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This information is current as of October 14, 2019.

*J Immunol* 2004; 173:1012-1022; ;  
doi: 10.4049/jimmunol.173.2.1012  
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# T Cell Immunity Induced by Live, Necrotic, and Apoptotic Tumor Cells<sup>1</sup>

Wolf C. Bartholomae,\*<sup>†</sup> Frauke H. Rininsland,\* Julia C. Eisenberg,\* Bernhard O. Boehm,<sup>†</sup> Paul V. Lehmann,\* and Magdalena Tary-Lehmann<sup>2\*</sup>

The rules that govern the engagement of antitumor immunity are not yet fully understood. Ags expressed by tumor cells are prone to induce T cell tolerance unless the innate immune system is activated. It is unclear to what extent tumors engage this second signal link by the innate immune system. Apoptotic and necrotic (tumor) cells are readily recognized and phagocytosed by the cells of the innate immune system. It is unknown how this affects the tumor's immunogenicity. Using a murine melanoma (B16m) and lymphoma (L5178Y-R) model, we studied the clonal sizes and cytokine signatures of the T cells induced by these tumors in syngeneic mice when injected as live, apoptotic, and necrotic cells. Both live tumors induced a type 2 CD4 cell response characterized by the prevalent production of IL-2, IL-4, and IL-5 over IFN- $\gamma$ . Live, apoptotic, and necrotic cells induced CD4 (but no CD8) T cells of comparable frequencies and cytokine profiles. Therefore, live tumors engaged the second signal link, and apoptotic or necrotic tumor cell death did not change the magnitude or quality of the antitumor response. A subclone of L5178Y-R, L5178Y-S cells, were found to induce a high-frequency type 1 response by CD4 and CD8 cells that conveyed immune protection. The data suggest that the immunogenicity of tumors, and their characteristics to induce type 1 or type 2, CD4 or CD8 cell immunity is not primarily governed by signals associated with apoptotic or necrotic cell death, but is an intrinsic feature of the tumor itself. *The Journal of Immunology*, 2004, 173: 1012–1022.

The activation state of the innate immune system is thought to play a critical instructive role in the decision whether an immune response is induced or tolerance develops after the (tumor) Ag is encountered. The induction of an immune response has been primarily linked to infectious nonself signals (1, 2), that is, to microbial products. In this process, receptors that are specific for various pathogen-associated-molecular patterns (3, 4), including TLR, recognize conserved microbial products (5, 6). The contamination of pure protein Ags with such microbial products (emulsifying the Ag in mycobacteria-containing adjuvant) has been the immunologist's "dirty little secret" for the induction of immunity, and of autoimmunity, for decades (7); Ag injections without such TLR-activating substances have served as classic protocols for the induction of tolerance (8). Because tumors clearly lack such microbial components that could serve as infectious nonself signals, they, like other cells of the body, are thought to be prone to induce tolerance.

It has been a matter of debate to what extent noninfectious danger signals, such as those associated with necrotic cell death can activate the innate immune system and can trigger the induction of

an autoimmune/antitumor immune response. Injured and stressed cells produce a variety of signals that can activate the innate immune system (9). Complement activation is invariably associated with necrotic (but not apoptotic) cell death (10). This leads to the local activation of dendritic cells (DC)<sup>3</sup> and macrophages. Moreover, tissue injury induces the expression of the MHC class I-like molecule MHC class I-related chain, which activates DC, NK cells, and  $\gamma\delta$  T cells via the NKG2D receptor (11). Also, heat shock proteins (hsp) expressed by damaged/stressed cells have been shown to activate DC (12, 13), whereby tumor-derived hsp70 (14) and hsp gp96 trigger DC receptors such as CD91 (15). It was shown that necrotic cells (unlike apoptotic cells) excel in inducing maturation of DC (16, 17). All of these DC/innate immune system-activating properties of necrotic cell death seem to suggest that necrotic cell death (unlike apoptotic cell death) is associated with danger signals that might contribute to the induction of an immune response. However, the development of autoimmunity after necrotic tissue injury is the rare exception (18). Does necrosis in tumors facilitate the induction of an antitumor response? Presently, this question cannot be answered definitively from the literature. In one recent study, DC were pulsed in vitro with sorted apoptotic or necrotic tumor cells. Both elicited similar immune responses (19). Such experiments leave open the question to what extent the in vitro manipulation of the DC results in DC activation on its own.

For a priori reasons, apoptotic cell death should not contribute to the induction of (auto/antitumor) immunity. Apoptotic cell death is the common way for a cell to die, and self-nonself discrimination (as we think of it today) would fail if dying cells would trigger an immune response against themselves (20). However, cells that undergo apoptosis are readily engulfed by DC (21) via  $\alpha_v\beta_5$ , CD36 (22), and PS receptor (23), and subsequently, the DC efficiently

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Received for publication June 26, 2003. Accepted for publication May 14, 2004.

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<sup>1</sup> This work was supported National Institutes for Health Grant AI47756 and American Cancer Society Grant RPG-95-092-03-LBC (to M.T.-L.). F.H.R. was supported by National Institutes of Health Training Grant in Tumor Immunology T32 AI-07427. W.C.B. is a fellow of the Studienstiftung des Deutschen Volkes, and J.C.E. is a fellow of the Evangelisches Studienwerk Viligst.

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; hsp, heat shock protein; TRP, tyrosinase-related peptide.

presents the Ags of the apoptotic cell to class I-restricted CTLs (21). In vivo, it is unclear whether this process leads to the induction of tolerance (in the absence of the second signal) or, defying the rules of immunology as we know them today, to immunity. It was shown that DC pulsed with apoptotic tumor cells induce a strong Ag-specific T cell response in vitro, and when injected into mice, such pulsed DC induced tumor-specific cytotoxic T cell responses and long-term protection from parental tumor challenge (24) (in these models, DC activation might result from the in vitro manipulation of the DC). It was also suggested that an increase of the apoptotic process in poorly immunogenic tumor cells can lead to a remission of established peritoneal carcinomatosis (25), and more recently, that apoptotic but not necrotic tumor cells are efficient vaccines in vivo (26). Using the RMA T cell lymphoma model, it was demonstrated that the injection of apoptotic tumor cells results in the priming of a functional long-lasting tumor-specific immune response, and that this occurs via cross-priming (27). Apparently contradicting these results, it has been reported that the presence of apoptotic cells during macrophage activation increases their secretion of anti-inflammatory mediators including TGF- $\beta$  (28) and decreases their secretion of proinflammatory cytokines (29, 30), and that apoptotic cells are actually able to induce active suppression of inflammatory responses (31). In line with this notion, there is evidence for the lack of immunogenicity (32) or reduced immunogenicity of apoptotic tumor cells as compared with viable counterparts (33). Given the predominance of apoptotic cell death in most common cancer treatment regimens (34), it is important to know whether the enhanced tumor Ag presentation associated with DC presenting these Ags will enhance the development of immunity, or of immune tolerance to the tumor.

Evidence that originated from our laboratory suggests that Ag encounter by the T cell in the absence of microbial danger signals tends to induce type 2 immunity (35–37). This leads to active (adoptively transferable) protection from autoimmune destruction (38). Also in tumor tolerance, therefore, it might be important to distinguish between the lack of an immune response and the induction of a nonrejecting immune response (21). As in autoimmunity, type 2 immunity might protect the tumor from immune destruction.

The low clonal sizes of Ag-specific T cells has made it challenging to obtain high-resolution data on the frequency and type 1/type 2 cytokine signature of tumor-specific T cells in vivo. This creates difficulty in answering the question to what extent live, apoptotic, and necrotic tumors are immunogenic, and what type of immune responses they induce. We used a new generation computer-assisted ELISPOT analysis to approach this (39). When freshly isolated cell material was tested in recall assays of 24-h duration (a time period too short for proliferation or cytokine differentiation to occur in vitro), the assay provided information on the frequencies (clonal sizes) and type 1/type 2 effector cell lineage of the (tumor) Ag-specific T cells in vivo, with a detection limit of 1 in a million (37, 39). Using this high-resolution approach, we first tested whether live tumors induce immunity. We selected L5178Y-R (referred to as LR) and L5178Y-S (LS) lymphoma cells that were subclones of the same tumor, but differed in their tumorigenicity: LR establishes autonomous growth in syngeneic DBA/2 mice, whereas LS does not establish uncontrolled growth in these mice. We extended these studies to B16 melanoma (B16m), which is lethal for syngeneic C57BL/6 mice. Subsequently, we compared the frequency and cytokine signature of CD4 and CD8 cells induced by the live tumors with those induced by the same number of apoptotic and necrotic cells.

