

# Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells

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If irradiated tumor cells could be rendered immunogenic, they would provide a safe, broad, and patient-specific array of antigens for immunotherapies. Prior approaches have emphasized genetic transduction of live tumor cells to express cytokines, costimulators, and surrogate foreign antigens. We asked if immunity could be achieved by delivering irradiated, major histocompatibility complex–negative plasmacytoma cells to maturing mouse dendritic cells (DCs) within lymphoid organs. Tumor cells injected intravenously (i.v.) were captured by splenic DCs, whereas subcutaneous (s.c.) injection led only to weak uptake in lymph node or spleen. The natural killer T (NKT) cells mobilizing glycolipid  $\alpha$ -galactosyl ceramide, used to mature splenic DCs, served as an effective adjuvant to induce protective immunity. This adjuvant function was mimicked by a combination of poly IC and agonistic  $\alpha$ CD40 antibody. The adjuvant glycolipid had to be coadministered with tumor cells i.v. rather than s.c. Specific resistance was generated both to a plasmacytoma and lymphoma. The resistance afforded by a single vaccination lasted >2 mo and required both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Mature tumor capturing DCs stimulated the differentiation of P1A tumor antigen-specific, CD8<sup>+</sup> T cells and uniquely transferred tumor resistance to naive mice. Therefore, the access of dying tumor cells to DCs that are maturing to activated NKT cells efficiently induces long-lived adaptive resistance.

The use of autologous tumor cells as vaccines dates back to the 1950s when it was found that chemically induced tumors of inbred mice, if injected as irradiated cells, could elicit protective immunity in syngeneic hosts (1). The prospect of rendering irradiated tumor cells immunogenic is important because this would deliver a large spectrum of epitopes to the immune system, including critical regression antigens that may be specific to an individual tumor (2). However, it has been difficult to induce protective immunity with inactive tumor cells (3–6). The injected cells can even expand suppressor cells leading to unresponsiveness (3). It remains a considerable scientific challenge to learn to improve the immunogenicity of safe nonreplicating tumor cell vaccines.

The online version of this article contains supplemental material.

Tumor cells have been transduced to express foreign proteins as surrogate antigens (7–10), but this approach does not address the capacity of the immune system to respond to a spectrum of intrinsic tumor antigens. Tumor cells have also been genetically modified to express individual costimulatory molecules from the B7 family (B7.1, B7H, B7-DC) (11–13) or the TNF superfamily (LIGHT) (14). These modifications can improve T cell–mediated antitumor responses, although there is evidence that the tumor cells must still be presented by host antigen-presenting cells (15). In addition, tumor cells have been transduced to secrete cytokines such as interferons, interleukins, and hematopoietins that increase systemic immunity (16–18). Tumors secreting IL-12 and GM-CSF are most effective in eliciting T cell–mediated tumor immunity (19–21). Both IL-12 and GM-CSF

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Abbreviations used:  $\alpha$ -Gal Cer,  $\alpha$ -galactosyl ceramide; CFSE, carboxyfluorescein diacetate succinimidyl ester; NKT, natural killer T.

can mediate the recruitment of DCs (21–23), which are specialized antigen-presenting cells for initiating immunity.

However, there are limitations to the clinical usefulness of genetically modified tumors. These approaches typically require the administration of live tumor cells, which raises a safety issue (4, 5). To prioritize responses to patient-specific antigens, tumor cell lines must be available from the patient for purposes of genetic transduction, and this may be impractical. In addition, genetically modified tumors depend on a few, often one, immune enhancing product.

An alternative to genetic transduction would be to identify mechanisms required to induce immunity to irradiated tumor cells. Two principles would seem valuable in this regard: (a) to learn to deliver dying whole tumor cells directly to DCs that are abundant in lymphoid organs, and (b) to render the DCs immunogenic through their differentiation or maturation. A key feature of DCs is the efficiency with which dying cells are processed for presentation on MHC class I and II products (24, 25). In vivo dying cells injected intravenously are selectively taken up by a subset of splenic DCs marked by expression of CD8 $\alpha$  and DEC-205. This leads to the presentation of a surrogate antigen, ovalbumin, from the dying cells (26, 27). Such “cross-presentation” of antigens by host cells, rather than direct presentation by the tumor cells themselves, primes protective T cells against several experimental tumors (28–30), and this has now been observed with a cellular human tumor vaccine as well (31). However, cross-presentation of model antigens from dying cells in the steady state leads to tolerance (26), whereas immunity develops when the DCs simultaneously mature, for example, in response to innate natural killer T (NKT) lymphocytes

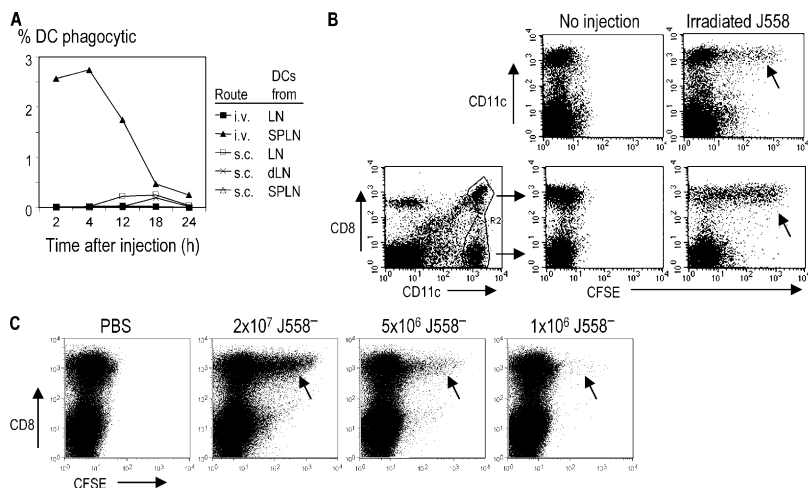
(32, 33). NKT cells not only act as adjuvants by maturing DCs but they also have additional valuable adjunct functions in tumor resistance. NKT cells can produce IFN- $\gamma$  and lyse tumor targets, whereas the interaction of NKT cells with DCs leads to the production of IL-12 and the activation of other innate NK cells (34–37). Thus, NKT cells are a potentially attractive means to link innate and adaptive immunity against tumors.

