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Synergy between Chemotherapy and Immunotherapy in the Treatment of Established Murine Solid Tumors[1]

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ABSTRACT

Cytotoxic chemotherapy is generally considered immunosuppressive, with neutropenia and lymphopenia being common adverse side effects. In this context, we have shown previously that the cytidine analogue, gemcitabine, abolishes humoral responses but, in contrast, augments antigen-specific cellular antitumor immunity. This augmentation occurs in the context of increased antigen cross-presentation, T lymphocyte expansion, and infiltration of the tumor. Here, we combine an immunotherapy (CD40 ligation using FGK45; 100 μg; i.p., q2dx3) with gemcitabine (120 μg/gram; i.p.; q3dx5) to treat established solid tumors. This protocol induced long-term cures in ≤80% of mice, and all of the cured mice were resistant to tumor rechallenge. Synergy between the drug and immunotherapy could not be established *in vitro* and was only seen in the context of tumor cell death. It was associated with an increase in both CD4 and CD8 T-cell infiltration of the tumor, but depletion studies clearly showed that CD4 T cells were not a necessary component of the cure. In contrast, CD8 T cells were absolutely required for the success of this treatment regimen. The priming effect of gemcitabine was not limited to debulking, because mice resected to an equivalent, or lesser residual tumor volume did not eradicate tumor with subsequent immunotherapy. This study provides evidence that chemotherapy has the capacity to augment cellular antitumor immunity, a finding with wider implications for the management of treatment-resistant solid tumors.

INTRODUCTION

Cytotoxic chemotherapy is an important mode of treatment in human malignancy. Unfortunately, delivery of cytotoxic agents is often limited by both acute and cumulative toxicities to normal tissues, limiting both the dose and duration of treatment. Treatment with combination chemoimmunotherapy could potentially exploit the debulking effects of chemotherapy to treat cancers, because the treatments have different mechanisms of action and different toxicities. However, cytotoxic chemotherapy is generally regarded as immunosuppressive because of its toxicity for dividing cells in the bone marrow and peripheral lymphoid tissue.

Gemcitabine (2′2′-difluorodeoxycytidine) is a nucleoside analogue of cytidine, which is active as a single agent and in combination with cisplatin and other drugs against many solid tumors (1–3). It becomes incorporated into DNA with the subsequent addition of one further base to the DNA strand, a process known as "masked chain termination" (4). It thus halts DNA synthesis and is invisible to DNA repair systems, leading the cell into the apoptotic pathway. Additionally, gemcitabine inhibits ribonucleotide reductase, a rate-limiting enzyme in DNA synthesis that converts ribonucleotide diphosphates into deoxyribonucleotide diphosphates. Gemcitabine, therefore, depletes the deoxynucleoside triphosphate pool, causing a competitively higher incorporation of itself, as compared with dCTP into nascent DNA.

We have demonstrated previously that gemcitabine profoundly suppresses the humoral immune response to a tumor neoantigen (5). Conversely, this drug is not detrimental to tumor antigen-specific cellular priming, because it increases tumor antigen cross-presentation, T lymphocyte expansion, and infiltration of the tumor (6). Furthermore, the increase in cross-presentation does not lead to functional or deletional tolerance. Gemcitabine treatment appears to prime the immune response as evidenced by the increased efficacy of postgemcitabine immunotherapy using viruses that express tumor antigens (6). In this study, we combine gemcitabine with subsequent nonspecific immunotherapy using the activating anti-CD40 antibody FGK45.

CD40 is a M_r 40,000 type I glycoprotein and member of the tumor necrosis factor receptor superfamily, which was initially identified on bladder carcinoma cells and later on normal and malignant B cells (7). It is expressed on DCs, monocytes, epithelial cells, endothelial cells, carcinomas of the lung, colon and breast, and leukemia (8, 9). Its ligand, CD40L (CD154), is preferentially expressed on mast and CD4 T cells shortly after TCR triggering. CD40-CD154 interactions have an important role in CTL priming (10, 11). The interaction is central to the decision whether CTLs become primed or tolerized. When the CD154 molecule on a CD4 T cell interacts with CD40 on an APC,[3] APC activation occurs, with production of interleukin-12 and up-regulation of B7–1 and B7–2, which are coactivators in the generation of CD8 effector cells (11, 12). This costimulatory role may be responsible for the initiation of T-cell responses against viruses and bacteria (13, 14), as well as antitumor immunity after tumor cell vaccination (14). When antigen presentation occurs in the absence of CD40 ligation, tolerance may occur (10, 16, 17). Indeed, antibody blockade of CD154 results in the failure of CTL generation (10). Exogenous CD40 ligation can, however, substitute for CD4 T-cell help, and CD40 activation of DCs can restore antigen-specific CTL responses in CD4-depleted mice (18). Depletion of CD8 T cells, however, abrogates the antitumor effect of CD40 ligation (19). These findings suggest that activating anti-CD40 antibody can replace or augment CD4 help in priming DCs to activate CD8 T cells.

Activation of cells using either activating monoclonal antibodies or rCD154 itself has been used against neoplastic B cells (20), as an adjuvant to vaccination (21), and to induce strong CTL priming to an otherwise tolerogenic peptide (22). It has also been shown to improve the efficacy of peptide-based vaccines and found to have antitumor effects in at least one tumor model (22). In our system, treatment with the activating anti-CD40 antibody, FGK45, alone causes a brief tumor regression over the treatment period, followed by rapid tumor outgrowth.[4]

Although CD40 is expressed by a variety of tumors, it is not expressed on the cell line used here. In this study, we show that CD40 ligation synergizes with a course of gemcitabine, resulting in cure of some mice with established solid tumors. We show that the drug must kill the tumor, making it unlikely that its effect is through modulating regulatory cells. Synergy does not require CD4 T cells but is crucially

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[3] The abbreviations used are: APC, antigen presenting cell; DC, dendritic cell; TCR, T-cell receptor; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

[4] R. Himbeck *et al.,* unpublished data.

dependent on CD8 T cells. We show that surgical resection does not synergize with immunotherapy, powerfully suggesting that synergy occurs because chemotherapy is an immunologically priming event.

