million T cells were transferred in each experiment, as indicated in Tables 1 and 2. The number of lung metastases in mice without T-cell transfer is >250. The mean number of lung metastases is from two or three experiments, as indicated in Tables 1 and 2. Bars, SE; for some group of mice, the error bar is too small to be seen.

Table 2 Delayed vaccination in RAG1 RLP mice results in less therapeutic T cells

Group	Immunotherapy <sup>a</sup> at day 3		Mean no. of pulmonary metastases <sup>b</sup> at day 13		
	T-cell donor	T cells × 10 <sup>6</sup>	Exp. 1	Exp. 2	Exp. 3
		None	>250	>250	>250
1	Normal	35	0 <sup>c</sup>	87 (50) <sup>c</sup>	16 (19) <sup>c</sup>
2	Normal	15	113 (66) <sup>c</sup>	ND	221 (24)
3	Normal	5	243 (11)	ND	245 (8)
4	RAG1/RLP	35	ND	0 <sup>c,d</sup>	1 (1) <sup>c,d</sup>
5	RAG1/RLP	15	38 (33) <sup>c</sup>	36 (27) <sup>c</sup>	96 (88) <sup>c</sup>
6	RAG1/RLP	5	192 (37) <sup>e</sup>	ND	>250 <sup>e</sup>

<sup>a</sup> RAG1 KO mice were reconstituted with 20 × 10<sup>6</sup> naïve spleen cells from wild-type C57BL6 mice. One week after reconstitution, both normal and reconstituted RAG1 mice were vaccinated with D5-G6 cells s.c. TVDLNs were harvested 8 days later and stimulated with anti-CD3/CD28 for 48 h. T cells were expanded in complete medium with 60 IU/ml IL-2 for 3 days. The indicated number of *in vitro* activated T cells (effector T cells) was adoptively transferred into tumor-bearing mice. After T-cell transfer, mice received 90,000 IU IL-2 daily for 3 days.

<sup>b</sup> Mice were injected i.v. with 2 × 10<sup>5</sup> D5 tumor cells to establish pulmonary metastases. A total of 5 mice were used for each experimental group. ND, not done.

<sup>c</sup> P < 0.05, compared with mice without T-cell transfer.

<sup>d</sup> P < 0.05, group 4 compared with group 1.

<sup>e</sup> P > 0.05, compared with group 3 and the group without T-cell transfer.

therapeutic compared with mice without T-cell transfer (Table 2 and Fig. 4B). These results indicate that vaccination efficacy in RLP mice is influenced by both proliferation rate of naïve T cells and space available for expansion of both antigen-specific and nonspecific T cells.

## Discussion

A major goal of current cancer immunotherapy strategies is to increase the immunogenicity of tumor cells. These strategies include mixing tumor cells with bacterial adjuvants, genetically modifying them to produce cytokines or express costimulatory molecules, and combining them as lysates or cell fusions with dendritic cells. In animal models, tumor vaccines have been shown to elicit immune responses that protect animals from tumor growth after subsequent tumor challenge. However, tumor vaccine strategies usually fail to elicit immune responses that are potent enough to eradicate established tumors. The delay in the development of an effective immune response and the overall weakness of the response when it finally develops are possible explanations for the observed lack of efficacy after active immunization of tumor-bearing hosts. Improved immunization strategies are required to produce therapeutic immune responses to tumor vaccines in tumor-bearing hosts. A major impediment continues to be the lack of potent tumor rejection antigens. In melanoma and many other tumors, the potential tumor rejection antigens are weak self-antigens. Because the tumor antigens are self-proteins, high-affinity T cells, which are likely to be the most effective antitumor T cells, will already have undergone negative selection in the thymus and/or silencing by other peripheral mechanisms. We know, however, that tumor-bearing hosts are not completely tolerant of these self-antigens, because vaccination with altered peptide ligands or xenogenic proteins (heteroclitic immunization) can result in a strong immune response to not only the modified antigen but the native antigen as well (12). For the melanoma antigen gp100, vaccination with modified peptide dramatically enhanced the immune response to the modified peptide but also to native peptides presented by melanoma cells (13).

For many cancers, the tumor-associated antigens are unknown, and whole tumor cells are often used as vaccines. One way to enhance an antitumor immune response would be to devise a situation in which tumor-specific T cells are "tuned" to become more sensitive to activation by weak tumor antigens. It has been reported that a naïve T-cell repertoire could be skewed to increase the relative percentage of antigen-specific T cells if antigen was given during immune reconstitution (14). Borrelle *et al.* (15) recently demonstrated enhanced efficacy of a GM-CSF gene-modified lymphoma vaccine in lethally irradiated mice reconstituted with T-cell-depleted bone marrow. Our findings extend the report of Borrelle *et al.* (15) in several important ways:

(a) Because vaccination is augmented in reconstituted RAG-1 mice that are not irradiated, it documents that the cytokine storm induced by lethal irradiation is not required for the increased antitumor effect.