Prior approaches to inducing immunity with safe irradiated tumor cells have not directly addressed the need for tumor cells to gain access to maturing DCs, to allow cross presentation and to induce strong T cell-mediated immunity. Here, we have studied the nonimmunogenic plasmacytoma cell line, J558, because it expresses a classical tumor antigen called P1A (38) for which TCR transgenic T cells have been made (39), and because J558 has an MHC class I-negative variant that is genetically incapable of direct presentation (40). We will describe that a single dose of irradiated J558 plasmacytoma cells, as well as A20 lymphoma cells, when targeted to DCs, leads to long-lasting adaptive immunity as long as the DCs are matured with innate NKT cells or a combination of the TLR ligand, poly IC, and agonistic anti-CD40 antibody.

## RESULTS

### Dying tumor cells are selectively captured by DCs when given by the i.v. route

We first verified that DCs in mouse spleen could take up the MHC class I-negative J558<sup>-</sup> variant of a mouse plasmacytoma. This variant has lost expression of cell surface MHC class I and multiple antigen presentation genes, including TAP-1, TAP-2, LMP-2, and LMP-7, due to malfunction of



**Figure 1. Intravenous delivery of dying tumor cells to CD8<sup>+</sup> CD11c<sup>+</sup> DCs in vivo.** (A) Kinetics of uptake of  $20 \times 10^6$  dying, irradiated, CFSE-labeled J558<sup>-</sup> tumor cells injected i.v. or s.c., by CD11c<sup>+</sup> spleen (SPLN), draining (LN), and distal (dLN) lymph node DCs and analyzed with flow cytometry as in B. (B) Flow cytometric assays to show selective uptake of irradiated CFSE-labeled J558<sup>-</sup> tumor cells by CD8 $\alpha$ <sup>+</sup>, CD11c<sup>+</sup>

splenic DCs 2 h later (arrows). Comparison of CD11c<sup>+</sup> and CD11c<sup>-</sup> spleen cells (top), and CD8 $\alpha$ <sup>+</sup> CD11c<sup>+</sup> to CD8 $\alpha$ <sup>-</sup>, CD11c<sup>+</sup> DC subsets (bottom). (C) Uptake by CD11c<sup>+</sup> DC subsets of graded doses of CFSE-labeled tumor cells i.v. 5, 20, or 10<sup>6</sup> irradiated CFSE-labeled J558<sup>-</sup> tumor cells were injected i.v. Uptake 2 h after injection is shown in gated CD11c<sup>+</sup> splenic DCs (arrows).

the proto-oncogene *pml* (40, 41). Because the tumor cells lack MHC class I, as we confirmed (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20050956/DC1>), and also fail to express MHC class II (not depicted), recipient antigen-presenting cells would have to capture and process J558<sup>-</sup> cells to elicit CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses. To verify that the J558<sup>-</sup> cells underwent cell death after 75 Gy  $\gamma$ -irradiation, we placed the cells in culture and showed that with time, they could be stained with annexin V and propidium iodide (see Materials and methods and Fig. S1 B). We followed the uptake of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled, irradiated, “dying” J558<sup>-</sup> cells in vivo by lymph node and splenic DCs after injection by the i.v. and s.c. routes, using flow cytometry. Fig. 1 A shows that the tumor cells, injected immediately after irradiation, were phagocytosed by CD11c<sup>+</sup> splenic DCs. In contrast, few CFSE-labeled cells were detected in splenic or lymph node DCs when the tumor cells were injected by the s.c. route (Fig. 1 A). CFSE-labeled tumor material was primarily detected in the CD11c<sup>+</sup> DC-enriched populations (Fig. 1 B, top) and, as expected from prior work (27), only the CD8 $\alpha$ <sup>+</sup> CD11c<sup>+</sup> DC subset endocytosed the injected CFSE-labeled tumor (Fig. 1 B, bottom). In experiments that are not depicted, we established that few CFSE-labeled cells were taken up by other CD11c<sup>-</sup> fractions of spleen, marked for either CD11b, B220, or CD4. Fig. 1 C shows that the uptake of J558<sup>-</sup> cells was limited by the dose of injected tumor. Furthermore, irradiated A20 (MHC I<sup>+</sup> MHC II<sup>+</sup>), J558 (MHC I<sup>+</sup> MHC II<sup>-</sup>), and J558<sup>-</sup> (MHC I<sup>-</sup> MHC II<sup>-</sup>) were phagocytosed by DCs to similar extents (unpublished data). Therefore, dying tumor cells are selectively captured by DCs in vivo as long as the i.v. route is used.

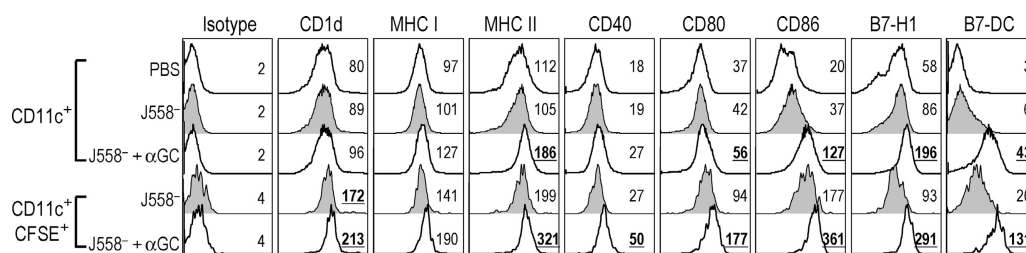
#### $\alpha$ -Gal Cer injection leads to rapid maturation of phagocytic DCs in vivo

$\alpha$ -Galactosyl ceramide ( $\alpha$ -Gal Cer) is a nonmammalian glycolipid that is presented by CD1d molecules to an invariant T cell receptor expressed by innate NKT lymphocytes (42). A single i.v. dose of  $\alpha$ -Gal Cer activates NKT cells to secrete IL-4 and IFN- $\gamma$ , but this activation also leads to the maturation of DCs in vivo, defined as the ability to initiate com-