## Materials and Methods

### *Mice and tumor models*

C57BL/6 and DBA/2J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the animal facility of Case Western Reserve University under specific pathogen-free conditions. Female mice were used at 6–10 wk of age. B16 melanoma BL6 (B16m), L5178Y-R (LR), L5178Y-S (LS), and P815 were obtained from American Type Culture Collection (Manassas, VA). B16m is a murine melanoma of a C57BL/6 background. LR is a murine leukemia, derived in 1952 from a thymic tumor induced in DBA/2 mice by methylcholanthrene. LR is highly tumorigenic. LS is a nontumorigenic subclone of LR. For tumor challenge, a total of  $2 \times 10^5$  tumor cells were injected in a volume of 500  $\mu$ l of PBS. B16m was injected i.v., which led to 100% lethality within 28–35 days. After 2 wk, the number of lung metastases was counted at  $\times 10$  magnification. For tumor challenge with LR and LS, the tumor cells were injected i.p. For LR, 100% lethality occurred within  $\sim 14$  days. Mice injected with LS showed no signs of tumor growth over a period of 6 mo. Mice were examined twice a day for survival beginning at the time of tumor injection. The mice injected with LR and LS were weighed daily after tumor injection.

### *Apoptosis and necrosis induction*

Necrosis was induced by osmotic shock treatment. Tumor cells were exposed to distilled water for 7 min, and then resuspended in medium and assessed under a fluorescence microscope using acridine orange/ethidium bromide staining. This treatment induced 100% cell death, yet cells were still discernible. The induction of apoptosis in these tumor cells required standardization. We treated the tumor cells with dexamethasone (10  $\mu$ M; for 4–24 h), the topoisomerase 1 inhibitor camptothecin (2  $\mu$ M; for 6 h), UV-B irradiation (for up to 1 h), and staurosporine (10  $\mu$ g/ml; for 4–24 h). Of these treatments, staurosporine induced the highest percentage of apoptotic cells. We tested different incubation periods with staurosporine and found that 6-h incubation with LR and 24-h incubation with B16m yielded the highest percentage of apoptotic cells. Results of such optimized staurosporine treatments are shown in Fig. 3. In each experiment, the level of apoptosis induced was evaluated using Annexin V<sup>FITC</sup>/propidium iodide staining. Fluorescein-conjugated annexin V (Annexin V<sup>FITC</sup>), propidium iodide staining solution, and binding buffer were purchased from BD Pharmingen (San Diego, CA). The tumor cells were adjusted at  $1 \times 10^6$  cells/ml. After washing twice with cold PBS, the cells were resuspended with  $1 \times$  binding buffer. One hundred microliters of the cell solution was transferred in a 5-ml culture tube and stained with 5  $\mu$ l of Annexin V<sup>FITC</sup> and 10  $\mu$ l of PI. After mixing gently and incubating for 15 min at room temperature in the dark, 400  $\mu$ l of  $1 \times$  binding buffer was added to each tube. The samples were then analyzed by flow cytometry, using a BD Biosciences (San Jose, CA) FACScan. For experiments using fixed cells, the designated cells were adjusted at  $5 \times 10^5$ /ml, and suspended in 1 ml of 1% paraformaldehyde for 20 min at room temperature in the dark. Then, the cells were washed twice with PBS and adjusted to the correct concentration for injection into the mice.

### *Cytokine ELISPOT assays*

These assays were performed as previously described (37). Briefly, ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH) were coated overnight at 4°C with the cytokine-specific capture Abs specified below. The plates were washed three times with PBS, and then blocked with 1% BSA in PBS for 2 h at room temperature. Freshly isolated spleen cells were plated at  $10^6$  cells/well in serum-free medium, HL-1 (BioWhittaker, Walkersville, MD), supplemented with L-glutamine and penicillin/streptomycin, in the presence or absence of 10,000-rad-irradiated B16m ( $2 \times 10^4$ /well) or LR and LS tumor cells ( $5 \times 10^4$ /well). As a positive control, anti-CD3 (2C11) was added to the wells at a concentration of 3  $\mu$ g/ml. After 24 h (for IFN- $\gamma$  and IL-2) or 48 h (for IL-4 and IL-5), cells were removed from the incubator and washed three times with PBS and four times with PBS containing 0.05% Tween (PBST). Then, the biotinylated detection Abs were added and incubated at 4°C overnight. The plates were then washed three times with PBST and streptavidin-HRP conjugate (DakoCytomation, Carpinteria, CA) was added at a 1/2000 dilution, incubated for 2 h at room temperature, and removed by washing twice with PBS. The spots were visualized by adding HRP substrate 3-amino-9-ethylcarbazole (Pierce, Rockford, IL). To stop the reaction, the plates were washed with distilled water, air dried, and analyzed with the Series 1 ImmunoSpot Image Analyzer (Cellular Technology). We used the following combinations of mAbs for the cytokines tested: IFN- $\gamma$  (R46A2; 4  $\mu$ g/ml), IL-2 (JES6-1A12; 4

$\mu\text{g/ml}$ ), IL-4 (11B11; 4  $\mu\text{g/ml}$ ), and IL-5 (TRFK5; 2.5  $\mu\text{g/ml}$ ). For detection, the following mAbs were used: IFN- $\gamma$  (XMG1.1-biotin; 0.25  $\mu\text{g/ml}$ ), IL-2 (JES6-5H4-biotin; 2  $\mu\text{g/ml}$ ), IL-4 (BVD6-24G2-biotin; 4  $\mu\text{g/ml}$ ), and IL-5 (TRFK4-biotin; 4  $\mu\text{g/ml}$ ).

#### Statistical analysis

All statistical tests were conducted using SigmaStat (version 7.0; SPSS, Chicago, IL) software. Statistical significance was set at  $p \leq 0.05$ . The overall significance of the differences was calculated with either  $t$  test or the Mann-Whitney rank sum test, as specified.

