Antitumor Immunity After Vaccination With B Lymphoma Cells Overexpressing a Triad of Costimulatory Molecules

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Background: The costimulatory molecules B7-1, intercellular adhesion molecule-1 (ICAM-1), and leukocyte function-associated antigen-3 (LFA-3) play pivotal roles in the activation of T cells. We investigated whether in vivo vaccination with lymphoma cells infected with a recombinant, nonreplicating fowlpox (FP) virus encoding this triad of costimulatory molecules (TRICOM) could stimulate lymphoma-specific immunity. Methods: TRICOM-infected A20 B lymphoma cells were analyzed for expression of B7-1, ICAM-1, and LFA-3. Mice (10 per group) were vaccinated with irradiated A20 cells infected with either the TRICOM vector or the wild-type FP virus (WT-FP), challenged with live A20 tumor cells, and followed for survival. Mice with established tumors were also treated with irradiated TRICOM-infected A20 cells. Survival curves were compared with the log-rank statistic. The mechanism of the antitumor effect was studied by in vitro depletion of CD4+ and CD8+ T cells and in vitro cytotoxicity assays. All statistical tests were two-sided. Results: A20 tumor cells infected with TRICOM expressed high levels of B7-1, ICAM-1, and LFA-3. Mice vaccinated with irradiated TRICOM-infected A20 cells had prolonged survival relative to mice vaccinated with WT-FP-infected cells (80% versus 20% survival at 110 days; P<.001). In mice with established tumors, tumor growth was slower in those treated with TRICOM-infected tumor cells than in those treated with WT-FP-infected cells, and this treatment provided a survival advantage (P<.001). Depletion of CD4+ or CD8+ T cells reduced the antitumor immunity provided by the tumor cell–TRICOM vaccine, and lymphocytes from vaccinated mice displayed in vitro cytotoxic activity toward A20 cells. Conclusions: Increasing expression of costimulatory molecules on B lymphoma cells by infection with a recombinant FP virus encoding B7-1, ICAM-1, and LFA-3 greatly enhanced antitumor immunity. Vaccination with genetically manipulated tumor cells represents an attractive therapeutic approach for patients with B-cell malignancies. [J Natl Cancer Inst 2003;95:548–55]

Vaccination with genetically manipulated tumor cells represents an attractive therapeutic approach for patients with B-cell malignancies. Because tumors of B-cell origin have the potential capability of antigen presentation, approaches that potentiate this process may be useful for the immunotherapy of B-cell lymphoma. However, B lymphoma cells often fail to activate T cells and induce T-cell proliferation in the autologous host (1). To function efficiently as antigen-presenting cells (APCs), tumor cells must express peptides bound to either class I or class II major histocompatibility complex (MHC) molecules and also express a number of costimulatory molecules that enhance T-cell activation and proliferation (2). Three of these molecules—B7-1, intercellular adhesion molecule-1 (ICAM-1), and leukocyte function-associated antigen-3 (LFA-3)—have been shown to play pivotal roles in T-cell stimulation (3). The failure of B lymphoma cells to function as efficient APCs has been attributed mainly to deficient expression of these costimulatory molecules (4). B-cell lymphomas can, however, be induced to function in vitro as APCs following CD40 ligation, which leads to increased expression of several costimulatory molecules, including B7-1, B7-2, ICAM-1, and LFA-3 (4).

However, detectable expression of costimulatory molecules by tumor cells does not necessarily lead to efficient activation of T cells. The capacity of an APC to activate T cells appears to depend on the level of expression of these costimulatory molecules (5). It has recently been shown that the capacity of professional APCs, such as dendritic cells (DCs), to stimulate T cells can be enhanced by transducing them with viral vectors encoding several costimulatory molecules (6–9). Even though mature DCs express sufficient costimulatory molecules to activate T cells, hyperexpression of B7-1, ICAM-1, and LFA-3 greatly enhanced their efficiency in doing so. Moreover, tumor cells modified to express high levels of these three costimulatory molecules can activate T cells to a greater extent than tumor cells transduced with any one or two of these molecules (10). Several reports (11–15) have shown that transfection of genes for costimulatory molecules into tumor cells may increase their immunogenicity in vivo and, once again, combinations of multiple costimulatory molecules provide better antitumor immunity.