bined CD4<sup>+</sup> and CD8<sup>+</sup> T cell immunity to coadministered antigens (32, 33, 43). We established that the coadministration of  $\alpha$ -Gal Cer with both J558 and A20 tumor cells did not increase the level of tumor cell uptake by DCs; actually, uptake could be slightly lower, probably because maturing DCs are known to decrease their level of endocytic activity (44). Also, in the presence of  $\alpha$ -Gal Cer, tumor cell uptake remained restricted to the CD8 $\alpha$ <sup>+</sup> subset of DCs. To evaluate the maturation status of the DCs that had phagocytosed dying tumor cells, we injected mice with CFSE-labeled, irradiated MHC-negative, J558<sup>-</sup> cells in the presence or absence of  $\alpha$ -Gal Cer. 5 h later, the DCs were analyzed by flow cytometry for the expression of a number of cell surface molecules that change during DC maturation. Injection of tumor cells alone had little effect on the phenotype of the total CD11c<sup>+</sup> splenic population relative to the PBS control (Fig. 2, compare first and second rows). However, injection of  $\alpha$ -Gal Cer (not depicted) or coinjection of tumor cells with  $\alpha$ -Gal Cer (Fig. 2, third row) resulted in the maturation of the total CD11c<sup>+</sup> DC population, as indicated by the up-regulation of MHC II, CD80, CD86, B7-H1, and B7-DC 5 h later (Fig. 2, third row). When we looked selectively at those DCs that had captured dying tumor cells (by examining cells positive for CD11c and CFSE), we found that the phagocytic cells, which represent <3% of the splenic DCs (Fig. 1 A), had higher levels of CD1d and other markers (Fig. 2, fourth row). The phagocytic cells strongly up-regulated the expression of all the measured antigen-presenting and costimulatory molecules in response to  $\alpha$ -Gal Cer (Fig. 2, compare fourth and fifth rows). Therefore, the administration of  $\alpha$ -Gal Cer allows DCs that capture tumor cells to exhibit numerous phenotypic changes typical of DC maturation.

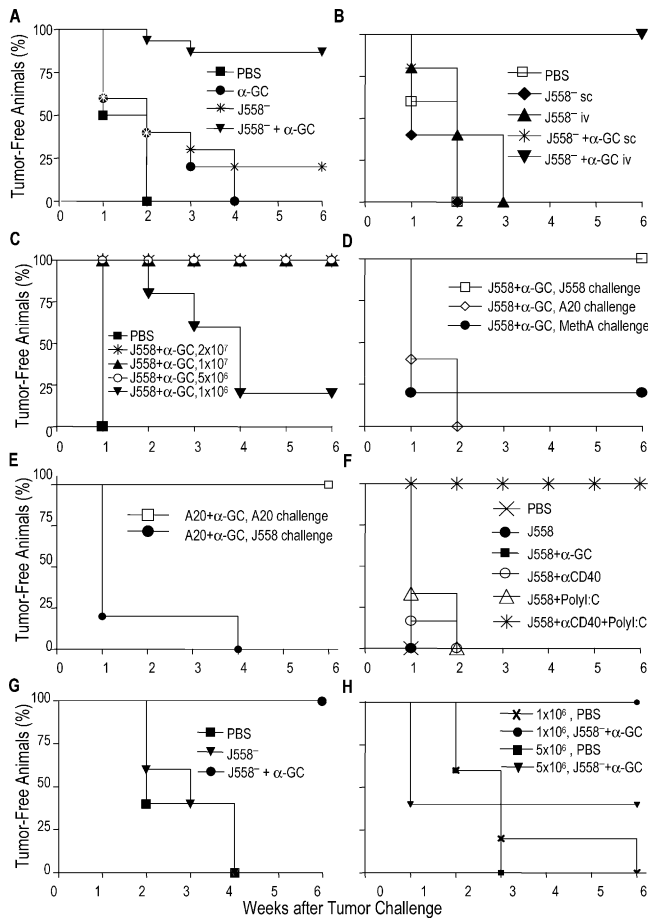
#### Injection of dying tumor cells together with $\alpha$ -Gal Cer induces tumor immunity

To determine if immunity was induced by delivery of dying tumor cells to DCs, we used a tumor protection assay. Different groups of naive BALB/c mice were injected with PBS,  $\alpha$ -Gal Cer,  $20 \times 10^6$  irradiated J558<sup>-</sup> tumor cells alone, or J558<sup>-</sup> together with  $\alpha$ -Gal Cer. 7 d after the vaccination, we challenged the mice with  $5 \times 10^6$  J558 live tu-



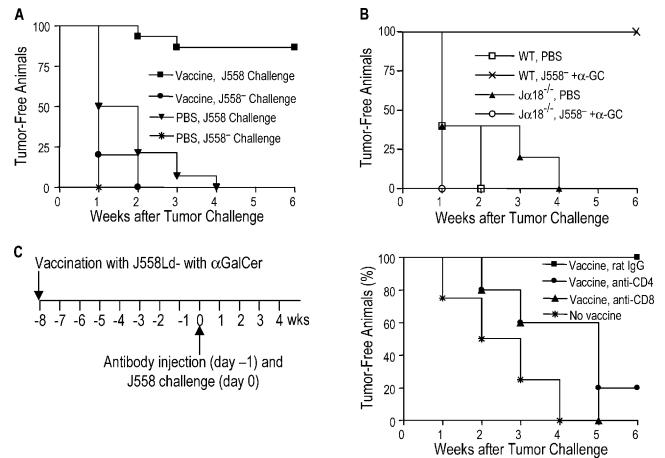
**Figure 2.**  $\alpha$ -Gal Cer injection rapidly matures CD1d-rich, splenic DCs capturing dying cells. BALB/c mice were injected with PBS or  $20 \times 10^6$  irradiated CFSE-labeled J558<sup>-</sup> tumor cells i.v. in the presence or absence of  $2 \mu\text{g}$   $\alpha$ -Gal Cer. 4.5 h later, bulk spleen cells were prepared

from the mice and stained with CD11c-allophycocyanin and PE-conjugated mAbs CD40, 80, 86, B7-H1, B7-DC, L<sup>d</sup>, I-A<sup>d</sup>, and CD1d. The data are shown for the CD11c<sup>+</sup> total DC population (top three rows) and CD11c<sup>+</sup> CFSE<sup>+</sup> phagocytic population (bottom two rows).