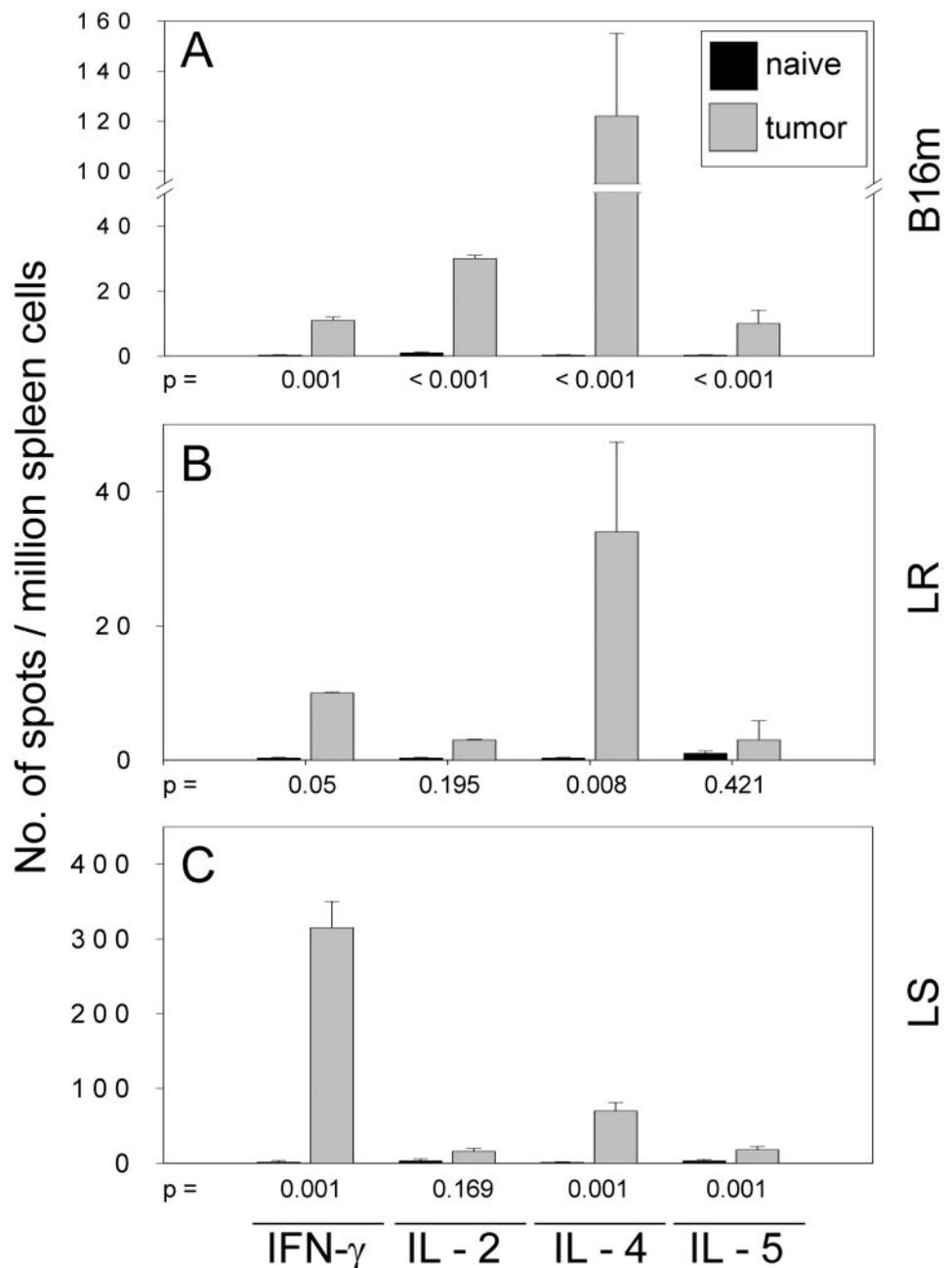
## Results

### The frequency of B16m, LR, and LS tumor-reactive cytokine-producing memory T cells is $<1,000,000$ in naive mice

It is presently unknown to what extent either cross-reactive environmental priming or autoreactivity (most tumor Ags are autoantigens) shape the preimmune tumor-specific T cell repertoire. We

used the single-cell resolution of the cytokine ELISPOT assay to address this question for B16m, LR, and LS. This assay permits accurate frequency measurements of Ag-specific cytokine-producing T cells in the 1:1,000,000 to 1:10,000 range (39). The production of IL-2 (as the cytokine produced by naive, Th0, and Th1 cells), IFN- $\gamma$ , IL-4, and IL-5 was measured in freshly isolated spleen cells of naive, PBS-preinjected mice. A 24- to 48-h coculture of spleen cells with the respective tumor did not induce cytokine production in naive mice exceeding this medium background (Fig. 1, ■; in the medium control, the numbers of spots for each of these cytokines was  $<1$  within the  $1 \times 10^6$  cells plated). These data show, first, that B16m, LR, and LS do not produce IL-2, IL-4, IL-5, or IFN- $\gamma$  on their own. Second, neither of these tumors induces the production of IL-2, IL-4, IL-5, or IFN- $\gamma$  in the cells of the innate immune system, although such cells can produce these cytokines and have the ability to recognize tumors. Therefore, the tumors did not induce a detectable activation of the

**FIGURE 1.** Frequency and cytokine profile of tumor-induced ELISPOTs in naive and tumor-primed mice. Tumors B16m (A), LR (B), and LS (C) were injected into syngeneic mice,  $2 \times 10^5$  tumor cells per mouse. Eight days later, the spleen cells of the tumor-injected mice (■) or of PBS control-injected naive mice (□) were tested directly ex vivo in ELISPOT assays for the tumor-induced production of cytokine. The frequency of IFN- $\gamma$ , IL-2, IL-4, and IL-5-producing cells is shown for individual mice that are representative for the six animals tested in each group. The mean and SD of spots in three replicate wells is shown. The data were reproduced in three independent experiments that also involved six mice per group in each experiment. The statistical significance indicated by the  $p$  values under each bar has been calculated for the difference between the numbers of spots induced in the total of 18 naive vs the total of 18 tumor-primed mice.



innate immune system. Third, the data show that the frequency of tumor-specific memory T cells that produce the cytokines in question is  $<1$  million in naive mice (being the detection limit of the assay as performed) (39).

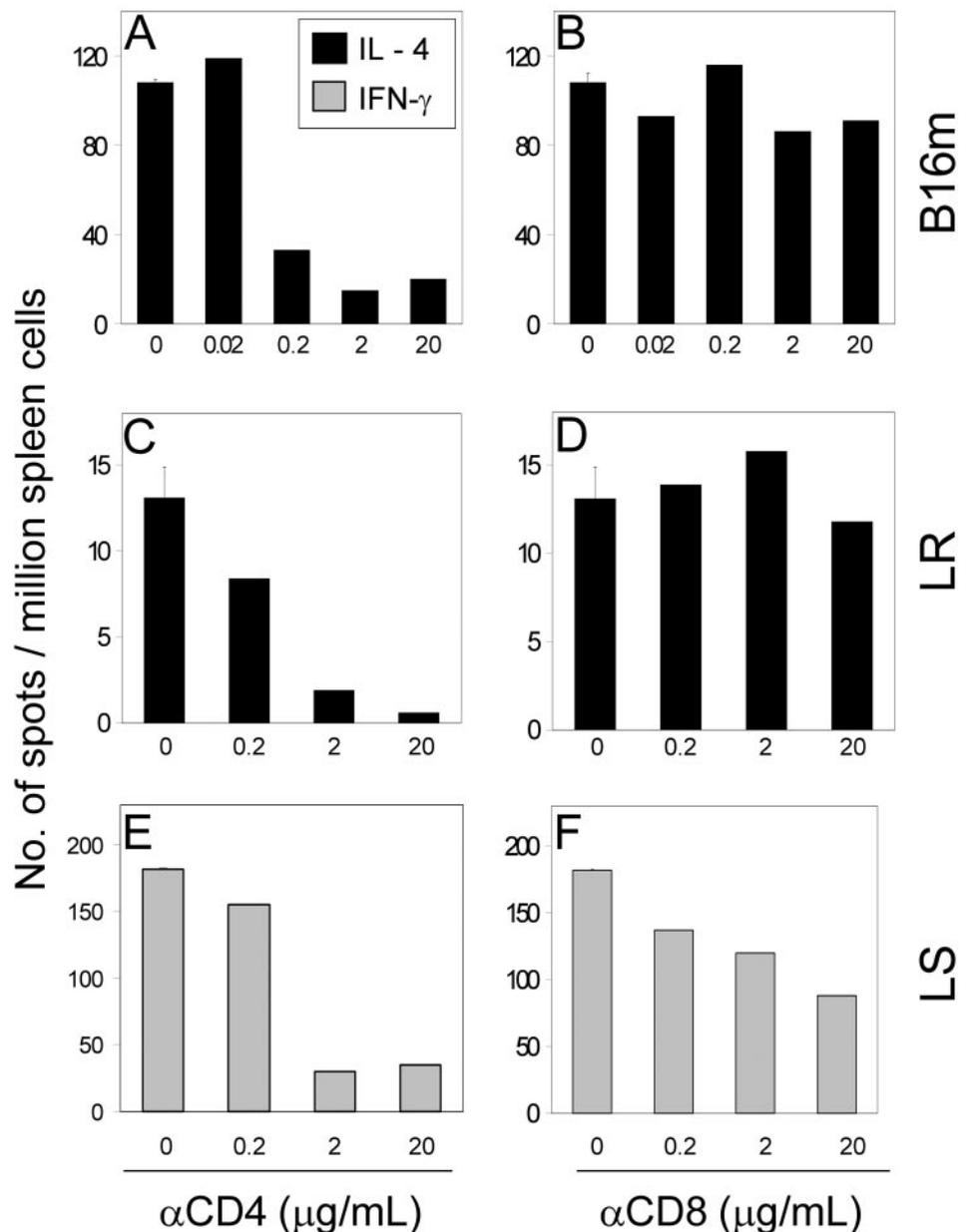
#### *B16m, LR, and LS are immunogenic in syngeneic mice*

B16m, LR, and LS were injected into syngeneic mice. Eight days later, their spleen cells were tested directly *ex vivo* in ELISPOT assays for the numbers of spots induced by the respective tumor. Representative data are shown in Fig. 1 (■). Of the 36 mice tested individually, all showed a clear-cut cytokine response against the tumor. Although the frequency of the tumor-induced cytokine-producing cells was low (in the  $1:10^6$  to  $1:10^4$  range), the numbers of spots induced were 3- to 300-fold higher than in the control wells (which contained either spleen cells of the tumor-injected mice in medium alone, or spleen cells of PBS-preinjected mice cultured with the tumor cells). This resulted in highly significant frequency elevations of some of the tumor-induced cytokine spots in the tumor-injected mice (specified in Fig. 1).