In this study, we investigated whether B lymphoma cells overexpressing costimulatory molecules could be used to provoke antitumor immunity in vivo. We vaccinated mice with tumor cells engineered to express high levels of the costimulatory molecules B7-1, ICAM-1, and LFA-3 and studied the effect on protective and therapeutic immunity in a murine B-cell lymphoma model. Our findings may have implications for the design of novel therapies for patients with B-cell lymphoma.

Materials and Methods

Mice

Female BALB/c mice (6–8 weeks old) were purchased from Harlan-Sprague-Dawley Laboratories (San Diego, CA) and were housed at the Laboratory Animal Facility at Stanford University Medical Center (Stanford, CA). All experiments were conducted in accordance with Stanford University Animal Facility and National Institutes of Health guidelines.

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See “Notes” following “References.”
Cell Lines

A20, the BALB/c B-cell lymphoma line (16) expressing MHC class I and II H-2d molecules, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Tumor cells were cultured in RPMI-1640 medium (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone Laboratories, Logan, UT), penicillin at 100 U/mL, streptomycin at 100 μg/mL (both from Life Technologies), and 2-mercaptoethanol at 50 μM (Sigma-Aldrich, St. Louis, MO), subsequently referred to as complete medium. Cells were grown in suspension culture at 37 °C in 5% CO2. Hybridomas (GK1.5 [anti-CD4], 53–6.72 [anti-CD8], and H22–15–5 [rat IgG2]), and the P815 mastocytoma cell line, which is syngeneic to the A20 line, were obtained from ATCC and cultured in complete medium.

In Vitro Transduction of Tumor Cells With Recombinant FP Viruses

Recombinant, nonreplicating FP viruses encoding the murine costimulatory molecules B7-1, ICAM-1, and LFA-3 (designated as TRICOM [triad of costimulatory molecules]) (10) and murine granulocyte–macrophage colony-stimulating factor (GM-CSF, designated as GM-FP) (11) were provided by Therion Biologics Corporation (Cambridge, MA). A nonrecombinant wild-type fowlpox (WT-FP) virus was used as a negative control (10). A20 cells cultured in a modified version of complete medium that contained only 2% FCS were infected with the TRICOM virus at a multiplicity of infection of 100 for 18 hours. In titration studies, this multiplicity of infection was found to provide optimal B7-1 expression on infected A20 cells as measured by flow cytometry (data not shown). After infection, cells were harvested, and the expression of costimulatory molecules was assessed by flow cytometry. For this purpose, the cells were washed twice in phosphate-buffered saline (PBS)–1% bovine serum albumin plus 0.05% sodium azide and stained for 30 minutes on ice with a panel of phycoerythrin (PE)-conjugated monoclonal antibodies specific for murine B7-1 (CD80), B7-2 (CD86), ICAM-1 (CD54), and CD48 (the mouse homologue of human LFA-3) (all from Pharmingen, San Diego, CA). Appropriate isotype controls were used in all experiments. After incubation, the cells were washed, fixed with 2% paraformaldehyde, and analyzed using a Becton Dickinson FACScan (Mountain View, CA) with CellQuest software (Becton Dickinson).