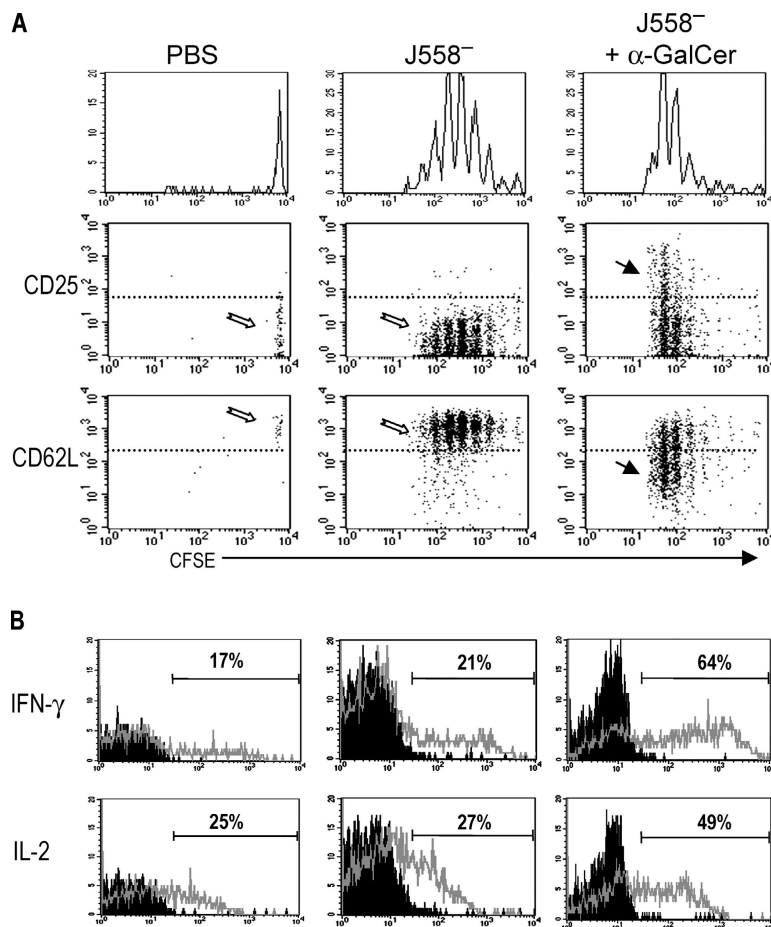
**Figure 3. Acquired resistance to J558 after vaccination with MHC I<sup>-</sup> tumor and  $\alpha$ -Gal Cer.** (A) Mice were vaccinated with PBS, 2  $\mu$ g  $\alpha$ -Gal Cer, 20 × 10<sup>6</sup> irradiated MHC I<sup>-</sup> J558<sup>-</sup> cells with or without 2  $\mu$ g  $\alpha$ -Gal Cer i.v. 1 wk later, mice were challenged with a lethal tumorigenic dose of 5 × 10<sup>6</sup> live MHC I<sup>+</sup> J558 tumor cells s.c. (B) Mice were vaccinated with PBS, 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> cells alone, or 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> cells with 2  $\mu$ g  $\alpha$ -Gal Cer i.v. or s.c. and, 1 wk later, challenged with 5 × 10<sup>6</sup> live MHC class I<sup>+</sup> J558 cells s.c. (C) Mice were vaccinated with 1, 5, or 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> cells plus 2  $\mu$ g  $\alpha$ -Gal Cer i.v. and, 1 wk later, challenged with 5 × 10<sup>6</sup> J558 s.c. (D) Mice were vaccinated with 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> cells plus 2  $\mu$ g  $\alpha$ -Gal Cer i.v. and, 1 wk later, challenged with 5 × 10<sup>6</sup> J558 or to establish tumor specificity, with 5 × 10<sup>6</sup> Meth A sarcoma or 5 × 10<sup>6</sup> A20 lymphoma cells s.c. (E) Mice were vaccinated with 5 × 10<sup>6</sup> irradiated A20 cells plus 2  $\mu$ g  $\alpha$ -Gal Cer i.v. and 1 wk later, challenged with 5 × 10<sup>6</sup> J558 or 5 × 10<sup>6</sup> A20 cells s.c. (F) Mice were vaccinated with 5 × 10<sup>6</sup> irradiated J558<sup>-</sup> cells and either 2  $\mu$ g  $\alpha$ -Gal Cer i.v., 50  $\mu$ g  $\alpha$ CD40 i.p., 50  $\mu$ g poly I:C i.p., or both  $\alpha$ CD40 and poly I:C and, 3 wk later, each group was challenged with 5 × 10<sup>6</sup> J558 s.c. (G) 8 wk after vaccination, mice were challenged with a lethal dose of 5 × 10<sup>6</sup> live J558 tumor cells. (H) To detect a therapeutic effect, mice were injected with 1 or 5 × 10<sup>6</sup> live J558 tumor cells s.c., and 3 d later treated with PBS, or 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> cells with 2  $\mu$ g  $\alpha$ -Gal Cer i.v. In all experiments, mice were monitored every other day for tumor growth and scored positive when the tumors were palpable. Each group included at least five mice, and one representative experiment of three is shown.

mor cells s.c. The tumor grew progressively within 7–10 d in mice that had received either PBS or  $\alpha$ -Gal Cer alone (Fig. 3 A). Injection with irradiated J558 tumor cells alone



**Figure 4. CD4<sup>+</sup> or CD8<sup>+</sup> T cell depletion abrogates tumor immunity at the time of challenge.** (A) Mice were vaccinated with 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> tumor cells plus  $\alpha$ -Gal Cer. 7 d after immunization, mice were challenged with 5 × 10<sup>6</sup> MHC class I positive or negative J558 cells s.c. (B) Wild-type BALB/c mice or J $\alpha$ 18<sup>-/-</sup> mice were vaccinated with 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> tumor cells plus  $\alpha$ -Gal Cer and, 3 wk after vaccination, the mice were challenged with 5 × 10<sup>6</sup> J558 cells s.c. (C) 8 wk after vaccination with 20 × 10<sup>6</sup> irradiated J558<sup>-</sup> tumor cells and  $\alpha$ -Gal Cer, mice received 1 mg anti-CD4, anti-CD8, or control rat IgG. 1 d later, all mice were challenged with 5 × 10<sup>6</sup> J558 tumor cells and monitored every other day for tumor growth. Mice were scored positive when the tumors were palpable. Each group included five mice; one representative experiment of two is shown.