Therefore, all three tumors induced an immune response in syngeneic mice.

#### *B16m and LR induce prevalent type 2 immunity, and LS triggers type 1 response*

As shown in Fig. 1C, LS cells that are not tumorigenic, induced IFN- $\gamma$ -producing memory T cells in the 300- to 400-per-million frequency range. The frequency of the LS-specific memory cells that produced IL-4 was  $\sim 4$ -fold lower. This IFN- $\gamma$  was produced by CD4 and by CD8 cells (Fig. 2, *E* and *F*). Therefore, the LS tumor induced a type 1-polarized CD4 and CD8 cell response. In contrast, the tumorigenic LR subline induced IL-4-producing memory cells in the 35-per-million frequency range with  $<10$ -per-million IFN- $\gamma$ -producing memory cells (Fig. 1B). This IL-4 was produced by CD4 cells (Fig. 2, *C* and *D*; as was the IFN- $\gamma$ , data not shown). The anti-LR CD4 cell response was therefore type 2 biased. For B16m, IL-4 production was seen in the 100-per-million frequency range in the absence of IFN- $\gamma$  (Fig. 1A), and this IL-4 was produced by CD4 cells (Fig. 2, *A* and *B*). Therefore, B16m induced a type



**FIGURE 2.** Definition of the tumor-induced cytokine-producing memory cells as CD4 or CD8 cells. Spleen cells of mice that were preinjected with B16m (*A* and *B*), LR (*C* and *D*), or LS (*E* and *F*) were challenged with the respective tumor, and the number of IL-4-producing (■) or IFN- $\gamma$ -producing (▒) cells was measured in the presence of anti-CD4 (*A*, *C*, and *E*) or anti-CD8 (*B*, *D*, and *F*) Abs that were included in the culture at the specified concentrations. The legend to Fig. 1 applies for the tumor priming of mice and for assay conditions. The data shown for individual mice are representative for a total of nine mice tested for each tumor in three repeat experiments.

2-polarized CD4 response. Overall, the high resolution analysis of the immune response induced by these three tumors showed a type 2-biased CD4 cell response for the two tumors whose growth is not controlled by the immune system, and a type 1-biased CD4 and CD8 cell response for the tumor cells that cannot establish uncontrolled growth in syngeneic mice.

*Apoptotic and necrotic tumor cells induce T cell responses with comparable clonal sizes and cytokine signature as those of live tumor cells*

Tumor cells in culture proved to be >99% viable as judged by acridine orange/ethidium bromide staining (data not shown). Such cells were injected as viable tumor cells. Treatment of such cells with hypotonic shock (7 min in distilled water) resulted in 100% dead cells as judged by acridine orange/ethidium bromide staining (data not shown). Such cells were injected as necrotic tumor cells. After comparing several apoptosis-inducing agents (see *Materials and Methods*), we selected staurosporine treatment: results of the optimized protocol are shown in Fig. 3. Such cells were injected as apoptotic LR and B16m cells (LS was resistant to apoptosis induction). Therefore, the apoptotic vaccine contained 4.3% live LR tumor cells and 11.8% live B16m cells, corresponding to a 95.7 and 88.2% purity for the desired apoptotic population, respectively. Sorting by flow cytometry not only did not provide higher purity but moved the early apoptotic population (that we intended to study) into the late apoptotic/dead stage. We argue that, if apoptotic cells behave immunologically different than live cells, then 95.7 and 88.2% apoptotic cells (contaminated with 4.3 and 11.8% live cells) should have a clear impact vs the injection of the live-cell control.

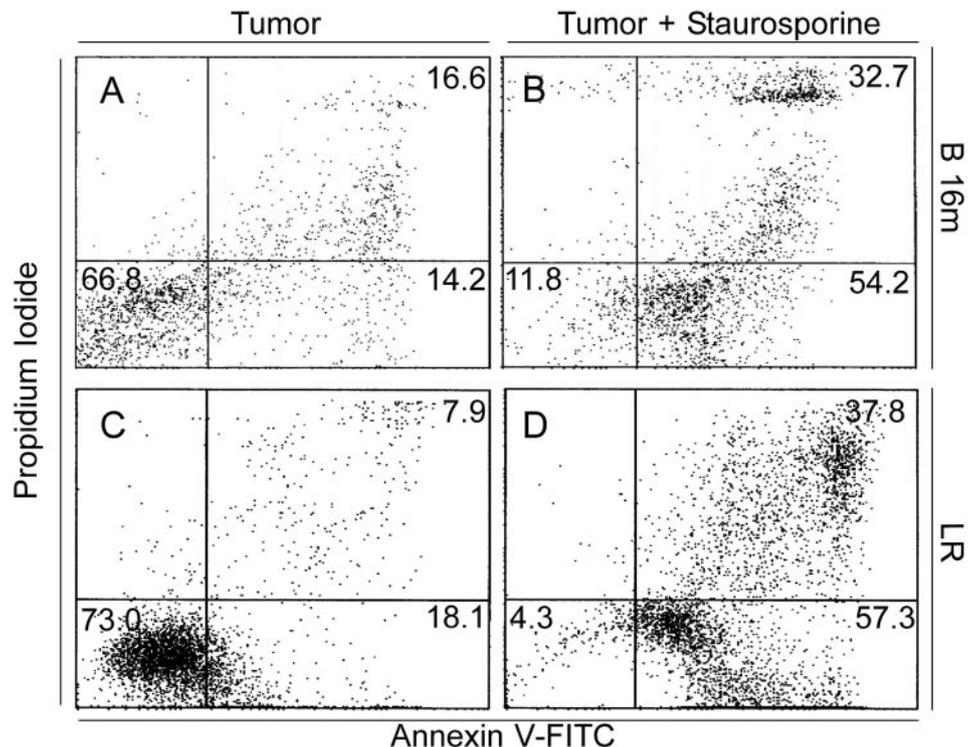
Equal numbers ( $2 \times 10^5$ ) of live, necrotic, and apoptotic B16m and LR cells were injected into syngeneic mice. Eight days later, the spleen cells were challenged with the live tumor, and the frequency of tumor-induced cytokine-producing cells was measured in ELISPOT assays. Results for individual mice are shown in Fig. 4. For B16m and LR each, six mice were tested per group, each in

three independent experiments. The comparison of all 18 mice in each group and for each cytokine showed that, overall, the frequencies of IFN- $\gamma$ -, IL-2-, IL-4-, and IL-5-producing cells were not significantly different among mice primed with live, apoptotic, and necrotic tumor cells. The exception was IL-2 production in the mice that were primed with apoptotic B16m cells vs the viable tumor-injected group ( $p = 0.002$ ), and IL-5 production in the mice injected with necrotic and apoptotic LR cells vs the group primed by the viable tumor ( $p = 0.014$  and  $p < 0.001$ , respectively).

We studied the CD4/CD8 lineage of the T cells induced in these experiments, because apoptotic cells are rapidly phagocytosed by DC, and this might have resulted in enhanced tumor Ag presentation on class I molecules. We used apoptotic, necrotic, and live tumor cells to recall the responses induced by the apoptotic necrotic or live tumor and included anti-CD4/CD8 Abs in each recall assay. The data shown in Fig. 2 for the viable tumor cells fully describe the results obtained with apoptotic and necrotic B16m and LR tumor: also the T cells primed in vivo by apoptotic and necrotic cells were blocked by CD4, but not by CD8 Abs (data not shown). Therefore, apoptosis or necrosis did not affect the CD4/CD8 class of the T cell response induced.