Vaccination and Tumor Challenge Experiments

The A20 tumor cells were thawed from a common frozen stock and grown in vitro in complete medium for 4 days before use. On the day of tumor inoculation, cells were washed three times in RPMI-1640 (with no supplements) and diluted to the appropriate concentration in RPMI-1640. Groups of mice (10 per group) were injected subcutaneously in one flank with a lethal inoculum of 2 × 105 cells in a volume of 0.2 mL and were followed daily for survival. For tumor protection experiments, mice were vaccinated twice subcutaneously in one flank at an interval of 2 weeks with 1 × 106 A20 cells (0.2 mL) that had previously been infected in vitro with TRICOM or with control WT-FP virus as described above, washed extensively with RPMI-1640, and irradiated (5000 cGy) immediately before injection. One week after the second vaccination, mice were challenged with live, non-irradiated tumor cells (i.e., by subcutaneous injection in the opposite flank of 0.2 mL containing 2 × 105 cells) and followed daily for survival. In some experiments, mice surviving this initial tumor challenge (i.e., that were tumor-free at 110 days) were subjected to a second identical lethal tumor inoculum and followed daily for survival.

Treatment of Pre-Existing Tumors

For therapeutic experiments, mice were first injected subcutaneously with 2 × 105 A20 cells in one flank. On days 5 and 11 after tumor cell inoculation, mice received two subcutaneous injections, in the opposite flank, of 1 × 106 irradiated A20 cells infected as described above with either TRICOM or WT-FP virus. Tumors were measured three times each week in two dimensions (length and width) with a caliper, and tumor volumes were calculated according to the following formula: width2 × length × 0.52. Final measurements were obtained on day 34 after tumor cell inoculation, before any deaths occurred, to ensure that all mice would be included in the analysis. Tumor volumes are reported as mean mm3 with 95% confidence intervals (CIs). Mice were followed daily for survival. In some experiments, mice with established A20 tumors were treated by direct intratumoral injection of free FP viruses. For this purpose, mice were first injected subcutaneously with 1 × 105 A20 tumor cells. When the tumors became palpable (i.e., on day 22, when they had reached 5–6 mm in maximal diameter), they were injected with 100 μL of PBS containing 105 plaque-forming units (pfu) of GM-FP or WT-FP virus, either alone or in combination with TRICOM, or with PBS alone. A second dose of virus(es) was given 5 days later. Tumor volumes were measured and reported as described above.

Tumor-Specific Cytotoxicity Assays

Ten days after the second vaccination, splenocytes and lymph node cells were isolated from two representative mice of each indicated group (Fig. 5), pooled, and restimulated in vitro for 6 days at 5 × 106 cells/mL with irradiated (5000 cGy) A20 cells (1 × 106/mL). Interleukin 2 (IL-2) at 10 U/mL (Chiron Corp., Emeryville, CA) was added to the cultures on day 3. Viable cells were harvested and tested in a 4-hour standard 51Cr release assay (15) for the ability to lyse A20 cells. Briefly, 51Cr-labeled A20 or control target P815 cells were incubated with the restimulated effector cells at different effector:target (E:T) ratios in triplicate wells, and 51Cr release was determined by analyzing the supernatants in a gamma counter (Wallac, Turku, Finland). The percentage of specific release was calculated according to the following formula: 100 × (experimental release – spontaneous release)/(maximal release – spontaneous release). Spontaneous release and maximum release were obtained from wells containing target cells incubated in medium alone or in 1% Triton-X-100, respectively. Data are represented as the mean of triplicate values ± standard deviation.

Measurement of Humoral Anti-Idiotype Response

Anti-idiotype antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Serum was collected from TRICOM-vaccinated mice 5 days after the second immunization and added to 96-well MaxiSorp plates (Nunc, Naperville, IL) coated with purified recombinant Fab fragment A20 idiotype protein (10 μg/mL) (kindly provided by H. Veelken, Freiburg University, Freiburg, Germany). Bound anti-idiotype antibody was detected using horseradish peroxidase-conjugated goat anti-
mouse IgG (Caltag Laboratories, Burlingame, CA). Serum from mice vaccinated with recombinant A20 idiotype protein conjugated to keyhole limpet hemocyanin and known to contain anti-A20 idiotype antibodies (provided by H. Veelken) served as positive control. Absorbance was determined at 405 nm using a Vmax microplate reader (Molecular Devices, Menlo Park, CA).