MATERIALS AND METHODS

Mice. BALB/c (H-2^d) mice were obtained from the Animal Resources Centre (Perth, Australia) and maintained under standard conditions. Two lines of TCR transgenic mice were used. Clone 4 TCR transgenic mice (CL4; Ref. 23) express a TCR recognizing the dominant class I restricted epitope of influenza HA. HNT mice express a class II-restricted receptor that recognizes the dominant class II HA epitope (24). All mice used in these studies were between 8 and 12 weeks of age.

Cell Lines. All cell lines were regularly tested and remained negative for *Mycoplasma spp*. The AB1 murine malignant mesothelioma cell line was generated by injecting crocidolite asbestos i.p. into BALB/c mice, and the peritoneal exudate was passaged *in vitro* and *in vivo* until stable clonal cell lines were obtained (25). Cell lines were maintained in RPMI 1640 (Invitrogen, Mount Waverly, Australia) supplemented with 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin (CSL, Melbourne, Australia), 50 μg/ml gentamicin (David Bull Labs, Warwick, UK), and 5% FCS (Invitrogen). AB1 is a class I^+, class II^- tumorigenic cell line. AB1 cells were transfected with the influenza HA gene (*AB1-HA*; Ref. 26). Transfected cells were maintained in media containing the neomycin analogue Geneticin (Invitrogen) at a final concentration of 400 μg/ml. Expression of HA was measured by FACS analysis before use in each experiment. Gemcitabine-resistant AB1-HA (AB1-HA-GR 250) was generated by culturing cells in media containing progressively increasing concentrations of the drug (Eli Lilly, Indianapolis, IN) to a final concentration of 1.67 μg/ml. The IC_{50} as assessed by the colorimetric MTT assay was >800 fold that of the parent cell line. AB1-HA-GR 250 maintained its expression of HA.

Experimental Protocol. Cells (1×10^6 AB1-HA) were inoculated s.c. on one side of the ventral surface in the lower flank region. In general, standard chemotherapy commenced 9 days later when a small palpable lump was evident, ranging from 1 to 2 mm in diameter. Mice were injected i.p. with 120 μg/gram gemcitabine every 3rd day for five doses (q3dx5), a regimen established previously as a maximal tolerated dose for BALB/c mice (27). Control mice received PBS vehicle alone. Mice receiving activating anti-CD40 antibody (FGK45; a gift of Dr. Antonius Rolink) received 100 μg in 100 μl of PBS i.v. three times in 6 days. Control mice received PBS alone. Tumor size was measured with calipers three times weekly during the course of chemotherapy and subsequently until tumor size reached $\sim 10 \times 10$ mm at which point mice were culled. In some experiments, single cell suspensions of TCR transgenic lymphocytes were infused i.v. Adoptive transfer consisted of 2×10^7 cells comprising equal numbers of HNT and CL4 lymphocytes in a total volume of 200 μl.

MTT Assays. The metabolic activity of tumor cells was determined *in vitro* using the colorimetric MTT assay. Optimal cell seeding densities were determined empirically. Thus, 3200 cells/well were seeded into 96-well flat-bottomed tissue culture plates in 100 μl of medium. Gemcitabine was serially diluted and added to wells in an additional 100 μl 24 h after cell seeding. Plates were incubated at 37°C for 72 h; then 50-μl MTT solution (2 mg/ml) was added to each well, and plates incubated for an additional 4 h. DMSO (100 μl) was used to solubilize the formazan, and optical densities were determined at 570 nm. Plates were blanked on wells containing cell free medium, which had been treated with MTT in a like manner. All experiments were performed in triplicate.

Immunohistochemical Staining. Tissues were snap frozen in compound-embedding medium (OCT; Miles, Inc., Elkhart, IN), and 10-μm sections were collected on poly-L-lysine-coated slides and allowed to air dry. Sections were fixed with either cold ethanol (5 min) and blocked with 1% (volume for volume) hydrogen peroxidase (5 min), followed by avidin/biotin block (10 min each). Sections were then incubated with the primary antibodies for 1 h, followed by incubation with a biotinylated secondary antibody (30 min). Immunostaining was visualized by incubating streptavidin horseradish peroxidase (DAKO, Glostrup, Denmark; 30 min) and diaminobenzidine-H_2O_2 (Sigma) for 5–10 min and counterstained with hematoxylin.

HA Tetramer Reagent and Staining. Cells were purified from tumors as single cell suspensions by chopping the mass with sterile instruments and mashing using two fully frosted glass slides in a small volume of PBS. The homogenates were transferred to conical tubes (Falcon, Becton/Dickinson, Bedford, MA) via plastic transfer pipette, topped up, and allowed time for cellular debris to settle. Supernatants were then transferred to a fresh tube before being centrifuged (12,000 rpm, 7 min) and resuspended in PBS.

Tetramers of H-2 K^d containing the CL4 peptide were obtained from Dr. J. Frelinger. For analysis, 1×10^6 lymphocytes were blocked in purified anti-mouse CD16/CD32 (FcγIII/II receptor; PharMingen, San Diego, CA), then stained with the HA tetramer for 2 h at room temperature. Samples were then incubated with FITC-labeled anti-CD8 antibodies for 30 min. Propidium iodide (1 μg/ml) was added in the final wash. Data were acquired on a FACScan flow cytometer and analyzed using CELLQuest software (Becton-Dickinson Immunocytometry Systems, San Jose, CA) gating on the small cell population.

Culture and Purification of Monoclonal Antibodies. The hybridoma FGK45 was grown in R5 containing low immunoglobulin FCS. Spent culture media was centrifuged at 2000 rpm for 10 min at room temperature. Supernatant was passaged through a Protein G purification column, and eluted fractions were pooled and dialyzed against PBS. Protein concentrations were determined by Bio-Rad protein assay. Biological activity was determined by FACS analysis of BALB/c spleen cells incubated with FGK45 and phycoerythrin-conjugated antirat antibody, double stained with B220-FITC. Typically, we recover 90% of the original activity with an overall purity as assessed by PAGE and quantitated by densitometry of better than 90% immunoglobulin.

The hybridomas YTS 191 (CD4+-depleting antibody; gift of Dr. A. Scalzo, Perth, Australia) and YTS 169 (CD8+-depleting antibody; gift of Dr. A. Scalzo, Perth, Australia) were grown in R5 and antibody purified as above. Biological activity was confirmed by FACS analysis of CD4+ and CD8+ lymphocytes from RBC-lysed whole blood from treated mice.