(b) Because we used spleen cells and vaccinated immediately after reconstitution, it suggests that the augmented antitumor response does not require differentiation of new T cells from bone marrow stem cells. These two findings are likely the most important and clinically relevant observations of our study, because lethal whole body irradiation and bone marrow transplant will likely prove to be roadblocks to widespread clinical application of this vaccine strategy for solid tumors.

(c) In contrast to their use of TCR transgenic T cells and a foreign model tumor antigen, influenza hemagglutinin, to detect an increase in antigen-reactive T cells, we were able to show strong CD4 and CD8

T-cell responses to native antigens presented by tumor cells. These results also infer that vaccination during a time of natural lymphocyte recovery from lymphopenia could increase the magnitude of the immune response to a vaccine. Because lymphocyte recovery occurs regularly in treated cancer patients (16), our data suggest that the development of transient lymphopenia after chemotherapy or irradiation may be exploited by vaccinating patients during these lymphopenic episodes to boost immunity and possibly induce tumor regression and prevent tumor recurrence.

Currently, little is known about the mechanisms that regulate the homeostasis of naïve T cells in lymphopenic hosts. Two nonexclusive explanations have been proposed to explain this phenomenon. One is the "fill up the space" hypothesis (17), which states that naïve and memory T cells occupy specific survival niches. The induction of lymphopenia empties these niches and allows naïve cells to expand and fill the empty "space." The other, regulatory, or suppressor cell hypothesis, proposes that regulatory cells counter self-reactive T cells and thereby prevent the development of organ-specific autoimmunity (18). Of course, the same process would inhibit the priming of tumor immunity (19). It is possible that regulatory cells were not transferred in sufficient number when RAG-1 mice were reconstituted with normal spleen cells to prevent the expansion and eradication of autoreactive T cells. The spleen cells used for reconstitution in our experiments were unfractionated; therefore, additional transfer experiments with spleen cells depleted of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells would be necessary to determine their role in priming therapeutic T cells during reconstitution of a lymphopenic host.

Neither hypothesis directly addresses the factors that drive naïve T cells to proliferate in the absence of exogenous antigenic stimulation. Our results can be explained best by the T-cell activation threshold tuning hypothesis proposed by Grossman and Paul (20). Their hypothesis states that the immune system is designed to be moderately self-reactive and that self-reactivity is controlled by the activation threshold. It proposes that the immune system responds to episodes of "system perturbation," such as episodes of infection or lymphopenia. In our experimental model, there are two system perturbations, lymphopenia and a tumor vaccine. As a population, T cells respond to lymphopenia by tuning their activation threshold down to a level at which a subgroup of naïve T cells are driven to proliferate, even in the absence of foreign antigens. If tumor antigens are also present, T-cell responses could be focused on these peptides, and tumor-specific T cells may be preferentially driven to proliferate to reach a level sufficient to confer resistance to tumor growth. Repopulation of both CD4 and CD8 niches with a T-cell repertoire skewed in favor of melanoma-reactive T cells after vaccination in reconstituted RAG1 mice would explain our results.

Previously, we demonstrated that priming of therapeutic CD8 effector T cells in normal hosts is CD4 independent and that CD8, but not CD4 effector T cells, mediated tumor regression (11). We observed a large increase in the number of Th1 CD4 effector T cells that appear to be MHC class II restricted and tumor specific in RLP RAG1 mice compared with normal hosts after vaccination with D5-G6. Because a large percentage of CD4 T cells were primed in vaccinated RLP RAG1 mice, we are currently reexamining the role of CD4 T cells during the priming and effector phases of adoptive immunotherapy. Preliminary experiments suggest that the adoptive transfer of either CD4 T cells derived from vaccinated RLP RAG1 mice but not from normal mice are highly therapeutic.<sup>4</sup> We also plan to determine whether these CD4 T cells are melanoma specific and identify the antigens they recognize. Future experiments will determine whether

<sup>4</sup> C. H. Poehlein and H-M. Hu, unpublished results.

tolerance in melanoma-specific CD4 T cells was broken by vaccination during reconstitution of lymphopenic hosts. Most interestingly, we observed both enhanced tumor protection and the rapid development of vitiligo after tumor challenge with the B16F10 melanoma cell line in reconstituted lymphopenic mice that had been vaccinated with irradiated unmodified B16F10 tumor cells.<sup>5</sup> This supports our hypothesis that vaccination of RLP hosts with whole tumor cell vaccines can break immune tolerance to tumor antigens. In conclusion, our study suggests that active vaccination of T-cell reconstituted lymphopenic hosts can jump start or reboot the immune system in favor of strong immune responses to self-antigens and promote protection against tumor outgrowth. This strategy will be tested in future clinical trials.