protected only 15% mice from J558 challenge (3 out of 20 mice in four experiments), whereas 88% of the mice (22 out of 25 in five experiments) were protected and remained tumor-free after vaccination with the combination of irradiated J558<sup>-</sup> with  $\alpha$ -Gal Cer (Fig. 3 A). We were not able to vaccinate mice with live J558<sup>-</sup> cells, which grew rapidly and killed the mice. Vaccination with irradiated J558<sup>-</sup> and  $\alpha$ -Gal Cer was effective only when tumor cells were injected by the i.v. and not the s.c. route (Fig. 3 B), as predicted by the data in Fig. 1 A in which tumor cells had to be injected i.v. to be picked up well by DCs. Corresponding to the uptake data in Fig. 1 C, the level of protection varied with the dose of injected tumor cells with 10<sup>6</sup> cells being ineffective (Fig. 3 C). To evaluate the specificity of the antitumor immune response, we challenged the mice with either live Meth A sarcoma cells s.c. or live A20 lymphoma cells after vaccination with irradiated J558 with  $\alpha$ -Gal Cer. Vaccination with dying J558 L<sup>d</sup> and  $\alpha$ -Gal Cer protected mice against a challenge of J558, but not Meth A or A20 (Fig. 3 D). Likewise, immunization with dying A20 with  $\alpha$ -Gal Cer led to protection against only A20, but not J558 (Fig. 3 E). To compare  $\alpha$ -Gal Cer with other DC maturation stimuli, we studied agonistic anti-CD40 monoclonal antibody as well as the TLR3 ligand, poly I:C. The glycolipid was the only adjuvant that could independently induce protective immunity to irradiated tumor injected i.v., but the combination of anti-CD40 and poly I:C was also active (Fig. 3 F). To assess memory, we verified that



**Figure 5. Proliferation and differentiation of tumor antigen-specific T cells in response to dying tumor cell delivery in vivo.** (A)  $3 \times 10^6$  CFSE-labeled, naive CD8<sup>+</sup> P1CTL T cells were injected i.v. into BALB/c mice. 1 d later,  $20 \times 10^6$  J558<sup>-</sup> cells were injected i.v. with or without 2  $\mu$ g  $\alpha$ -Gal Cer coinjection. 3 d later, mice were killed and spleen cells were stained with CD8-allophycocyanin and CD25 or CD62L-PE. (B) Spleen cells obtained as in A were cultured at  $10^7$ /ml with 1  $\mu$ g/ml P1A 35–43

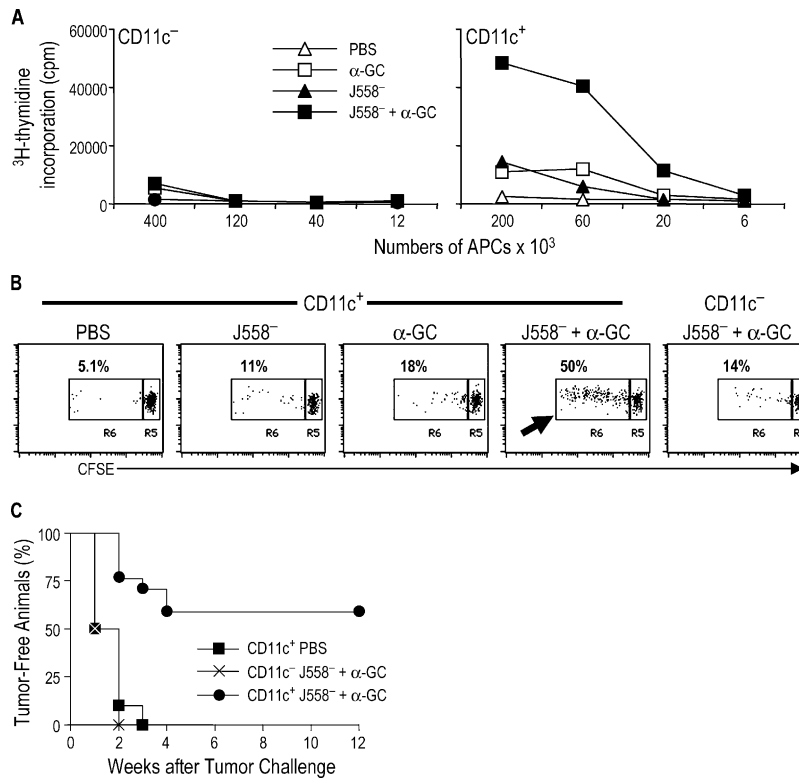
peptide (LPYLGWLVF) for 4 h at 37°C in the presence of 5  $\mu$ g/ml BFA. The depicted cells were from gated CD8<sup>+</sup>CFSE<sup>+</sup> P1A transgenic T cells, and intracellular IFN- $\gamma$  and IL-2 were identified with PE-conjugated mAb after fixation and permeabilization. Black lines are staining with isotype control antibody; gray lines are staining with anti-IFN- $\gamma$  or IL-2. Percentages are the fraction of transgenic T cells expressing intracellular cytokine above background.

vaccinated mice were protected against J558 challenge 2 mo (Fig. 3 G) and 4 mo (not depicted) after vaccination. We also induced tumor resistance if we performed an immunotherapy-type experiment and vaccinated 3 d after the injection of the tumor cells (Fig. 3 H). However, therapeutic immunity was manifest only when the tumor dose was  $10^6$  and not  $5 \times 10^6$  cells, whereas protective immunity was evident in mice challenged with both doses. Thus, long-lived tumor immunity can be elicited by a single i.v. vaccination with irradiated J558 and  $\alpha$ -Gal Cer, which also has a therapeutic effect.

#### Innate and adaptive T cells are required to generate resistance after vaccination with dying tumor and $\alpha$ -Gal Cer

To identify resistance mechanisms for the observed protective tumor immunity, we vaccinated mice with dying MHC class I<sup>-</sup> J558<sup>-</sup> plus  $\alpha$ -Gal Cer and challenged them with live MHC class I<sup>-</sup> negative or positive J558 tumor cells. The vac-

inated mice were protected against the MHC class I<sup>-</sup> positive J558 cells, but not the MHC class I<sup>-</sup> negative J558<sup>-</sup> tumor cells (Fig. 4 A), suggesting that CD8<sup>+</sup> T cells were required for resistance. As expected, NKT cells were also required for effective vaccination because J $\alpha$ 281<sup>-/-</sup> mice (also termed J $\alpha$ 18<sup>-/-</sup>), which cannot respond to  $\alpha$ -Gal Cer because they lack NKT cells (45), failed to develop immunity to dying cells plus glycolipid (Fig. 4 B). To further evaluate the type of T cells required for protective immunity 8 wk after a single vaccination, the immune mice were injected with depleting antibodies specific for CD4, CD8, or control IgG and challenged with J558 cells 1 d later. We verified by FACS that anti-CD4 and anti-CD8 antibodies depleted the respective cell populations within 2 d, and that the depletion lasted 2 wk, when they began to repopulate slowly as reported (46). As shown in Fig. 4 C, mice injected with control IgG remained resistant to J558 challenge. However, de-