***In Vivo* CD4+ and CD8+ Depletion.** Mice received 100 μg of antibody in 100 μl of PBS i.p. (YTS 191 or YTS 169) for three consecutive days and then subsequently on the day of other treatments (gemcitabine or FGK45).

Surgical Debulking of Tumors. Mice were anesthetized with chloral hydrate 0.1 ml/10 grams mouse i.p. and inhaled methoxyflurane as necessary. s.c. tumor was removed via an incision, which was closed using surgical staples. Mice received 2.5 mg/kg buprenorphine i.p. in the recovery phase as postoperative analgesia.

Statistical Analysis. Data comparing differences between groups were assessed using a Student *t* test. Differences between growth curves were compared using ANOVA. Survival curves were compared by the Log-rank test. Differences were considered significant when the P was <0.05. Statistical analysis was conducted using the SPSS for Windows program and Graph Pad Prism program.

RESULTS

Appropriate Combination of Chemotherapy and Immunotherapy Can Cause Regression and Cure of an Established Tumor. Treatment protocols were initiated 9 days after tumor inoculation, when an s.c. mass became just palpable, to 2×2 mm. Mice treated with the activating anti-CD40 antibody, FGK45 alone, showed a small growth delay (Fig. 1*A*), as compared with control mice ($P < 0.05$), but this treatment did not improve survival (Fig. 1*B*). Mice treated with FGK45 followed by gemcitabine regrew tumors faster than mice treated with gemcitabine alone ($P < 0.05$). There was no significant difference in survival between mice treated with gemcitabine alone and those treated with FGK45 before or concurrent with chemotherapy; however, 2 of 5 mice treated with concurrent gemcitabine and FGK45 died from treatment toxicity, and 1 of the remaining 3 mice survived long term tumor free. When mice were treated with gemcitabine and subsequently given FGK45, 4 of 5 mice failed to regrow tumor. These results were confirmed in subsequent experiments, with the proportion of long-term survivors ranging from 40 to 80% in experiments using the same treatment dose and schedule.

Mice Cured after Treatment with Combination Therapy Are Resistant to Tumor Rechallenge. To test whether mice cured of tumor using gemcitabine followed by FGK45 had developed immu-

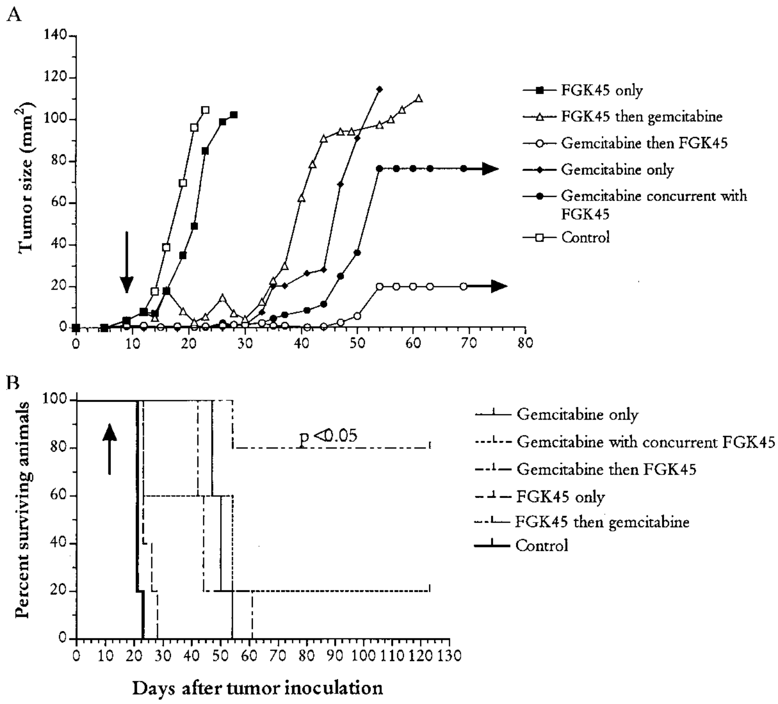

Fig. 1. Mice (5/group) were injected with AB1-HA tumor and then treated with 120 μg/gram gemcitabine i.p. q3dx5 and/or 100 μg of FGK45 i.p. three times over 6 days, in different groups as shown. Control animals were treated with PBS. Tumor growth rates (*A*) and survival (*B*) were monitored. *P* (*B*) is the result of a Log-rank test for survival difference between mice treated with gemcitabine alone *versus* gemcitabine followed by FGK45. *Arrows*, start of treatment. The experiment was performed once and repeated in part using 10 mice/group for treatment with gemcitabine alone *versus* gemcitabine followed by FGK45, with similar results.

nological memory, 5 such mice were reinoculated with 1×10^6 tumor cells in the contralateral flank 120 days after their initial tumor inoculation. No tumor development in either site was noted for $\leq$160 days after the second tumor challenge. All control mice rapidly developed tumor (data not shown).

Combination Therapy Does Not Work against a Gemcitabine-resistant Tumor. There is some evidence that B cells and their products may inhibit the induction of T cell-dependent antitumor immunity. Immune sera have been shown to enhance tumor growth, most likely by blocking access of tumor-specific lymphocytes to their target (28), and B cell-deficient mice have been shown to control tumor growth more readily than their normal littermates (29). We have shown previously that gemcitabine is selectively toxic to B cells and markedly decreases antibody production (5). To clarify whether the synergistic effects of FGK45 and gemcitabine required killing of tumor, the therapy was tested against a gemcitabine-resistant tumor (AB1-HA-GR250). This line has been described previously; it grows *in vivo* at the same rate as the parent with or without gemcitabine treatment (5).

Using the standard protocol, this experiment was limited by rapid tumor growth in the absence of effective chemotherapy, so that by the end of chemotherapy, animals were unable to receive FGK45. A shorter course of gemcitabine (120 μg/gram i.p. q3dx4) was therefore initiated earlier (7 days after tumor inoculation). Immunotherapy (100 μg of FGK45 i.v. three doses over 6 days) was applied as before, but in this case, the tumors were ~80 mm^2. Tumors grew rapidly with no evidence of a decrease in tumor growth rate after combined treatment (data not shown).