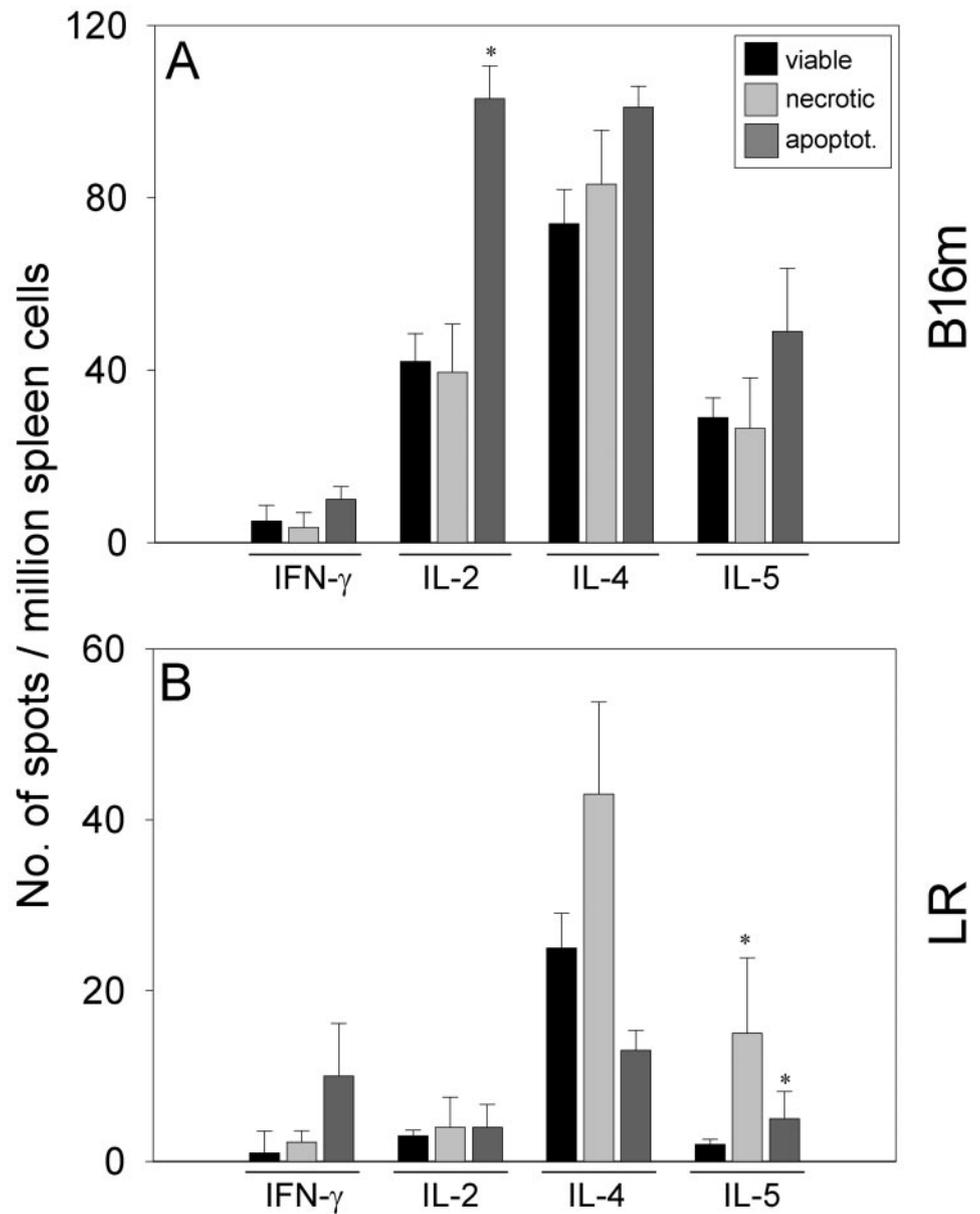
*Apoptotic or necrotic B16m or LR tumor does not excel over live tumor in inducing protective immunity*

Intraperitoneal injection of  $2 \times 10^5$  live LR cells resulted in a characteristic weight change in the recipient mice: during the first 5–7 days, there was an initial weight gain due to ascites induction; subsequently, although the ascites continued to increase in volume, the weight declined as the mice developed cachexia (Fig. 5A) and died between days 10–13 (B). The growth of B16m was assessed by counting the number of lung metastases that were readily visible under  $\times 10$  magnification (Fig. 5C). Injection of  $2 \times 10^5$  osmotic shock-treated LR or B16m cells did not lead to detectable tumor growth over an observation period of up to 2 mo, confirming the effective induction of necrotic cell death.



**FIGURE 3.** Staurosporine-induced apoptosis. B16m and LR tumor were cultured with staurosporine or in medium as specified in *Materials and Methods*. The cells were stained with Annexin V<sup>FITC</sup> and propidium iodide. The percentage of early apoptotic cells is shown in the *bottom-right quadrant*. Cells in the *upper-right quadrant* are late apoptotic and necrotic.

**FIGURE 4.** Frequency and cytokine profile of tumor-induced ELISPOTs in mice primed with viable, necrotic, and apoptotic tumor. Viable, apoptotic, and necrotic tumor cells were prepared as specified in *Materials and Methods*, and  $2 \times 10^5$  cells were injected into syngeneic mice. Eight days later, the spleen cells of the tumor-injected mice were tested directly ex vivo in the ELISPOT assays specified for the tumor-induced production of cytokine. The frequency of IFN- $\gamma$ -, IL-2-, IL-4-, and IL-5-producing cells is shown for individual mice that are representative for the six animals tested in each group. The mean and SD of spots in three replicate wells is shown. The data were reproduced in three independent experiments that also involved six mice per group in each experiment.

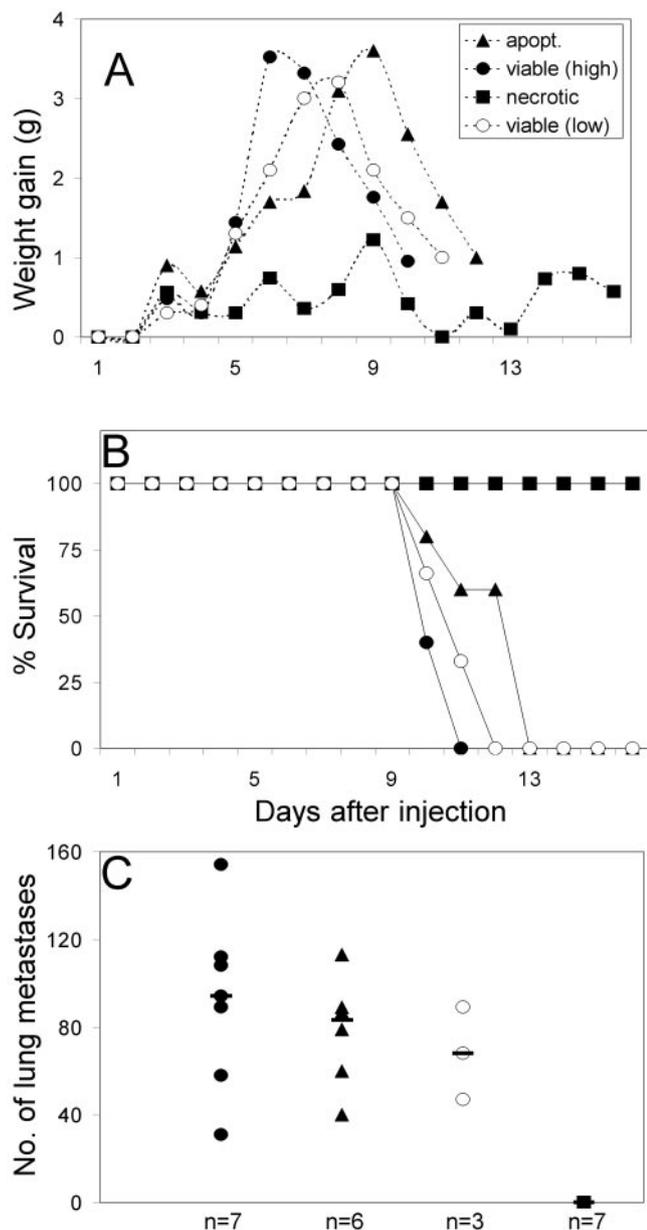


When apoptotic LR cells were injected (which typically contained  $\sim 10\%$  viable cells; see Fig. 3), the weight curve and the survival curve was shifted to the right by 3 days (Fig. 5, *A* and *B*). Because a moderate delay in tumor progression was seen when lower numbers of viable tumor cells were injected (Fig. 5), this right shift seen with apoptotic cells might merely reflect the reduced number of viable cells injected. Also with B16m, mice injected with the apoptotic cells developed similar numbers of lung metastases to those of the mice injected with the viable cells (Fig. 5C; the frequency of viable cells within the apoptotic B16m inoculum was  $\sim 15\%$ ). Therefore, in both models, the majority of apoptotic cells present in the inoculum did not succeed in eliciting a T cell response that would have prevented or substantially delayed the outgrowth of the minority of viable tumor cells present in the same inoculum.