**Figure 6. Maturing DCs mediate P1A antigen presentation and elicit tumor immunity in vivo.** Mice were given  $\alpha$ -Gal Cer or PBS together with irradiated J558<sup>-</sup> cells. 4 h later, CD11c<sup>+</sup> and CD11c<sup>-</sup> cells were isolated and used to stimulate CD8<sup>+</sup> TCR transgenic T cells from P1CTL mice in vitro (A) or in vivo (B). In A, in vitro T cell proliferation was measured by a [<sup>3</sup>H]thymidine pulse at 40–50 h. In B, in vivo proliferation of CFSE-labeled P1CTL T cells

was measured 3 d later. (C) Splenic CD11c<sup>+</sup> and CD11c<sup>-</sup> cells were isolated from mice injected with irradiated J558<sup>-</sup> cells and  $\alpha$ -Gal Cer 4 h earlier.  $10^6$  CD11c<sup>+</sup> or  $5 \times 10^6$  CD11c<sup>-</sup> cells were then transferred i.v. into naive BALB/c mice. 1 wk later, the mice were challenged with live  $5 \times 10^6$  J558<sup>-</sup> cells. Mice were monitored every 3 d for tumor growth. Each group includes 12–17 mice pooled from three experiments.

pletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells from vaccinated mice significantly abrogated tumor immunity elicited by J558 with  $\alpha$ -Gal Cer (Fig. 4 C). Therefore, both innate NKT cells and adaptive CD4<sup>+</sup> and CD8<sup>+</sup> T cells contribute to the tumor resistance induced by DCs capturing dying cells in vivo.

#### $\alpha$ -Gal Cer coinjection with dying cells better activates antigen-specific CD8<sup>+</sup> T cells

To document the consequences of  $\alpha$ -Gal Cer for the quality of an antigen-specific T cell response to irradiated tumor, we took advantage of the P1CTL mouse, a CD8<sup>+</sup> TCR transgenic line specific for P1A tumor antigen presented on L<sup>d</sup> MHC class I molecules (39).  $20 \times 10^6$  irradiated MHC class I-negative, J558<sup>-</sup> cells with or without  $\alpha$ -Gal Cer were injected into the mice that had received CFSE-labeled P1CTL cells 1 d earlier. T cell proliferation and phenotype were analyzed 3 d later with flow cytometry. In the absence of  $\alpha$ -Gal Cer, DCs could cross-present P1A from dying tumor cells to CD8<sup>+</sup> P1CTL T cells, driving the T cells into multiple cycles of proliferation (Fig. 5, top row). This is consistent with the capacity of CD8<sup>+</sup> DCs to present antigens on both MHC class I and II products from dying cells in the steady state (26, 27). However, the proliferating P1CTL T cells re-

tained markers typical of naive cells, i.e., low CD25 and high CD62L (Fig. 5 A, white arrows). In contrast, in the mice that had received J558<sup>-</sup> plus  $\alpha$ -Gal Cer, the T cells proliferated more extensively and many began to show an activation phenotype within 3 d, as indicated by the up-regulation of CD25 and down-regulation of CD62L (Fig. 5 A, compare black and white arrows). Furthermore, T cells that had been stimulated in the presence of  $\alpha$ -Gal Cer adjuvant in vivo were able to produce significantly more IFN- $\gamma$  and IL-2 upon brief restimulation with P1A peptide in vitro (Fig. 5 B, compare right with middle and left panels). Therefore, DCs process antigens from tumor cells and induce the proliferation of antigen-specific T cells in vivo, but a maturation stimulus is required for the differentiation of effector T cells (Fig. 5) as well as protective immunity (Figs. 3 and 4).

#### Evidence that mature DCs present tumor antigen and transfer tumor immunity

To verify that mature DCs were responsible for the presentation of antigens from the captured dying tumor cells and also elicited tumor immunity, we first isolated DCs from mice injected with dying J558<sup>-</sup> tumor cells without or with  $\alpha$ -Gal Cer. The CD11c<sup>+</sup> DC-enriched and CD11c<sup>-</sup> DC-depleted

cells from spleen were added as stimulators in culture of naive CD8<sup>+</sup> P1CTL TCR transgenic T cells without further antigen. When  $\alpha$ -Gal Cer had been coadministered, the isolated DCs were much more effective at stimulating proliferation of naive CD8<sup>+</sup> T cells in culture, whereas CD11c<sup>-</sup> cells were inactive (Fig. 6 A, right, closed squares). Next, we isolated CD11c<sup>+</sup> DCs from spleens 4 h after immunization and transferred the DCs to naive animals to test their capacity to stimulate proliferation of CFSE-labeled CD8<sup>+</sup> P1CTL T cells in vivo (Fig. 6 B). 3 d later, we detected P1CTL proliferation only in response to DCs from mice given irradiated tumor cells with  $\alpha$ -Gal Cer (Fig. 6 B, black arrow). CD11c<sup>-</sup> non-DCs from the same mice were not able to stimulate P1CTL T cells, and DCs from mice that had received J558<sup>-</sup> without  $\alpha$ -Gal Cer failed to stimulate P1CTL above the background (Fig. 6 B). Finally, to test if the antigen-presenting mature DCs were critical for inducing protective tumor immunity, we transferred DCs or non-DCs from the vaccinated mice into naive mice and then challenged them with live J558 tumor cells. As shown in Fig. 6 C, when naive mice had been given  $1.5 \times 10^6$  CD11c<sup>+</sup> DCs from donor mice injected with dying J558<sup>-</sup> cells together with  $\alpha$ -Gal Cer, 58% of mice (10 out of 17 mice tested;  $P < 0.01$ ) were fully protected. CD11c<sup>-</sup> non-DCs and CD11c<sup>+</sup> DCs from mice injected with PBS or dying J558<sup>-</sup> cells alone, failed to transfer protection to naive mice (0/12, Fig. 6 C). These data provide direct evidence that mature antigen-capturing DCs are responsible for presentation of tumor antigen and the adjuvant action of  $\alpha$ -Gal Cer in vivo.