The experiment was again redesigned in an endeavor to address the problem of initiating immunotherapy against a large tumor mass. Chemotherapy was therefore started 1 day after tumor inoculation (120 μg/gram i.p. q3dx4) and followed 2 days later by immunotherapy (100 μg of FGK45 i.v. three doses over 6 days). Tumor size was ~30 mm^2 when treatment with FGK45 started. There was no effect on tumor growth nor any change in overall survival under these conditions (Fig. 2).

FGK45 Is Not Cytotoxic to AB1-HA, nor Does it Interact with Gemcitabine *in Vitro*. To determine whether the synergy between therapies could be attributable to a direct effect on the tumor, an MTT assay was used to assess whether FGK45 inhibited AB1-HA metabolism with or without gemcitabine. FGK45 alone did not inhibit tumor cell metabolism (Fig. 3*A*). Gemcitabine was then titrated in the presence of increasing concentrations of FGK45. The IC_{50} of the drug (the concentration required to inhibit tumor metabolism by 50%) did not change at any concentration of FGK45 (Fig. 3*B*).

Combination Therapy Augments T-cell Infiltration of the Tumor. Growing AB1-HA tumors become progressively depleted of intratumoral CD4 and CD8 cells with increasing tumor size (26). Gemcitabine treatment reverses this phenomenon, with tumors remaining small and extensively infiltrated with both CD4 and CD8 cells. In the absence of further treatment, this infiltrate persists during tumor regrowth (data not shown). Macrophage infiltration is always extensive in this tumor and does not change with time or treatment. To assess lymphocyte infiltration in the context of combination therapy,

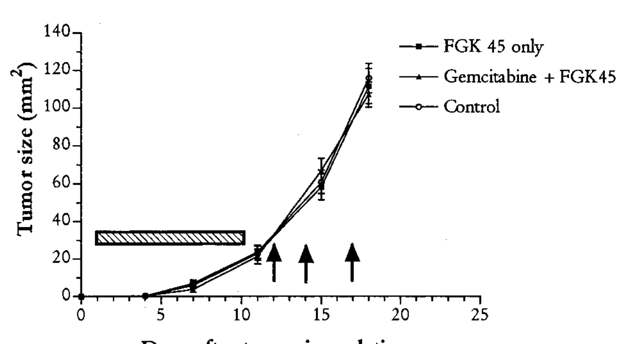

Fig. 2. Mice (5/group) were injected with AB1-HA-GR250 tumor and 1 day later started a shortened course of 120 μg/gram gemcitabine i.p. q3dx4 or PBS as control. One group of gemcitabine-treated mice was then given FGK45 antibody as described in Fig. 1. Tumor growth was measured as the product of bidimensional measurements. *Cross-hatch,* the period of gemcitabine treatment. *Arrows,* the timing of FGK45 injections.

tumors were removed and examined for CD4, CD8, and F480 2 days after the end of all treatment. There was a marked increase in lymphocyte infiltration over the increase observed with gemcitabine alone (Fig. 4). The level of macrophage infiltration was unchanged (data not shown). The specificity of the infiltrating lymphocytes was assessed by tetramer staining 2 days after the end of combination therapy. There was no difference in the percentage of tetramer-positive cells in the tumors of mice treated with gemcitabine compared with those of mice treated with combination therapy (Fig. 5, *A* and *B*). Of note, in 1 of 5 mice treated with the combination, there was no tumor present to analyze.

CD4 T Cells Are Not Required for Successful Combination Therapy. The effects of FGK45 have been reported as independent of CD4 T cells (18). To test the requirement for this population in our system, treated mice were depleted of CD4 cells at different times during the treatment cycle: (*a*) at the start of gemcitabine treatment; (*b*) at the start of FGK45 treatment; or (*c*) after FGK45 treatment. Although the proportion of survivors varied slightly from group to group, these differences were not significant (Fig. 6*A*). In fact, non-depleted control mice had the lowest survival, with only 40% of animals surviving long term. Groups of mice depleted of CD4 cells exhibited 60–80% survival after combination therapy, a finding consistent with the hypothesis that the requirement for these cells can be substituted by CD40 ligation. In those animals where tumors regrew, there was no change in the rate of growth between different protocols.

CD8 T Cells Are Essential for Successful Combination Therapy. Mice participating in the standard protocol were treated with a CD8-depleting antibody for the duration of active treatment. There was no significant difference in the rate of tumor growth between these animals and controls during the course of gemcitabine treatment. One mouse from each group died early (day 25) as a result of treatment toxicity. At the end of combination therapy, tumor growth was noted in animals depleted of CD8 T cells, whereas tumors remained small in control mice. Five mice from the control group (50%) remained tumor free long term. All 9 remaining mice (100%) from the CD8-depleted group developed tumors (Fig. 6*B*).

Combination Therapy Does Not Work Simply because Gemcitabine Causes Tumor Debulking. It was possible that combination therapy was successful because immunotherapy was effective at eliminating small volumes of residual tumor. This hypothesis could be tested by starting immunotherapy early, when tumors are small.

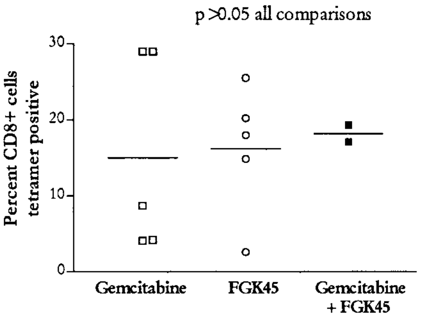

Fig. 5. Mice (5/group) were injected with AB1-HA tumor and treated as shown. Two days after the end of treatment, tumors were removed, made into a single cell suspension, and stained with HA tetramer before analysis with FACS. Analysis was gated on the lymphocyte region. The figures show the percentage of CD8+ T cells that were +ve for HA-tetramer. Three mice in the combination treatment group had no tumor to analyze. This experiment was performed once.