We then tested whether vaccination with apoptotic or necrotic LR or B16m cells would protect from a subsequent challenge with the respective live tumor. Mice were injected with  $2 \times 10^5$  necrotic cells, and the same numbers of paraformaldehyde-fixed viable cells, or fixed apoptotic cells (in these experiments, we needed

to vaccinate with fixed cells to avoid mice succumbing to the live tumor cells contained in the vaccine). Nine to 10 days later, these mice were challenged with  $2 \times 10^5$  viable tumor cells. The tumor growth was monitored. The tumor progressed in all of these mice at a comparable rate (no statistical difference between the groups), as it did in PBS-preinjected control mice (Fig. 6). Therefore, the challenge experiments provided no evidence for apoptotic or necrotic LR or B16m cells being more potent tumor vaccines than the viable cells.

We characterized the tumor-specific T cells in these vaccinated/challenged mice. In Fig. 7A, data are shown for mice that were vaccinated with fixed-apoptotic LR cells, challenged with the live LR tumor 10 days later, and tested for anti-LR reactivity on day 18. The clonal sizes of the LR-specific T cells and their cytokine signatures were essentially the same as after a primary tumor challenge (Fig. 7A vs 1B). The CD4 lineage of the response (Fig. 7B, vs 2, C and D) was also identical. Results supporting the same conclusion were obtained in mice that were first injected with necrotic LR cells, and challenged with viable LR cells (data not shown). In the B16m model, a primary vaccination with apoptotic

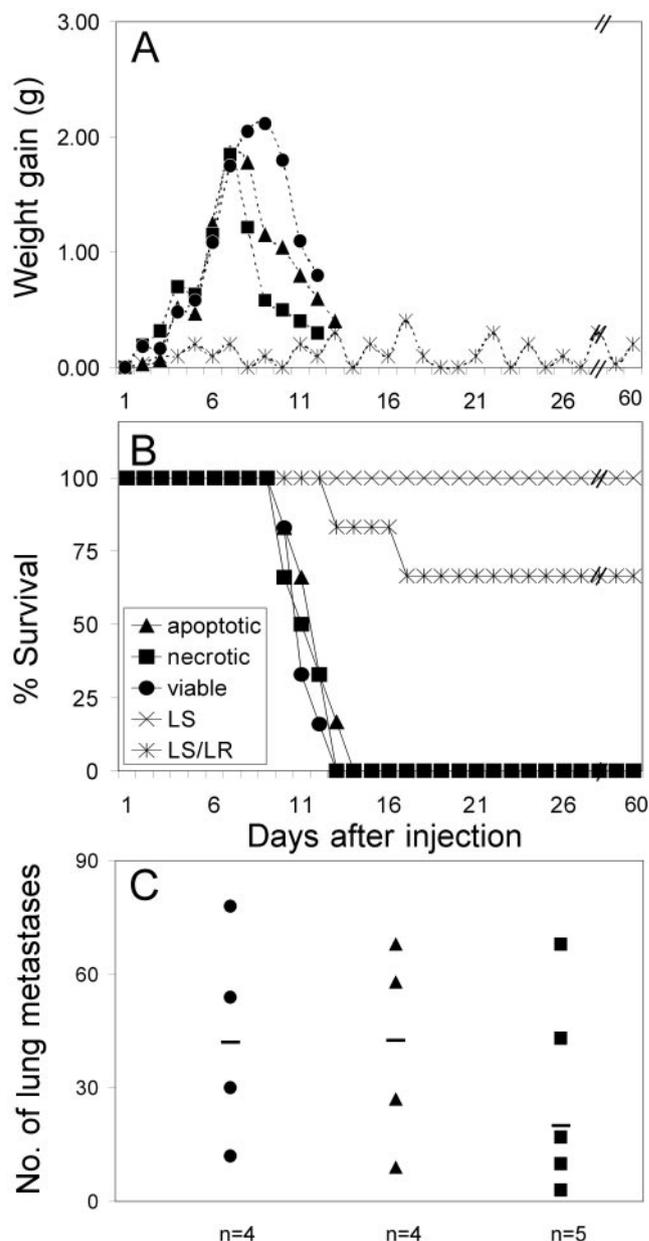


**FIGURE 5.** Tumor growth after primary inoculation of viable, apoptotic, and necrotic tumor cells. Apoptotic and necrotic cells were injected at  $2 \times 10^5$  cells per mouse. Viable cells were injected at the same dose (viable high) and at  $1 \times 10^5$  (viable low). **A**, Weight of mice after i.p. injection of LR tumor. **B**, Survival curve for LR-injected mice. **C**, Lung metastases counted on day 14 in B16m-injected mice. Viable, apoptotic, and necrotic cells at the  $2 \times 10^5$  dose were injected in two experiments, three to four mice per group; live cells of the  $1 \times 10^5$  dose were injected in one experiment, three mice per group. The data summarize the results from both experiments.

or necrotic cells did not affect the low clonal sizes and type 2 cytokine signatures in the rechallenged mice vs primarily injected mice (data not shown).

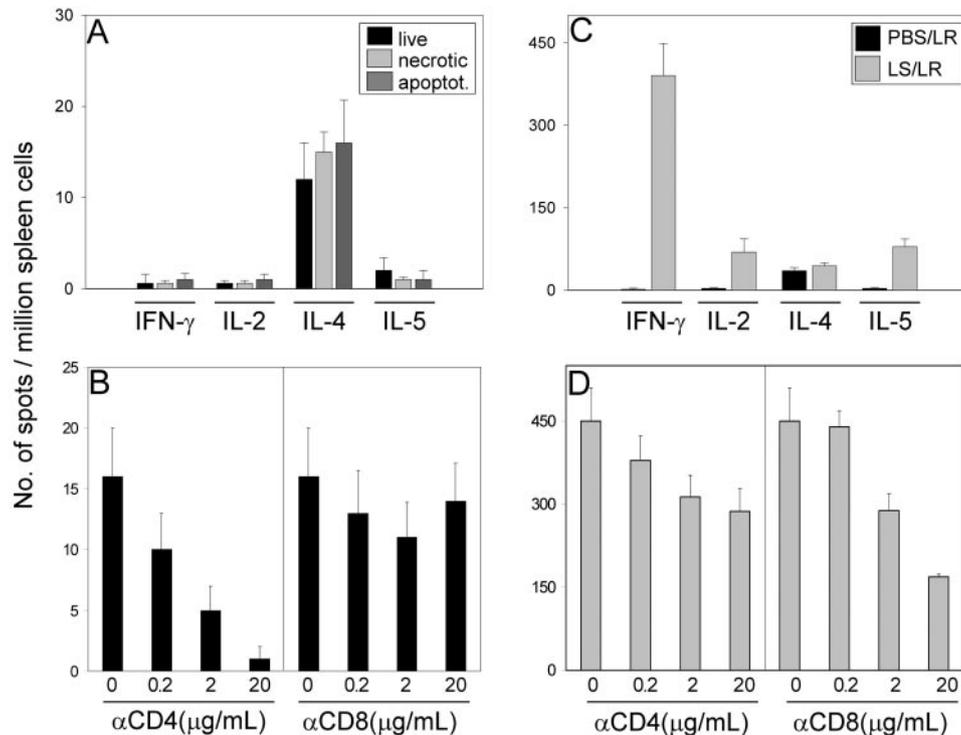
#### *Immunity induced by vaccination with LS tumor is protective against LR tumor*