**T-Cell Depletion Experiments**

Mice (10 per group) were vaccinated twice, 2 weeks apart, with irradiated TRICOM-infected A20 tumor cells and challenged with $2 \times 10^5$ live, non-irradiated tumor cells 1 week after the second vaccination. Mice were depleted of CD4$^+$ and CD8$^+$ cells by intraperitoneal injection of anti-CD4 (GK1.5 hybridoma) or anti-CD8 (53–6.72 hybridoma) ascitic fluid. These monoclonal antibodies (200 µL of diluted ascitic fluid per dose) were injected on days −3, −2, −1, and 0, relative to the tumor challenge, and then every other day for 1 week, followed by four weekly injections. A group of mice received the irrelevant rat IgG2 antibody (H22-15-5 hybridoma) as a control. These depletion conditions were validated by flow cytometry analysis of splenocytes using PE-conjugated anti-CD4 (Caltag) and anti-CD8 (clone 53-5.8; Pharmingen) monoclonal antibodies; 99% of the relevant T-cell subset was depleted, whereas all other lymphocyte subsets remained within normal levels (data not shown).

**Statistical Analyses**

Statistical significance of differences in survival between groups of mice was determined by the log-rank test using Prism software (GraphPad Software, Inc., San Diego, CA). For tumor measurement data, mean values of measured tumor volumes and 95% CIs are reported. $P$ values were determined using a two-tailed Mann–Whitney nonparametric test, and a $P$ value of less than .05 was considered to be statistically significant.

**RESULTS**

**In Vitro Expression of Costimulatory Molecules on TRICOM-Infected A20 Cells**

To determine whether infection of A20 cells with the TRICOM vector could induce expression of costimulatory molecules, A20 cells were infected with either TRICOM or WT-FP virus and cell surface expression of B7-1, ICAM-1, and LFA-3 was assessed by flow cytometry. As shown in Fig. 1, cells infected with WT-FP virus lacked expression of B7-1 but did express ICAM-1 and LFA-3. However, after infection with TRICOM, more than 95% of the cells expressed B7-1 and also expressed higher levels of both ICAM-1 (mean fluorescence intensity; WT-FP versus TRICOM, 56.5 versus 140.5, respectively) and LFA-3 (166 versus 372) than did cells infected with WT-FP. However, the expression of other surface molecules, such as B7-2 and Fas, was not affected (data not shown).

**In Vivo Antitumor Effect of Irradiated TRICOM-Infected Tumor Cells**

To attempt to stimulate systemic immunity against lymphoma, we vaccinated mice with irradiated A20 tumor cells that had been engineered to express high levels of B7-1, ICAM-1, and LFA-3 by infection with the TRICOM vector. First, we analyzed whether the irradiated TRICOM-infected tumor cells could confer protective immunity against challenge with lymphoma cells. For this purpose, groups of 10 mice were vaccinated twice, at an interval of 2 weeks, with irradiated tumor cells infected in vitro with TRICOM and were then challenged 1 week later with a lethal dose of parental A20 tumor cells. Controls included naive unvaccinated mice, mice vaccinated with irradiated A20 cells not infected with virus, and mice vaccinated with irradiated A20 cells infected with WT-FP virus. As shown in Fig. 2, A, mice vaccinated with TRICOM-infected irradiated...
tumor cells had prolonged survival relative to mice vaccinated with WT-FP-infected cells (80% [95% CI = 49% to 94%] versus 20% [95% CI = 6% to 51%] survival at 110 days; \( P < .001 \) by log-rank test). In contrast, mice vaccinated with tumor cells infected with WT-FP virus had no survival advantage relative to unvaccinated mice (\( P = .6 \) by log-rank test). To test whether vaccination with TRICOM-infected tumor cells could induce long-term (i.e., >100 days) immunity against the parental tumor, animals that had rejected the first tumor challenge (\( n = 8 \); Fig. 2, A) were re-challenged with the same lethal dose at 110 days after the initial challenge. Seven of eight mice (87.5%, 95% CI = 52% to 97%) remained tumor-free, whereas all naive control animals receiving this same challenge (\( n = 10 \)) developed disease and died by day 57 (\( P < .001 \)) (Fig. 2, B).