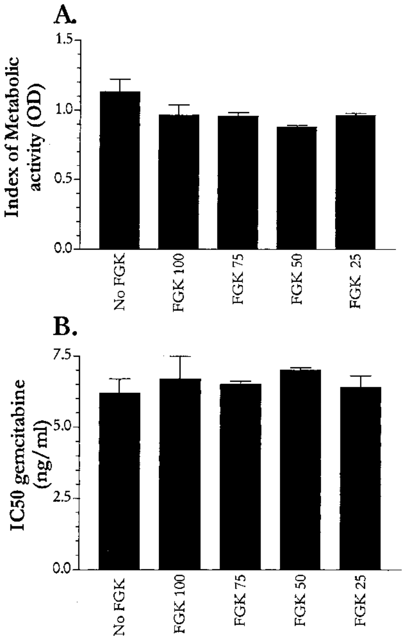

Fig. 3. The sensitivity of AB1-HA to FGK45 with or without gemcitabine *in vitro* was established using the colorimetric MTT assay. FGK45 (25–100 μg/ml) and gemcitabine (0.0001–1 ng/ml) were added to 96-well plate cultures of tumor cells (4×10^5/well). Plates were incubated for 48 h before adding MTT for an additional 4-h incubation. Dye accumulation was estimated spectrophotometrically and expressed as the mean of the absorbance of triplicate wells for FGK45 (*A*) or concentration of gemcitabine (nanograms/milliliter) required to get IC_{50} *in vitro* (*B*). Data were derived from one experiment performed in triplicate wells.

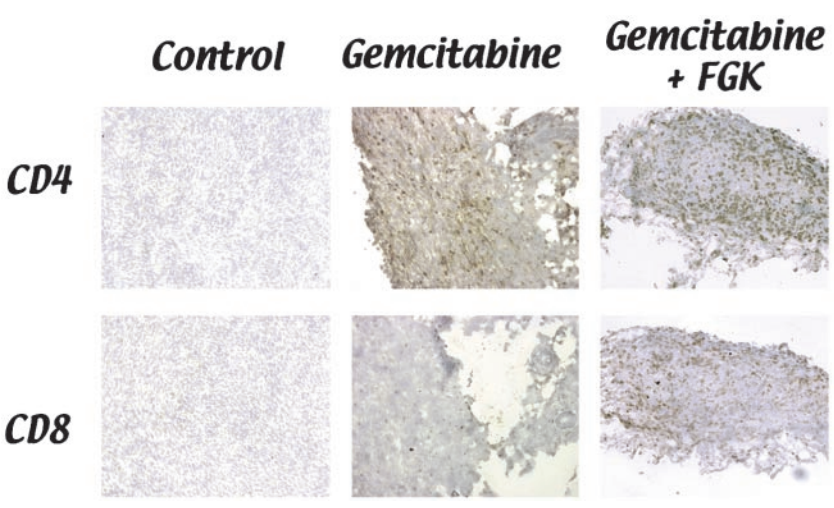

Fig. 4. Groups of 3 mice carrying AB1-HA tumors were treated as described in Fig. 1. Two days after the end of treatment, mice were culled, and their tumors were sectioned and stained for CD4 and CD8 expression. Representative sections are shown from untreated control and gemcitabine- and combination-treated mice. Original magnification: ×200.

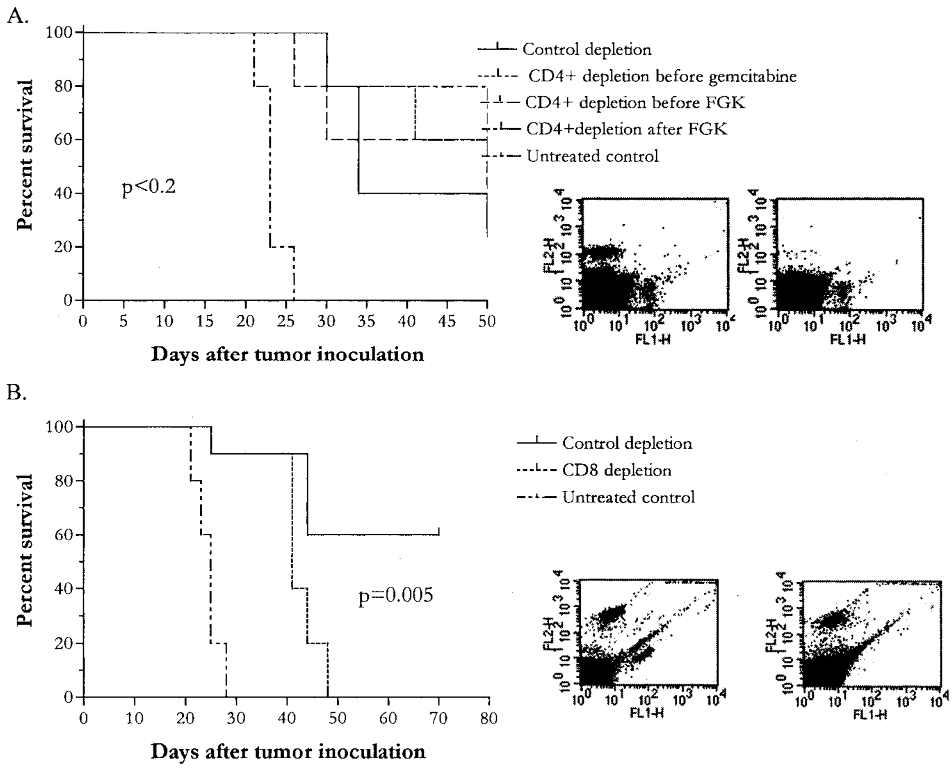

Fig. 6. Mice (5/group) were injected with AB1-HA tumor and treated as described in Fig. 1. Animals were depleted of CD4 T cells using the depleting antibody YTS191 (100 μg i.p. daily × 3), commencing depletion as shown. Survival of CD4-depleted mice was monitored (*A*). The *P* is derived from a Log-rank test comparing the survival curves of the treated groups with the control group. This experiment was repeated twice with similar results. CD4 depletion (>90% in all cases) was confirmed by FACS analysis of peripheral blood (*inset* showing CD4 *versus* CD8 profile). In *B*, mice (10/group) were treated as above. One group was depleted of CD8 T cells using the depleting antibody YTS169 (100 μg i.p. daily × 3, then q3d), commencing depletion just before gemcitabine treatment and continuing until the end of the treatment protocol. The figure shows survival curves, and the *P* is derived from a Log-rank test. This experiment was repeated twice with similar results. CD8 depletion was confirmed by FACS analysis of peripheral blood and >90% in all cases.

However, we know that there is limited antigen presentation and CTL activity in this model before day 10 (26, 30). Thus, we decided to reduce tumor bulk at a time when the amount of antigen available to the immune system was similar to that in gemcitabine-treated animals.