## DISCUSSION

Genetic transduction of tumor cells is being used to increase the adaptive immune response to tumor antigens, including strategies in which cytokines and chemokines are introduced to recruit DCs to the tumor. An alternative would be to learn to deliver dying whole tumor cells to maturing DCs within lymphoid tissues because these cells have dozens of specializations pertinent to the initiation of immunity, including ready access to the recirculating pool of T cells. Here, we provide some mechanisms to address this goal.

The earliest attempts to increase the immunogenicity of whole tumor cells and stimulate T cell-mediated tumor immunity used bacterial components such as BCG or *C. parvum* as adjuvants (47–49). Although these approaches have achieved only limited success (50), they imply a need for coordination between innate immunity and adaptive immunity against tumors. Interestingly, innate NKT cells are required for the anti-tumor effects of both IL-12 and GM-CSF (45, 51), the two cytokines that are most often successful in improving immunity to genetically modified tumor cells. Our findings indicate that NKT cells can serve as superior adjuvants for protective antitumor immunity. In prior studies, we studied the capacity of NKT cells to mature DCs that were presenting the foreign antigen, ovalbumin (32, 43), but in the current paper, we have examined the generation of long-lived T cell memory and protection to a poorly immunogenic syngeneic tumor.

We find that a single dose of irradiated nonmodified tumor cells, when directed to maturing DCs in vivo, here by the i.v. route, leads to long-lived protective and combined CD4<sup>+</sup> and CD8<sup>+</sup> T cell immunity. We compared the efficacy of targeted i.v. delivery of J558 tumor to maturing DCs with the use of DCs that were derived from bone marrow precursors and loaded with J558 tumor ex vivo as described previously (6). However, two doses of 200,000 tumor loaded DCs given 1 wk apart by the s.c. route, failed to protect the mice to challenge with J558 s.c. The  $\alpha$ -Gal Cer maturation stimulus we used to mature splenic DCs in vivo induced superior protective immunity relative to other DC stimuli, such as ligation of CD40, TLR4, and TLR3. This glycolipid has been manufactured in a nontoxic form for human use (52). It is presented on CD1d molecules to activate NKT lymphocytes (42, 45), which then mature the DCs as mentioned (32, 33). When NKT cells are activated with a single dose of  $\alpha$ -Gal Cer, there is a production of immune enhancing cytokines, specifically TNF $\alpha$  and IL-12 by the DCs, and IFN- $\gamma$  by NKT and NK cells, and there is an up-regulation of CD40L on the NKT cells. To elicit protective immunity to a syngeneic tumor, we find that the combination of a proinflammatory TLR ligand, poly IC, and agonistic anti-CD40 antibody is able to mimic the effects of  $\alpha$ -Gal Cer. Interestingly, each stimulus (poly IC, anti-CD40,  $\alpha$ -Gal Cer) induces similar phenotypic changes of maturation; i.e., increased expression of CD40, CD86, MHC class II, B7-H1, B7-H2, and decreased interferon- $\gamma$  receptor or CD119, but for protective immunity, either  $\alpha$ -Gal Cer or the combination of poly IC and  $\alpha$ CD40 is required.

When DCs were isolated from mice injected with irradiated tumor cells, we could establish that the DCs from glycolipid-treated mice were better stimulators of CD8<sup>+</sup> T cells specific for the P1A tumor antigen in vitro. Importantly, transfer of these maturing DCs to naive mice protected ~60% of them, whereas direct vaccination of dying tumor cells with  $\alpha$ -Gal Cer protected 80–100% of the mice. The DC transfer experiments might not have been optimal because they entailed the transfer of only ~10–30,000 DCs bearing tumor cells (only a small percent of the CD8<sup>+</sup> DCs take up tumor cells in Fig. 1), which also might not lodge in the recipient lymphoid tissues with high efficiency.

The harnessing of DCs in this paper offers some advantages over genetic modification of tumor cells to improve immunogenicity. First, maturing DCs express a plethora of cytokines, chemokines, and accessory molecules (32, 53, 54) as illustrated in Fig. 2. Second, because of the capacity of DCs to cross-present cell-associated antigens in vivo (26, 55), the tumor cells become an effective source of antigen, potentially a spectrum of antigens for a broad immune attack. Third, by delivering tumor cells to the DCs, one enhances antigen presentation beyond the presenting capacities of the tumor cells themselves, because DCs express such efficient processing pathways for MHC class I (26), class II (55), and CD1 (56), even if the tumor cells have dampened their own antigen-presenting activities, as is often the case (57).

This is illustrated by our findings with the presentation of P1A. This is a classical tumor antigen, one of the first specific antigens to be described by Van den Eynde et al. (38). The experiments in Fig. 5 reveal the processing and presentation of the nonmutated P1A antigen from the tumor cells.

Nonetheless there are limitations to our experiments. We would like to mention three. First, the delivery of tumor cells to DCs, while enhanced by the use of the i.v. route of injection, requires a relatively large dose of irradiated tumor in mice, at least  $5 \times 10^6$  cells. Efficacy might be further increased, for example, by opsonizing the tumor or otherwise increasing the frequency of antigen-capturing DCs. Likewise, human research will be needed to determine the effective dose that will result in immunization of patients (e.g., with hematologic malignancies like myeloma). Second, we have tested only one CD1d-binding glycolipid,  $\alpha$ -Gal Cer, and there are newer synthetic and natural glycolipids that may prove to be more effective (58, 59). As mentioned,  $\alpha$ -Gal Cer has already been found to be nontoxic in humans, even at relatively high doses. Third, our studies do not address the potentially important regulation imparted by suppressor T cells which, beginning with the work of R. North and colleagues, has been shown to be able to regulate the function of protective or effector T lymphocytes (60, 61).

The approach in this paper takes full advantage of the positive feedback between NKT cells and DCs that occurs when a single dose of  $\alpha$ -Gal Cer is given together with irradiated tumor cells. We have observed specific immunity to two different hematopoietic tumors, the J558 plasmacytoma and the A20 lymphoma. We would like to suggest that vaccination with dying tumor cells, under conditions where the tumor cells are captured by DCs maturing in response to innate NKT cells, be evaluated in humans, and that this be initiated with hematologic malignancies such as the tumors tested here.

## MATERIALS AND METHODS

**Mice.** 6–8-wk-old BALB/c females were obtained from Taconic. BALB/c transgenic mice expressing a TCR specific for the tumor antigen P1A35–43:L<sup>d</sup> complex have been described previously (39). J $\alpha$ 18<sup>-/-</sup> mice on BALB/c background were gifts from M. Tsuji (New York University School of Medicine, New York, NY). Mice were maintained under specific pathogen-free conditions. All experiments were conducted according to institutional guidelines.