We next studied the capacity of the vaccine to stimulate therapeutic antitumor immunity in mice bearing established tumors. Mice (groups of 10) were first inoculated with \( 2 \times 10^5 \) A20 cells and then treated on days 5 and 11 after tumor cell inoculation with \( 1 \times 10^6 \) irradiated TRICOM-infected tumor cells. Tumors in mice injected with TRICOM-infected tumor cells grew more slowly than those in mice injected with WT-FP-infected cells; on day 34, mean tumor sizes were 758 mm\(^3\) (95% CI = 265 to 1252 mm\(^3\)) and 1945 mm\(^3\) (95% CI = 1555 to 2335 mm\(^3\)), respectively (\( P = .0017 \)) (Fig. 3, A). More important, mice receiving TRICOM-infected tumor cells had a survival advantage (20%, 95% CI = 6% to 51%) relative to control mice receiving WT-FP-infected or uninfected tumor cells (0%, 95% CI = 0% to 28% for both; log-rank \( P = .0023 \) and \( P < .001 \), respectively) (Fig. 3, B).
To investigate whether direct intratumoral injection of FP viruses could also slow the growth of pre-existing tumors, groups of 10 mice were inoculated with $10^5$ A20 cells and then injected intratumorally 5 and 10 days later with free recombinant viruses (TRICOM plus WT-FP; TRICOM plus GM-FP; or WT-FP, GM-FP, or PBS alone). GM-FP was used because GM-CSF delivery to tumor sites can augment antitumor immunity by inducing local accumulation and activity of APCs, including DCs (11).

Although tumors in mice injected with the combination of TRICOM plus WT-FP virus were not statistically significantly smaller than tumors in mice injected with WT-FP virus alone (at day 35, mean was 875 mm$^3$ [95% CI = 359 to 1391 mm$^3$] versus 1192 mm$^3$ [95% CI = 552 to 1832 mm$^3$], respectively; $P = .36$), tumors in mice injected with the combination of TRICOM plus GM-FP virus were smaller than those in mice injected with WT-FP virus (at day 35, mean was 436 mm$^3$ [95% CI = 203 to 670 mm$^3$] versus 1192 mm$^3$ [95% CI = 552 to 1832 mm$^3$]; $P = .018$) (Fig. 4). However, there was no survival advantage for mice treated with the combination of TRICOM and GM-FP virus compared with mice in other treated groups (data not shown).

**Cellular and Humoral Immune Responses in Vaccinated Mice**

To examine the generation of cytotoxic T-lymphocyte (CTL) responses against parental tumor cells, lymph node cells and splenocytes from mice vaccinated with irradiated TRICOM-infected tumor cells were cultured with irradiated parental A20 tumor cells for 6 days. As shown in Fig. 5, the effector cells from the vaccinated mice were able to lyse parental A20 tumor cells. This lysis was tumor-specific because the same effector cells were not able to lyse the syngeneic tumor cell line P815. No tumor-specific CTL activity was detected in mice vaccinated with tumor cells infected with the WT-FP virus.

We also sought evidence for a humoral response against the A20 idiotype protein in mice vaccinated with irradiated TRICOM-infected A20 cells to assess any role of this tumorspecific antigen in the observed tumor protection. In contrast to serum from mice vaccinated with recombinant A20 idiotype protein conjugated to keyhole limpet hemocyanin, no antiidiotype antibodies were detected in serum from mice collected 5 days after the second TRICOM-A20 cell vaccine (data not shown).