The standard treatment protocol was followed and compared with surgery as a substitute for chemotherapy. Tumors grew rapidly in untreated control animals and animals planned for surgical treatment (Fig. 7*A*). On the day of surgery, two animals from the surgery FGK45 group and three animals from the surgery control group died as a direct result of the procedure or anesthetic, and one additional animal in the surgery alone group died 3 days later. The remaining animals had recovered completely by the next day. Mice receiving gemcitabine all showed a reduction in tumor mass, which persisted for the duration of treatment. Resected mice all regrew tumors, and this was slightly but significantly delayed ($P = 0.005$) when compared with mice treated with gemcitabine alone, indicating that the efficiency of surgical debulking was at least equivalent to that of chemical debulking, although this did not translate to an improvement in survival ($P > 0.05$). There was no evidence of growth delay ($P = 0.76$) or increased survival ($P = 0.3$) when surgically debulked mice were treated with FGK45. Animals treated with gemcitabine followed by FGK45 showed a significant slowing of tumor outgrowth as compared with resected animals receiving FGK45 ($P = 0.002$), and 4 of 10 animals in the combined treatment group became long-term survivors. This survival difference was statistically significant when compared with all other groups (Fig. 7*B*; $P = 0.02$ for gemcitabine FGK45 *versus* surgery FGK45). However, when data were analyzed separately for mice in the combination chemoimmunotherapy group, it was evident that mice that went on to develop tumors did so at the same rate as mice treated with gemcitabine alone. Thus, treatment outcome does not have an intermediate phenotype.

Combination Therapy Is Successful even when the Tumor Does Not Express HA. To test the importance of the strong viral antigen HA in the success of combination therapy, mice were inoculated with the parent AB1 tumor and then treated with the same combination

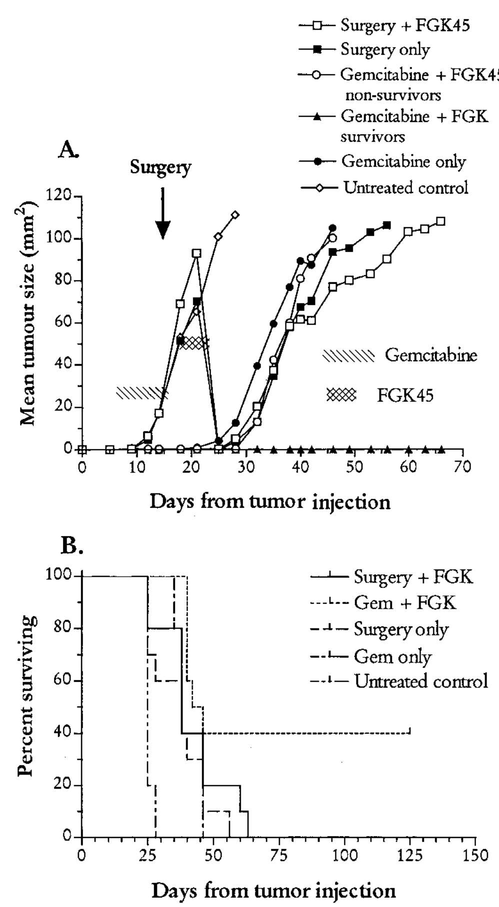

Fig. 7. Five groups of 5 (gemcitabine-only control, untreated control) or 10 (all other groups) mice were injected with AB1-HA and either left untreated, treated with gemcitabine, or surgically debulked before treatment with FGK45 or control vehicle. All treatments were given as described in Fig. 1. Animals were monitored for tumor growth (*A*) and survival (*B*). *Arrow* (*A*), the time of surgery. This experiment was performed once.

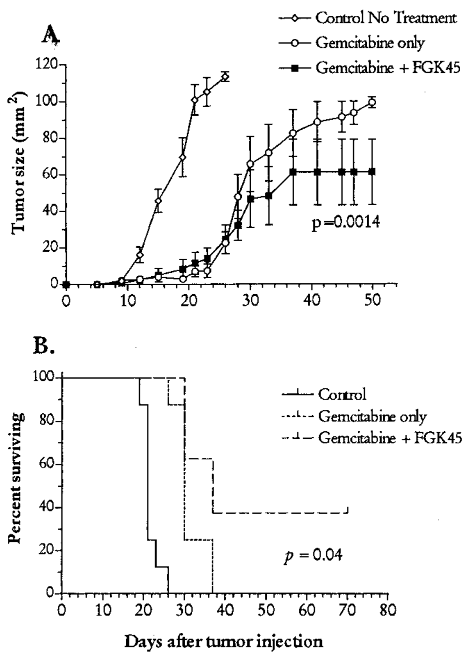

Fig. 8. Two groups of 8 mice were injected with 1×10^6 cells of AB1 tumor and, 9 days later, treatment with gemcitabine alone or gemcitabine followed by FGK45 as described in Fig. 1. Mice were monitored for tumor growth rates (*A*) and survival (*B*). The *P*s were derived by ANOVA (*A*) and Log-rank test (*B*). This experiment was performed once.

protocol or gemcitabine alone. Mice treated with gemcitabine showed delayed tumor growth as compared with untreated control mice. Mice treated with gemcitabine followed by FGK45 had slower mean tumor growth relative to those treated with gemcitabine alone ($P = 0.0014$), with 3 of 8 mice surviving long term, a significant increase in survival ($P = 0.04$; Fig. 8).

Mice Cured of Established AB1-HA Resist Subsequent Tumor Challenge with the Parent AB1. Mice cured of AB1-HA tumor using gemcitabine followed by FGK45 resist rechallenge with the same tumor. To further test the importance of HA in this immune response, 5 mice that were inoculated with AB1-HA, treated with combination therapy, and remained free from tumor as described were then inoculated with 1×10^6 AB1 cells in the contralateral flank 120 days after their initial tumor inoculation. No tumor development at either site was noted for ≤160 days after the second tumor challenge. All control mice rapidly developed tumor.

DISCUSSION

There have been few studies combining immunotherapy and chemotherapy in cancer, probably because it has been generally assumed that chemotherapy is immunosuppressive and would therefore be likely to negate the benefits of immunotherapy. Contrary to these expectations, we have shown that gemcitabine is not detrimental to cellular antitumor immunity (6). Here, we have gone on to show that this drug can synergize with nonspecific immunotherapy, mediated by CD40 ligation, to cure mice with established solid tumors and that the success of the therapy is schedule dependent in that it is successful, only when immunotherapy follows chemotherapy.