**Cell lines.** The plasmacytoma J558 and the MHC class I mutant cell line J558L<sup>d-</sup> have been described previously (40). The Meth A fibrosarcoma was provided by Z. Li (University of Connecticut Health Center, Farmington, CT). The A20 lymphoma was provided by H.I. Levitsky (Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD). The cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS, 100  $\mu$ g/ml penicillin/streptomycin, and 2 mM glutamine. All lines tested negative for *Mycoplasma* by Hoechst staining and PCR reaction (American Type Culture Collection).

**Reagents.** Rat mAbs for MHC class II (TIB120, M5/114.15.2), granulocytes (RB6–8C5, Gr-1), B220 (TIB146, RA3–3A1), F4/80 (HB198), CD4 (GK 1.5), and CD8 (TIB211, 3.155) were obtained from the American Type Culture Collection. Anti-CD16/32, PE-conjugated anti-CD8 $\alpha$ , CD11b, B220, CD4, V $\alpha$ 8.3, IFN- $\gamma$ , IL-2, H-2L<sup>d</sup>/H-2D<sup>b</sup>, CD62L, CD69, B7–H1, B7–DC, and allophycocyanin-CD11c were obtained from BD Bio-

sciences or eBioscience. Sheep anti-rat IgG conjugated to magnetic beads were obtained from Dynal. Anti-CD11c and CD8 microbeads were obtained from Miltenyi Biotec. The other reagents were RPMI 1640 (GIBCO BRL), FCS (GIBCO BRL), CFSE (GE Healthcare), ACK buffer (BioSource30% BSA solution (Sigma-Aldrich).  $\alpha$ -Gal Cer (2S, 3S, 4R–1-O( $\alpha$ -galactopyranosyl)-2(N-hexacosanoylamino)-1,3,4-octadecanetriol) was provided by Kirin Brewery and diluted in PBS.

**Induction of cell death.** Tumor cells were harvested, washed twice with RPMI 1640, resuspended to  $10 \times 10^6$ /ml in RPMI 1640, and irradiated with 75 Gy. Detection of apoptotic tumor cells used the annexin V-FITC Apoptosis Detection Kit (BD Biosciences), after which flow cytometry (FACS Vantage SE, Becton Dickinson) was performed. Within 24 h, 24% of the tumor cells were apoptotic, i.e., annexin V positive but PI negative (Fig. S1 B). By 48 h, 57% of the irradiated cells underwent secondary necrosis; and 72 h later, 84% of them were necrotic (PI<sup>+</sup>). Therefore, we refer to the irradiated cells that we injected as “dying cells.”

**Tumor-specific TCR transgenic T cells.** CD8<sup>+</sup> P1A-specific, T cells were prepared from meshed cell suspensions of TCR transgenic lymph nodes and spleen by depleting B220, CD4, F4/80, and MHC class II-expressing cells using sheep anti-rat IgG Dynabeads. For CFSE labeling (GE Healthcare), the cells at  $10^7$ /ml in PBS were incubated with 5  $\mu$ M CFSE for 10 min at 37°C. The reaction was stopped by washing three times with PBS.

**In vivo delivery of dying tumor cells, DC maturation stimuli, and tumor protection assay.**  $2 \times 10^7$  irradiated J558-L<sup>d-</sup> cells were injected i.v. or s.c. into BALB/c mice with or without  $\alpha$ -Gal Cer as a DC maturation stimulus. In some experiments, we compared  $\alpha$ -Gal Cer to agonistic anti-CD40 mAb (1C10, 25  $\mu$ g i.p.) or the Toll-like receptor ligands, poly IC (50  $\mu$ g i.p., Invivogen) or lipopolysaccharide (20  $\mu$ g i.p., Sigma-Aldrich). The mice had been given CFSE-labeled P1CTL CD8<sup>+</sup> T cells i.v. 1 d earlier, or were naive animals. In some experiments, mice were killed 3 d later, and T cell division and activation in spleen were analyzed by flow cytometry. Additionally, 7 d or 2 mo later,  $5 \times 10^6$  live J558 tumor cells were inoculated subcutaneously.  $5 \times 10^6$  Meth A fibrosarcoma was used as a control tumor for challenge. Tumor cell growth was measured with calipers every other day. Mice were scored positive for tumor as soon as tumors became palpable and grew progressively. Mice were killed when tumor size exceeded 400 mm<sup>2</sup>.

**Flow cytometry.** T cell division, phagocytosis of CFSE-labeled tumor cells, and acquisition of cell surface-activation markers were determined by flow cytometry. In brief, spleens were harvested and low density splenocytes were stained with CD11c-allophycocyanin and CD8 $\alpha$ -PE, CD11b-PE, B220-PE, or CD4-PE for the up-take examination. For in vivo proliferation of P1CTL T cells, spleens were harvested and the splenocyte suspension was staining with CD8 $\alpha$ -Cy, V $\alpha$ 8.3-PE, CD25-PE, and CD62L-PE. For intracellular cytokine staining, splenocytes were stimulated in vitro with 1  $\mu$ g/ml P1A peptide in 5  $\mu$ g/ml brefeldin A (Sigma-Aldrich) at 37°C for 4 h. The cells were first stained with CD8 $\alpha$ -Cy-Chrome, fixed, permeabilized with cytofix-cytoperm buffer (BD Biosciences), and stained with PE-conjugated mAbs to IL-2 or IFN- $\gamma$ .

**In vivo depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes.** Mice vaccinated with J558 and  $\alpha$ -Gal Cer were injected with ascites containing 1 mg of rat monoclonal anti-CD4 (clone GK 1.5) or anti-CD8 (clone 53–6.72). The mice received three daily injections, the first one i.v. 1 d before the challenge, and two i.p. injections on the day of the challenge and 1 d after the challenge. Control mice received 1 mg of rat IgG (Jackson ImmunoResearch Laboratories). The depletion was monitored by staining with anti-CD4 and anti-CD8 antibodies followed by flow cytometry (BD Biosciences).

**Online supplemental material.** Fig. S1 A shows lack of MHC class I expression on J558 cells relative to parental J558 plasmacytoma. Fig. S1 B



depicts death of J558 cells, untreated or 24/48/72 h after  $\gamma$ -irradiation, as assessed by staining with annexin V and propidium iodide. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20050956/DC1>.

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