**Involvement of CD4+ and CD8+ T Cells in the Antitumor Effect of Irradiated TRICOM-Infected Tumor Cells**

We next determined which subpopulations of effector T cells were involved in the *in vivo* antitumor effect after vaccination with tumor cells infected with TRICOM. After the vaccine was administered, and before the tumor cell challenge, mice (groups of 10) were depleted of CD4+ or CD8+ T cells by treatment with monoclonal antibodies. Antibodies were also given after the tumor challenge to ensure continued depletion of the relevant...
T-cell subsets. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were important for the antitumor effect induced by the vaccine, because at 75 days, 60% (95% CI = 31% to 83%) of mice treated with control antibody were still alive compared with 20% (95% CI = 6% to 51%) of the anti-CD4-treated mice and 0% (95% CI = 0% to 28%) of the anti-CD8-treated mice; log-rank P = .023 and P<.001, respectively, by log-rank test (Fig. 6).

DISCUSSION

In recent years, efforts to increase the immunogenicity of tumors have involved the transfer of genes encoding costimulatory molecules. Even if a tumor expresses a host of immunogenic tumor-associated antigens, however, it may fail to induce stimulation of T cells if its expression of costimulatory molecules is not adequate (2). The importance of costimulation to tumor immunity was demonstrated by showing that transfection of costimulatory molecules into a subset of murine tumors confers immunogenicity (12,15,17). B7-1 has been one of the most extensively studied costimulatory molecules; through its interaction with CD28 on T cells, it is able to enhance T-cell activation (18). However, it is now clear that at least two other molecules expressed by APCs—ICAM-1 and LFA-3—play essential roles in providing T-cell stimulation (19–21). Moreover, all three of these molecules behave synergistically to amplify the activation of T cells (10,22). B-cell lymphomas themselves can be induced to function as APCs and present tumor antigens directly to T cells (23,24). For example, cross-linking of CD40 on the cell surface by local expression of CD40 ligand increases the immunogenicity of the murine A20 lymphoma by increasing the expression of costimulatory molecules (25), a concept that has been recently tested for human B-cell malignancies as well (26,27).

In this study, we have shown that murine A20 lymphoma cells infected with TRICOM vector express high levels of the costimulatory molecules B7-1, ICAM-1, and LFA-3 and can be used as a vaccine to promote immunity against parental lymphoma cells. Whereas parental A20 cells express no B7-1 and only moderate levels of ICAM-1 and LFA-3, after infection with the TRICOM vector, more than 95% of the cells expressed B7-1, and increased levels of both ICAM-1 and LFA-3 were observed. Eight of 10 mice immunized with the irradiated TRICOM-infected A20 cells were able to reject a challenge with parental A20 tumor cells (Fig. 2, A). In addition, seven of the eight protected animals were able to reject a second challenge with parental tumor cells long after being immunized, which is consistent with the development of a memory antitumor immune response.

Several lines of evidence indicate that the level of expression of costimulatory molecules regulates the immunogenicity of a tumor and the effectiveness of an antitumor response (28). First, hyperexpression of B7-1, ICAM-1, and LFA-3 by TRICOM-infected DCs greatly enhances their capacity to stimulate T cells (6,9). Furthermore, fibroblasts engineered to express high levels of B7-1, ICAM-1, and LFA-3 along with a class I MHC molecule are able to stimulate antigen-specific cytotoxic T lymphocytes to a level even greater than that induced by mature DCs (29). These data further emphasize that a high level of expression of costimulatory molecules is desirable when attempting to induce T-cell immunity.

Although B-cell lymphoma represents a malignancy of professional APCs, our data indicate that lymphoma immunogenicity can be augmented by the forced overexpression of B7-1, ICAM-1, and LFA-3. The relative contributions of each of these costimulatory molecules to induction of the observed antitumor immunity is not known, but it has been shown that maximal activation of T cells by TRICOM vector-infected DCs requires the activity of all three molecules (29). Furthermore, the costimulatory signals provided to T cells via the B7-1/CD28, ICAM-1/LFA-1, and LFA-3/CD2 pathways are known to provide complementary effects promoting cellular adhesion, cytokine production, and proliferation (20). Hence, all of these pathways may play important roles in the generation of antilymphoma immunity.