CD40 is central to the decision whether CTLs become primed or tolerated. When CD8 cells recognize antigen on DCs without the help of CD4 T cells and the coligation of CD40 by CD154, tolerance may occur (10, 16, 17). Exogenous CD40 ligation can, however, substitute for this CD4 T-cell help, although antibody blockade of CD154 results in the failure of CTL generation, which can then be overcome by CD40 triggering (11). CD40-activated DCs can also restore antigen-specific CTL responses in mice which have been depleted of CD4 T cells (18). However, CD8 T cells are still necessary to mediate killing of solid tumors, because depletion of this lymphocyte subset abrogates the antitumor response (19). All these findings suggest that a major role of activating anti-CD40 antibody is to replace or augment CD4 help in priming DCs to activate CD8 T cells. Activated T cells also have a role in regulating the growth, differentiation, and immunoglobulin class switching of B cells via CD40 ligation (reviewed in Ref. 31). B cell-deficient mice also demonstrate induction of CTL responses to tolerizing peptide vaccines with CD40 activation, indicating that B cells are not required for CD40-mediated restoration of CTL priming (22).

Ligation of CD40 has been investigated previously for antitumor effects. On neoplastic B cells, it can result in growth inhibition and cell death (20). It has been shown *in vivo* that CD40 ligation can improve the efficacy of peptide-based vaccines, inducing therapeutic CTLs in mice with established tumors, resulting in cure in some mice (22). However, in the tumor system we are using, CD40 ligation alone causes a brief and minor tumor regression over the period of treatment, followed by rapid tumor outgrowth.

We have shown previously that gemcitabine induces tumor cell apoptosis and that if the tumor expresses HA, the drug primes the host for a strong response to a second, virus-generated and HA-specific signal (6). These experiments show that tumor cell apoptosis *in vivo* neither sequesters tumor antigens nor cross-tolerizes tumor-specific CD8 cells. Taken together with the current study, our findings strongly suggest that gemcitabine induces immunological priming in that subsequent signaling via molecules like CD40 leads to an effective antitumor immune response. Although this method of apoptosis induction increases the amount of tumor antigen cross-presented in the draining lymph node, this is not likely to be the only mechanism by which T-cell responses are enhanced, because progressive tumor growth itself increases tumor antigen cross-presentation but does not necessarily increase priming. Thus, it is likely that additional signals are being received by the APC that is processing antigens from apoptotic tumor cells, and these signals increase the priming of specific T cells, an effect that is profoundly augmented by CD40 signaling. Mice cured by combination therapy are resistant to tumor rechallenge, and this contrasts with the failure of typical immunization protocols using irradiated tumor cells, nominally characterizing this tumor as poorly immunogenic.

Synergy requires apoptotic tumor cell death and does not occur as a consequence of perturbations in immunological regulatory circuits. This was clear from the finding that the gemcitabine-resistant cell line was not susceptible to combination therapy. There are minor *caveats* to this interpretation: (*a*) an abbreviated course of gemcitabine was given; nevertheless, it seems unlikely that eliminating one of five doses of gemcitabine would abolish all of the effect; and (*b*) the growth kinetics necessitated that treatment commence at an earlier time point. And in the absence of growth retardation, there was considerable variability in tumor size at the commencement of immunotherapy. Nevertheless, the absence of any growth delay when drug-resistant tumors were exposed to combination therapy strongly suggests that tumor cell death is a prerequisite for success. This conclusion is also supported by the data comparing mice debulked with gemcitabine or surgery. It was suggested that immunotherapy worked because of the small size of tumors after chemotherapy. Effective surgical debulking is neither curative *per se,* nor does it interact with immunotherapy.

There are several inherent problems in trying to address the mechanisms that mediate the success of this protocol. Firstly, when to look? We chose to assess cellular responses 2 days after the completion of treatment, because we expected to find the greatest differences between treatment groups at this time point. This necessitated the commencement of treatment at different times and meant that at the end of treatment, tumors had been present for differing durations. A second problem was the lack of tumor, or very small tumor size, in animals treated with combination therapy, and the fact that these animals were culled for the experimental readout meant it was not possible to determine which of the individual animals would have survived.

Notwithstanding these problems, lymphocyte depletion studies showed that CD4 cells were not necessary for the success of combination therapy. Depletion at three different stages during treatment showed that they were not necessary during early tumor growth, drug-mediated priming, nor the immunotherapy-mediated eradication phase. In contrast, CD8 cells were absolutely required for the success of combination therapy.

The interaction between therapies is clearly finely balanced. The proportion of survivors in treatment groups varied between experiments, ranging between 40 and 80%. We have shown that the parent cell line (AB1) that does not express the strong tumor antigen HA can also be cured by combination therapy. The data also suggest that the immune response in animals with HA-expressing tumors that are cured by combination therapy is not principally directed against HA, because these mice are equally resistant to challenge with AB1. These observations may explain why we were unable to show any change in HA-specific lymphocyte infiltration of the tumor nor any change in HA-directed CTL activity.

There was a high degree of variability in the number of HA-specific cells recovered from tumors of different mice after their treatments. We can assume from previous experiments that none of the animals treated with single therapy would have survived, but we cannot know whether the two animals that were recordable in the combination therapy group were destined to survive or relapse. One possibility for the variability is that we are reflecting the highly individual nature of specificity that is a common feature of the immune response. Our own work in humans suggests that the antitumor response is dominated by private specificities (32). Although these mice have identical genetic background and the tumor is identical, the hierarchy of the response may still be variable.

It is unclear whether gemcitabine is unique in its immunomodulatory properties or whether the priming effect is shared by other drugs that cause tumor cell death by apoptosis. As gemcitabine is most commonly used in combination with other agents in the clinic, it is also important to ensure that combination with other drugs does not abrogate the benefits of chemoimmunotherapy. This work will be vital in determining whether the combination of cytotoxic chemotherapy and activating anti-CD40 antibodies can progress to clinical trials.