Based on the fundamentally different type of T cell response that the nontumorigenic LS cells induced vs the tumorigenic LR cells (they are subclones of the same tumor), we hypothesized that the T cell response seen with LS (the relatively high-frequency tumor-



**FIGURE 6.** Tumor growth in mice vaccinated with paraformaldehyde-fixed viable, apoptotic, and necrotic tumor cells; LS induced protection to LR challenge. Live, apoptotic, and necrotic cells were prepared as in Fig. 5, but additionally paraformaldehyde treated. A total of  $2 \times 10^5$  cells of each type was injected per mouse. On day 10, mice were challenged with  $2 \times 10^5$  viable cells. Weight (**A**) and survival curves (**B**) of mice in the LR/LS model.  $\blacktriangle$ ,  $\blacksquare$ , and  $\bullet$ , Mice primed with apoptotic, necrotic, or viable LR tumor that were challenged with LR tumor. X, Mice injected once with live LS. \*, Mice primed with live LS and challenged with live LR tumor. **C**, Lung metastases counted in vaccinated mice on day 14 after challenge with B16m. Symbols for vaccination with apoptotic, necrotic, and viable B16m cells are as specified in the inset. The data summarize the results from two experiments with three to four mice per group.

specific CD4 and CD8 cells that produce IFN- $\gamma$ ; Figs. 1C, and 2, E and F) might be successful tumor surveillance. We therefore vaccinated DBA/2 mice with  $2 \times 10^5$  LS tumor cells (viable cells), and 9 days later, we challenged them with  $2 \times 10^5$  LR cells. Although this dose of LR tumor resulted within 12 days in 100% lethality in the control mice and in mice vaccinated with apoptotic and necrotic LR cells, 67% of the mice vaccinated with live LS



**FIGURE 7.** Cytokine recall response in vaccinated and challenged mice. *A*, Live, apoptotic, and necrotic LR cells were prepared and injected as in Fig. 6 and as specified in the *inset*. On day 10, mice were challenged with  $2 \times 10^5$  viable LR cells. Eight days later, the spleen cells were tested directly *ex vivo* in the ELISPOT assays specified for the recall response to LR. Single injection controls showed responses similar to those shown in Fig. 4*B*. *B*, The IL-4 response was tested as specified in *A*, and in the presence of blocking anti-CD4 or anti-CD8 Ab, which was included in the culture at the specified concentrations. *C*, Mice were primed with  $2 \times 10^5$  viable LS cells (▨) or control-injected with PBS (■), and 10 days later, challenged with LR cells. Eight days later, the spleen cells were tested in the ELISPOT assays for the recall response to LR. The primary response in PBS/LR-injected mice is compared with the secondary response of the LS/LR-injected mice. *D*, The IFN- $\gamma$  response was tested as specified in *C*, and in the presence of blocking anti-CD4 or anti-CD8 Ab, which was included in the culture at the specified concentrations. The mean and SD of spots in three replicate wells is shown for individual mice. The data were reproduced in two independent experiments that each involved three to five mice per group.

tumor survived the 60-day observation period (Fig. 6, *A* and *B*) without showing signs of tumor growth. This protection was specific, because LS-preinjected mice were not protected from a challenge with P815 cells (an unrelated tumor of DBA/2 origin; data not shown). Therefore, the type of immune response induced by LS tumor in the vaccinated mice effectively prevented the growth of the related LS tumor.

#### *The successfully vaccinated mice display high clonal sizes of LR-specific CD4 and CD8 cells*

We characterized the anti-LR response in the LS-vaccinated mice that survived the LR challenge. A representative experiment is shown in Fig. 7*C*. The frequencies of LR-specific IFN- $\gamma$ -producing cells were in the 400-per-million level in these protected mice,  $\sim$ 40-fold higher than in those induced by a primary LR challenge (Fig. 7*C*). Although the primary anti-LR response was type 2 polarized, the anti-LR response in these protected mice was type 1 polarized (Fig. 7*C*). Ab blocking showed that the LR-induced IFN- $\gamma$ -producing cells in these protected mice were both CD8 and CD4 cells (Fig. 7*D*). Therefore, the protected mice displayed a high-frequency, tumor-specific type 1 response that encompassed CD8 and CD4 cells.

## Discussion

Our first set of experiments addressed the question whether B16m, LR, and LS tumors induce immunity in syngeneic mice. The answer was an unambiguous “yes” for all three tumors. Natural self-tolerance mechanisms might have contributed to depletion of the

high-affinity end of the T cell repertoires specific for the peptides presented by these tumors. However, there were precursor T cells present that could be engaged to recognize the tumor. This situation seems to be reminiscent of autoimmune responses, whereby precursor T cells escape negative selection because of their low affinity for the autoantigen, and persist as ignorant naive cells in the host. They can be induced to recognize the same Ag after they become primed following engagement of the second signal link (in that case, by injecting the peptide in adjuvant containing microbial Ags) (40, 41). This change of the T cell’s perception of the autoantigen has been related to a shift in the T cell activation threshold, whereby the memory cells become activated at lower MHC-peptide ligand density than does the naive T cell.

Based on present concepts of immunogenicity, it might come as a surprise that live tumor cells induce an immune response at all. There is clearly no infectious nonself signal associated with them that would engage the second signal link and lead to the activation of DC. The observation that a CD4 cell response was primarily engaged by B16m and LR, while both of these tumors are class II negative, suggests that cross-priming is involved. That is, DC rather than the tumor cells themselves were involved in the priming of the naive T cells. Apparently, the live tumor cells activated DC either directly or indirectly. For example, NK cell recognition of the tumors might have resulted in local cytokine release and DC activation (15).

Tyrosinase-related peptide (TRP)-2 has been identified as a class I-restricted tumor-associated Ag for B16m (42). We tested

for the presence of TRP-2 peptide-reactive T cells in B16m-injected mice (after injecting live, apoptotic, and necrotic tumor), but did not detect cytokine recall responses to this peptide (data not shown). These data further support the notion that CD4 cells (but not CD8 cells) were induced by live, apoptotic, and necrotic B16m tumor. The exact nature of the class II-restricted peptides on B16m, LR, and LS that were immunogenic for CD4 cells will need to be defined.

B16m and LR, which both grow in an apparently uncontrolled manner in syngeneic mice, induced Th2 immunity. Induction of this class of response seemed to be intrinsic to both tumors: Th2 responses were seen when the B16 m or LR cells were injected as live, apoptotic, or necrotic cells. Our previous studies suggested that immune responses engaged in the absence of TLR-activating microbial substances (such as mycobacterium, CpG, LPS, pertussis toxin) tend to be type 2 by default (Refs. 35–37, 40, and 43). Tumor cells in general might fit this category. An alternative possibility is that the tumors induce Th1 and Th2 cells indiscriminately, but eventually Th2 cells are selected because they are more resistant to Fas/Fas ligand-mediated apoptosis than are Th1 cells (44). The induction of a Th2 response to the B16m and LR tumor did not seem to be suited to control the tumor growth. In autoimmunity and transplantation, Th2 immunity usually results in the protection of the target tissue, rather than its destruction (38). Several mechanisms contribute to this effect, including the inactivation of macrophages and DC at the site by cytokines such as IL-10 and IL-4, and the local IL-4 release by the first generation of effector T cells creating a microenvironment in the target organ that perpetuates type 2 immunity (Th2 determinant spreading) (45). Such intramolecular determinant spreading has also been described for antitumor responses (46). In the case of tumors, the angiogenesis-promoting effects of IL-4 might even promote tumor growth (47). Berzofsky and colleagues (48) reported that CD4 cells can promote tumor cell growth by decreasing the expression of mRNA that encodes the tumor Ag in the tumor cell. This mechanism was not IL-4 dependent, but seemed to be mediated by a type 2 cytokine.