In this study, we have also shown that vaccination with irradiated tumor cells expressing high levels of costimulatory molecules can induce statistically significant antitumor effects in animals that have pre-existing lymphoma (Fig. 3, B). Although these effects are modest, further refinements, such as increased vaccine dosage or co-injection of GM-CSF-producing FP virus, may improve the therapeutic efficacy of our approach in the clinically relevant setting of established tumors. We attempted to induce therapeutic antitumor immunity by injecting TRICOM, GM-CSF-encoding, or control wild-type FP viruses directly into established tumors. In this scenario, the tumors injected with the free TRICOM vector had a trend toward slower growth, but this effect did not reach statistical significance (Fig. 4). This result may be explained, in part, by the relatively low dose of virus used because it has been shown that the effect of intratumoral administration of recombinant viruses expressing immune stimulatory proteins is largely dose-dependent (30). However, a statistically significant inhibition of tumor growth was found when direct intratumoral administration of free TRICOM was combined with that of an FP virus-encoding murine GM-CSF, a combination that has been previously shown to cooperate in the

![Fig. 6. Effect on survival of in vivo depletion of T-cell subsets on survival following vaccination with TRICOM (triad of costimulatory molecules)-infected tumor cells. Groups of 10 mice were vaccinated subcutaneously twice, at intervals of 2 weeks, with 1 x 10<sup>6</sup> irradiated A20 cells that had been infected in vitro with TRICOM. They were then challenged with 2 x 10<sup>5</sup> parental A20 tumor cells 1 week after the second vaccination. Mice were depleted of T cells just before tumor challenge by intraperitoneal administration of GK1.5 (anti-CD4) (▲) or 53–6.72 (anti-CD8) (▼) antibodies. A group of mice received an irrelevant rat IgG (H22-15-5) as a control (□). Another group of naive mice received tumor challenge only (●).](image-url)
generation of an antitumor effect (31). Nevertheless, because no survival advantage was incurred with the direct intratumoral injection, immunization with tumor cells infected ex vivo with TRICOM remains our favored strategy for future studies.

The tumor protection induced by vaccination with irradiated TRICOM-infected A20 tumor cells appeared to require T lymphocytes. That is, in vivo depletion of CD8\(^+\) T cells completely abrogated the development of a protective immune response, and depletion of CD4\(^+\) T cells reduced it—findings that are consistent with those of previous studies (25, 32) using the A20 tumor model. These results support our in vitro finding that mice vaccinated with tumor cells infected with TRICOM vector, but not with a control virus, generated effector T cells able to specifically lyse parental A20 tumor cells. The requirement for both subsets of effector T cells may be related to the fact that tumor A20 cells express both class I and class II MHC molecules (16), thus making them potential targets for both CD4\(^+\) and CD8\(^+\) T cells. Alternatively, CD4\(^+\) T cells may also be involved indirectly in mediating tumor protection in the effector phase (i.e., after establishment of the immune response) by helping to maintain the number of or activity of CD8\(^+\) effector T cells.

Although cellular immune mechanisms appear to be responsible for the antitumor effect observed with whole tumor cell vaccines, the role of a humoral response against antigens expressed by the tumor cells is less clear. For example, we cannot rule out the existence of antibodies against unknown tumor antigens, but our study suggests that antibodies directed against one known B-cell lymphoma tumor antigen (the idiotype) may not play an important role in the protection we observed. However, because we did not measure T-cell responses against A20 idiotype protein, a role for anti-idiotypic T cells in the observed antitumor effects cannot be ruled out.

In summary, our data suggest that autologous cellular vaccines engineered to express high levels of costimulatory molecules are able to stimulate potent cellular immune responses against parental lymphoma cells. This approach may ultimately prove useful in developing treatments for patients with B-cell lymphoma.

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NOTES

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