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REFERENCES

1. Oettle, H., Arnold, D., Hempel, C., and Riess, H. The role of gemcitabine alone and in combination in the treatment of pancreatic cancer. Anticancer Drugs, *11:* 771–786, 2000.
2. Manegold, C. Chemotherapy for advanced non-small cell lung cancer. Semin. Oncol., *28:* 1–6, 2001.
3. Orlando, M., and Mandachain, M. Gemcitabine in ovarian cancer. Semin. Oncol., *28:* 62–69, 2001.
4. Storniolo, A. M., Allerheiligen, S. R., and Pearce, H. L. Preclinical, pharmacologic, and phase I studies of gemcitabine. Semin. Oncol., *24:* S7-2–S7-7, 1997.
5. Nowak, A. K., Robinson, B. W., and Lake, R. A. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. Cancer Res., *62:* 2353–2358, 2002.
6. Nowak, A. K., Lake, R. A., Marzo, A. L., Scott, B., Heath, W. R., Collins, E. J., Frelinger, J. A., and Robinson, B. W. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. J. Immunol., *170:* 4905–4913, 2003.
7. Paulie, S., Ehlin-Henriksson, B., Mellstedt, H., Koho, H., Ben-Aissa, H., and Perlmann, P. A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. Cancer Immunol. Immunother., *20:* 23–28, 1985.
8. Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J. P., van Kooten, C., Liu, Y. J., Rousset, F., and Saeland, S. The CD40 antigen and its ligand. Annu. Rev. Immunol., *12:* 881–922, 1994.
9. Karmann, K., Hughes, C. C., Schechner, J., Fanslow, W. C., and Pober, J. S. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. Proc. Natl. Acad. Sci. USA, *92:* 4342–4346, 1995.
10. Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and Heath, W. R. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature (Lond.), *393:* 478–480, 1998.
11. Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., and Melief, C. J. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature (Lond.), *393:* 480–483, 1998.
12. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J. Exp. Med., *184:* 747–752, 1996.
13. Kamanaka, M., Yu, P., Yasui, T., Yoshida, K., Kawabe, T., Horii, T., Kishimoto, T., and Kikutani, H. Protective role of CD40 in Leishmania major infection at two distinct phases of cell-mediated immunity. Immunity, *4:* 275–281, 1996.
14. Yang, Y., and Wilson, J. M. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. Science (Wash. DC), *273:* 1862–1864, 1996.
15. Mackey, M. F., Gunn, J. R., Ting, P. P., Kikutani, H., Dranoff, G., Noelle, R. J., and Barth, R. J., Jr. Protective immunity induced by tumor vaccines requires interaction between CD40 and its ligand, CD154. Cancer Res., *57:* 2569–2574, 1997.
16. Guerder, S., and Matzinger, P. A fail-safe mechanism for maintaining self-tolerance. J. Exp. Med., *176:* 553–564, 1992.
17. Kurts, C., Kosaka, H., Carbone, F. R., Miller, J. F., and Heath, W. R. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. J. Exp. Med., *186:* 239–245, 1997.
18. Ridge, J. P., Di Rosa, F., and Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature (Lond.), *393:* 474–478, 1998.
19. Todryk, S. M., Tutt, A. L., Green, M. H., Smallwood, J. A., Halanek, N., Dalgleish, A. G., and Glennie, M. J. CD40 ligation for immunotherapy of solid tumours. J. Immunol. Methods, *248:* 139–147, 2001.
20. Funakoshi, S., Longo, D. L., Beckwith, M., Conley, D. K., Tsarfaty, G., Tsarfaty, I., Armitage, R. J., Fanslow, W. C., Spriggs, M. K., and Murphy, W. J. Inhibition of human B-cell lymphoma growth by CD40 stimulation. Blood, *83:* 2787–2794, 1994.
21. Mendoza, R. B., Cantwell, M. J., and Kipps, T. J. Immunostimulatory effects of a plasmid expressing CD40 ligand (CD154) on gene immunization. J. Immunol., *159:* 5777–5781, 1997.
22. Diehl, L., den Boer, A. T., Schoenberger, S. P., van der Voort, E. I., Schumacher, T. N., Melief, C. J., Offringa, R., and Toes, R. E. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. Nat. Med., *5:* 774–779, 1999.
23. Morgan, D. J., Liblau, R., Scott, B., Fleck, S., McDevitt, H. O., Sarvetnick, N., Lo, D., and Sherman, L. A. CD8(+) T cell-mediated spontaneous diabetes in neonatal mice. J. Immunol., *157:* 978–983, 1996.
24. Scott, B., Liblau, R., Degermann, S., Marconi, L. A., Ogata, L., Caton, A. J., McDevitt, H. O., and Lo, D. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. Immunity, *1:* 73–83, 1994.
25. Davis, M. R., Manning, L. S., Whitaker, D., Garlepp, M. J., and Robinson, B. W. Establishment of a murine model of malignant mesothelioma. Int. J. Cancer, *52:* 881–886, 1992.
26. Marzo, A. L., Lake, R. A., Robinson, B. W., and Scott, B. T-cell receptor transgenic analysis of tumor-specific CD8 and CD4 responses in the eradication of solid tumors. Cancer Res., *59:* 1071–1079, 1999b.
27. Braakhuis, B. J., Ruiz van Haperen, V. W., Boven, E., Veerman, G., and Peters, G. J. Schedule-dependent antitumor effect of gemcitabine in in vivo model system. Semin. Oncol., *22:* 42–46, 1995.
28. Hellstrom, K. E., and Hellstrom, I. Lymphocyte-mediated cytotoxicity and blocking serum activity to tumor antigens. Adv. Immunol., *18:* 209–277, 1974.
29. Qin, Z., Richter, G., Schuler, T., Ibe, S., Cao, X., and Blankenstein, T. B cells inhibit induction of T cell-dependent tumor immunity. Nat. Med., *4:* 627–630, 1998.
30. Marzo, A. L., Lake, R. A., Lo, D., Sherman, L., McWilliam, A., Nelson, D., Robinson, B. W., and Scott, B. Tumor antigens are constitutively presented in the draining lymph nodes. J. Immunol., *162:* 5838–5845, 1999.
31. Aversa, G., Punnonen, J., Carballido, J. M., Cocks, B. G., and de Vries, J. E. CD40 ligand-CD40 interaction in Ig isotype switching in mature and immature human B cells. Semin. Immunol., *6:* 295–301, 1994.
32. Robinson, C., Callow, M., Stevenson, S., Scott, B., Robinson, B. W., and Lake, R. A. Serologic responses in patients with malignant mesothelioma: evidence for both public and private specificities. Am. J. Respir. Cell Mol. Biol., *22:* 550–556, 2000.

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