In striking contrast to the tumorigenic B16 m and LR cells, the nontumorigenic LS cells induced type 1 immunity. Because LS is class II negative, LS-specific CD4 cells must have been induced by DC cross-priming. Clear evidence has been provided that tumor cells can directly activate tumor-specific T cells without cross-priming *in vitro* and *in vivo* (49). Therefore, it is unclear whether the induction of the LS-specific CD8 cells involved cross-priming and whether it was dependent on the help provided by the concurrently induced CD4 cell. Jointly, the LS-induced CD4 and CD8 cells conveyed protection from a lethal dose of the closely related LR tumor. In the protected mice, the frequency of LR-specific, IFN- $\gamma$ -producing CD4 and CD8 cells was boosted, but also type 2 components emerged after the challenge. Because cytokine commitment of T cells is rather firmly imprinted, it seems that the type 1 cells induced during the vaccination with LS both maintained their phenotype after LR challenge and expanded further. The emergence of the type 2 component in these mice suggests, however, that LR additionally exerted its own type 2-polarizing activity. The data are consistent with observations made in other cancer models suggesting that the induction of CD8 cells is frequently required for tumor rejection (50, 51). The protected mice also displayed CD4 cell immunity; this might have additionally contributed to the tumor rejection either by sustaining the expansion of the tumor-specific CD8 cells (52) or by the recruitment and activation of macrophages and NK to the tumor site. CD4 cells can adoptively transfer antitumor immunity (53–55) and facilitate tumor rejection by CD8 cells (56). Our detection of IFN- $\gamma$ -producing

memory cells in the protected mice is consistent with the theory that type 1 immunity affords better protection from tumors than does type 2 immunity, although this issue is controversial. It was shown, for example, that OVA-transfected A20 tumor cells are rejected by Th1- or Th2-polarized OVA-specific TCR transgenic CD4 T cells equally well, but by different mechanisms. Th1 cells eradicated the tumor by inducing cellular immunity, whereas Th2 cells destroyed the tumor by inducing tumor necrosis. Both the Th1 and the Th2 cells required CD8 cells to fully exert these effects (57).

It will be of interest to more closely define why the two closely related tumors, LR and LS, differ so fundamentally in the type of T cell response they induce. The LS subclone was selected because of its multidrug resistance. It is likely that, unlike LR or B16m, LS displays a cell surface molecule that activates pattern recognition receptors on cells of the innate immune system triggering IL-12 production, and thereby elicits a danger/infectious-nonself reaction that is typical of infectious organisms.

LS is resistant to apoptosis induction. The propensity of live LR cells to induce a protective class of immune response is therefore clearly unrelated to apoptosis. If anything, it might be related to these cells' resistance to it. When apoptosis or necrosis was induced in B16m or LR cells, moderate changes in cytokine profiles were seen, but these did not translate into immune protection. For B16m, effector cytokines IFN- $\gamma$  (Th1), IL-4, and IL-5 (Th2) were not affected, but we detected the induction of an increased IL-2 response by the apoptotic vaccine (Fig. 4), which points toward a propensity of apoptotic B16m cells to augment Th0 immunity. However, when such apoptotic B16m cells are injected, the growth of the contaminating 12% live cells contained in the vaccine (Fig. 3) was uninhibited by this increase in IL-2: mice injected with apoptotic B16m cells developed a similar number of lung metastases as did mice injected with live B16m cells (Fig. 5C), showing that there is no protective immunity associated with the increased IL-2 response. For LR, both necrotic and apoptotic tumor cells induced a weak but significant IL-5 response compared with mice injected with the viable tumor cells (Fig. 4B). Testing for the actual tumor growth provided no evidence that the IL-5 response induced by the apoptotic population results in protective immunity: mice injected with the apoptotic LR vaccine succumbed to the contaminating 5% live cells with only a minor delay vs the mice injected with the viable tumor control (Fig. 5, A and B). Also, rechallenge experiments showed no significant differences in protection induced by live, necrotic, and apoptotic LR cells (Fig. 6, A and B). Therefore, the (moderate) cytokine differences between these groups did not seem to be leading to functionally significant differences in immune protection.

The induction of tumor-specific CD4 cells in the absence of CD8 cells was unchanged, nor did the apoptotic cells trigger protective immunity. Apparently, the rate at which the live tumor cells (or the tumor cells that spontaneously undergo apoptosis) are phagocytosed and presented by the local DC has already reached its maximum with the live tumor; injection of a surplus of apoptotic cells does not lead to enhanced Ag presentation. It was shown in human breast carcinoma tissue that immature DCs reside within the tumor, whereas mature DCs are located in the peritumoral areas (58). Growing tumors were shown to contain abnormally elevated numbers of cells undergoing apoptosis (31). Apoptosis is a common reaction in tissue differentiation and injury and, so far, has not been linked to the induction of autoimmune T cell responses. Because it is so common, it is tempting to postulate that the immune system has evolved under the evolutionary pressure to avoid the induction of destructive classes of immune response at sites of increased apoptotic cell death. However, in our models,

apoptotic cells were immunogenic, but their immunogenicity was indistinguishable from that of necrotic cells. This argues against an apoptosis-related immune privilege. However, apoptosis might protect tissues via a mechanism that does not involve T cells, because exposure of macrophages to apoptotic tumor cells has been reported to result in impaired macrophage-mediated tumor destruction *in vivo* and even to support tumor cell growth (59).

Tumor cells undergoing necrosis might have been predicted to show increased immunogenicity. Cell death by necrosis is typically associated with inflammation, in contrast to apoptosis (59). Necrotic cells lead to the activation of DC *in vitro* as detected by cell surface marker expression, whereas apoptotic or live cells do not have such an effect (16). Similar results were obtained by Schwamberger and colleagues (59), who showed that macrophage exposure to necrotized tumor cells caused pronounced stimulation of macrophage antitumor activity. In different studies, Bhardwaj and colleagues (17) showed that, although DC phagocytose apoptotic and necrotic cells alike, only apoptotic cells induce phenotype maturation in DC and heighten their T cell-stimulatory capacity *in vitro* (16, 17). Furthermore, it was shown that the injection of OVA-pulsed necrotic cells induced a delayed-type hypersensitivity response, whereas OVA-pulsed apoptotic cells did not. Although all of the above observations suggest that necrotic cell death should facilitate the induction of immunity, possibly biasing the response toward the type-1 differentiation pathway, we did not observe such an effect.

One possible explanation why our tumors behaved differently may lie in the very nature of the Ags studied. Tumor Ags are self-Ags that induced negative selection. The induction of a destructive type 1 response against tumor Ags might need more vigorous means than against foreign Ags. For example, in mice immunized with the purified melanocyte differentiation Ag, TRP-1 failed to elicit an Ab or CTL response even when different adjuvants or cytokines were included. However, immunization with allogeneic TRP-1 readily elicited an immune response (60). Also, using the protooncogene HER-2/neu tumor Ag system has shown that significant CTL activity could be induced only after immunization with an altered peptide, but not with the wild-type peptide itself (61). Along the same lines, immunization with the foreign Ag OVA as naked DNA, with OVA peptide-pulsed DC or with OVA in adjuvant readily induced a protective immune response against OVA-transfected B16m cells. However, the autoantigen TRP-2 peptide induced protection against the melanoma only after peptide-pulsed DCs were injected, not when immunization was attempted according to other protocols (62). Furthermore, protection against B16m could only be induced after genetic vaccination with a xenogeneic human TRP, not after vaccination with murine TRP (63).

Contrary to what might have been expected, we did not observe striking differences in the immunogenic behavior of apoptotic or necrotic tumor cells. This outcome might have been affected by the experimental procedure itself. Injecting the tumors *i.p.* or *i.v.* may result in their preferential capture by macrophages. In spontaneous tumor biology, tumor Ag presentation by DCs may be more prevalent. Because apoptotic vs necrotic tumor cells exert differential influences on DCs (9), the ensuing immune response might differ. However, in experimental tumor vaccinations, these differences may not manifest themselves unless the tumors are selectively targeted to DCs.

In summary, our data show that the tumors we studied engaged the second signal link and induced an immune response on their own. However, the T cells engaged by the malignant tumors, being CD4 and Th2, were of a nonprotective class. This outcome was independent of whether live, apoptotic, or necrotic cells were in-

jected. In contrast, a nonmalignant live tumor induced type 1 CD4 and CD8 cells and protective immunity. Therefore, the immunogenicity of tumors and their propensity to induce type 1 or type 2, CD4 or CD8 cell immunity is not primarily governed by danger signals associated with apoptotic or necrotic cell death, but is primarily an intrinsic feature of the live tumor itself.

## Acknowledgments

We thank Dr. Peter Heeger for insightful discussions, and Carey Shive and Tameem Ansari for excellent technical assistance.

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