### Acknowledgments

We acknowledge Drs. A. D. Weinberg and J. W. Smith II for helpful discussions, Dr. Dan L. Longo for critical reading of the manuscript, and T. Ruane for maintaining the mouse colonies. We thank Michal Hogaboom for assistance in preparation of the manuscript.

### References

1. Surh, C. D., and Sprent, J. Homeostatic T cell proliferation: how far can T cells be activated to self-ligands? *J. Exp. Med.*, *192*: F9–F14, 2000.
2. Germain, R. N., and Stefanova, I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol.*, *17*: 467–522, 1999.
3. Ernst, B., Lee, D. S., Chang, J. M., Sprent, J., and Surh, C. D. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity*, *11*: 173–181, 1999.
4. Goldrath, A. W., Bogatzki, L. Y., and Bevan, M. J. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J. Exp. Med.*, *192*: 557–564, 2000.
5. Murali-Krishna, K., and Ahmed, R. Cutting edge: naive T cells masquerading as memory cells. *J. Immunol.*, *165*: 1733–1737, 2000.
6. Cho, B. K., Rao, V. P., Ge, Q., Eisen, H. N., and Chen, J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J. Exp. Med.*, *192*: 549–556, 2000.
7. Goldrath, A. W., and Bevan, M. J. Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity*, *11*: 183–190, 1999.
8. Oehen, S., and Brduscha-Riem, K. Naive cytotoxic T lymphocytes spontaneously acquire effector function in lymphocytopenic recipients: a pitfall for T cell memory studies? *Eur. J. Immunol.*, *29*: 608–614, 1999.
9. Kieper, W. C., and Jameson, S. C. Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. *Proc. Natl. Acad. Sci. USA*, *96*: 13306–13311, 1999.
10. Gudmundsdottir, H., and Turka, L. A. A closer look at homeostatic proliferation of CD4(+) T cells: costimulatory requirements and role in memory formation. *J. Immunol.*, *167*: 3699–3707, 2001.
11. Hu, H. M., Winter, H., Urba, W. J., and Fox, B. A. Divergent roles for CD4+ T cells in the priming and effector/memory phases of adoptive immunotherapy. *J. Immunol.*, *165*: 4246–4253, 2000.
12. Dyall, R., Bowne, W. B., Weber, L. W., LeMaout, J., Szabo, P., Moroi, Y., Piskun, G., Lewis, J. J., Houghton, A. N., and Nikolic-Zugic, J. Heteroclitic immunization induces tumor immunity. *J. Exp. Med.*, *188*: 1553–1561, 1998.
13. Lee, K. H., Wang, E., Nielsen, M. B., Wunderlich, J., Migueles, S., Connors, M., Steinberg, S. M., Rosenberg, S. A., and Marincola, F. M. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to *in vitro* stimulation but does not lead to tumor regression. *J. Immunol.*, *163*: 6292–6300, 1999.
14. Mackall, C. L., Bare, C. V., Granger, L. A., Sharrow, S. O., Titus, J. A., and Gress, R. E. Thymic-independent T cell regeneration occurs via antigen-driven expansion of peripheral T cells resulting in a repertoire that is limited in diversity and prone to skewing. *J. Immunol.*, *156*: 4609–4616, 1996.
15. Borrello, I., Sotomayor, E. M., Rattis, F. M., Cooke, S. K., Gu, L., and Levitsky, H. I. Sustaining the graft-versus-tumor effect through posttransplant immunization with granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing tumor vaccines. *Blood*, *95*: 3011–3019, 2000.
16. Mackall, C. L., Fleisher, T. A., Brown, M. R., Magrath, I. T., Shad, A. T., Horowitz, M. E., Wexler, L. H., Adde, M. A., McClure, L. L., and Gress, R. E. Lymphocyte depletion during treatment with intensive chemotherapy for cancer. *Blood*, *84*: 2221–2228, 1994.
17. Tanchot, C., Rosado, M. M., Agenes, F., Freitas, A. A., and Rocha, B. Lymphocyte homeostasis. *Semin. Immunol.*, *9*: 331–337, 1997.
18. Shevach, E. M., McHugh, R. S., Thornton, A. M., Piccirillo, C., Natarajan, K., and Margulies, D. H. Control of autoimmunity by regulatory T cells. *Adv. Exp. Med. Biol.*, *490*: 21–32, 2001.
19. Suttmuller, R. P., van Duivenvoorde, L. M., van Elsas, A., Schumacher, T. N., Wildenberg, M. E., Allison, J. P., Toes, R. E., Offringa, R., and Melief, C. J. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J. Exp. Med.*, *194*: 823–832, 2001.
20. Grossman, Z., and Paul, W. E. Self-tolerance: context dependent tuning of T cell antigen recognition. *Semin. Immunol.*, *12*: 197–203; discussion 257–344, 2000.

<sup>5</sup> H-M. Hu. A poorly immunogenic tumor induces protective antitumor immunity in mice subjected to sublethal irradiation and immune reconstitution, manuscript in preparation.