In Vivo Priming of Two Distinct Antitumor Effector Populations: The Role of MHC Class I Expression

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Summary

Downregulation of major histocompatibility complex (MHC) class I expression is an important mechanism by which tumors evade classical T cell–dependent immune responses. Therefore, a system was designed to evaluate parameters for active immunization against MHC class I^- tumors. Mice were capable of rejecting a MHC class I^- tumor challenge after immunization with an irradiated granulocyte/macrophage colony-stimulating factor (GM-CSF) transduced MHC class I^- tumor vaccine. This response was critically dependent on $CD4^+$ T cells and natural killer (NK) cells, but minimally on $CD8^+$ T cells. A strong protective response against MHC class I^+ variants of the tumor could be elicited when mice were immunized with irradiated MHC class I^+ GM-CSF–secreting tumor cells. This response required $CD4^+$ and $CD8^+$ T cells, and in addition, elimination of NK cells resulted in outgrowth of tumors that had lost expression of at least one MHC class I gene. Finally, class I MHC expression on the vaccinating cells inhibited the response generated against a MHC class I^- tumor challenge. These results demonstrate that the host is capable of being immunized against a tumor that has lost MHC class I expression and reveal conditions under which distinct effector cells play a role in the systemic antitumor immune response.

The immune system is capable of recognizing and responding to tumors via several effector mechanisms. Recent evidence demonstrates that tumor-specific CTL can be generated which recognize tumor antigens presented on the cell surface together with MHC class I molecules (1, 2). Such neoantigens are peptides derived from endogenously synthesized proteins which are seen as foreign by the host, either as a consequence of mutations that arise during malignant transformation or tumor expression of gene products not normally expressed in adult tissues. The predicted consequence of such a T cell–mediated selective pressure is outgrowth of tumors that have lost expression of these antigens, or equivalently, loss of the class I MHC molecules themselves. Indeed, reports of diminished class I expression on clinically advanced tumors and metastases are consistent with this idea (3–5). Likewise, in the majority of experimental models tested, introduction of MHC class I genes into MHC class I^{low} tumor cells to increase their surface expression results in a decrease in in vivo tumorigenicity (6–9).

In contrast to T cell–mediated, MHC-restricted recognition of tumor, NK cells are known to be able to kill certain tumor cell lines in spite of the fact that they express little or no MHC class I antigen. The nature of the recognition molecules involved in NK–target cell interaction is not well understood, although emerging data support the hypothesis that one pathway of NK cell activation occurs as a consequence of encountering cells that have lost self-MHC expression (10). Mechanistically, target expression of class I MHC would either mask the NK receptor ligand, precluding activation, or alternatively, NK cell engagement with target MHC would result in an inactivation signal. In vitro data demonstrate that many class I^-, NK-sensitive targets are rendered resistant to lysis by restoring or increasing MHC class I expression (11–13). This recognition appears to be at least partially haplotype specific, as expression of allogeneic class I molecules on the target still results in sensitivity to NK lysis. Recent studies of cloned human NK cell line allospecificity demonstrate that the trait of susceptibility to NK lysis is inherited in an autosomal recessive fashion and segregates with MHC haplotypes, compatible with the activation signal being the failure to encounter self-MHC (14, 15). This pattern of NK activation does not appear to be restricted to tumor recognition, as NK cell–mediated bone marrow graft rejection has been shown to occur when an otherwise MHC-identical marrow graft lacks a single MHC locus of the recipient (16, 17).

Seemingly at odds with the in vivo consequence of enhanced tumor MHC expression mentioned above, studies of

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several MHC class I–deficient tumors report rejection of small tumor inocula by syngeneic recipients, whereas the MHC class I^+ wild-type counterparts are tumorigenic (12, 18, 19). Similarly, in the B16 murine melanoma model, the ability to form lung metastases has been shown to be inversely related to the class I MHC expression on the tumor (20). In these models, elimination of NK cells results in the loss of the response to MHC class I^- tumors.

To examine the interplay of these distinct effector pathways in a single tumor system, we used a strategy of immunization with genetically modified tumor vaccines followed by live tumor challenge to dissect the role of these different effector cells in tumor rejection in vivo as a function of class I MHC expression.

Materials and Methods

Tumor Cells. B78H1 cells were obtained from L. Graf, Jr. (University of Illinois College of Dentistry, Chicago, IL; 21). The F10 subline of B16 melanoma cells (22) was obtained from the National Institutes of Health Depository of Cells and Tissues. Cells were cultured in vitro in RPMI media, supplemented with 10% FCS, penicillin/streptomycin (50 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and nonessential amino acids (0.1 mM), and grown at 37°C, 5% CO_2. All cells were periodically tested for, and found to be free of mycoplasma contamination.

Detection of MHC Class I mRNA. B78H1 and B16F10 were grown in vitro with or without the addition of recombinant murine IFN-γ (100 U/ml for 3 d). Cell were trypsinized and washed in serum-free HBSS, and total cellular RNA was isolated from 10^7 cells that were denatured in the presence of guanidinium thiocyanate (23). cDNA was prepared by reverse transcriptase reaction using standard procedures (24). 1 μg of cDNA was amplified by PCR utilizing a 5′ consensus primer for $H\text{-}2D^b$ and $H\text{-}2K^b$ (5′-CGC GAC GCT GCT GCG CAC AG-3′), and 3′ primers specific for $H\text{-}2D^b$ (5′-TAC AAT CTC GGA GAG ACA TT-3′) or $H\text{-}2K^b$ (5′-TAC AAT CTG GGA GAG ACA GA-3′). Amplification of dihydrofolate reductase message served as a positive control using the primers 5′-CTC AGG GCT GCG ATT TCG CGC CAA ACT and 3′-CTG GTA AAC AGA ACT GCG TCC GAC TAT C. 40 cycles of amplification was performed on a thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT; 94°C melt, 65°C anneal, 72°C extension temperatures for 1 min each time). Reaction products were electrophoresed and stained with ethidium bromide.

Gene Transfer. MHC class I variants were created by introduction of DNA as a coprecipitate with calcium phosphate (25). $H\text{-}2D^b$ expression was achieved using plasmid pMo/D^b (26) which contains a $H\text{-}2D^b$ genomic segment driven off a MoLTR promoter. For $H\text{-}2K^b$ expression, plasmid $pK^bT.8$ (27, 28) containing $H\text{-}2K^b$ cDNA under a ubiquitin promoter was used. Both vectors contain the neomycin-resistance gene. Control transfections with the vectors alone (minus the MHC genes) were also performed. A total of 5 μg of plasmid vectors was precipitated, and cells were exposed to the precipitate for 14–16 h, washed once with HBSS without Ca^{2+} or Mg^{2+}, refed with DMEM with 10% FCS, and incubated at 37°C. Selection in G418 at 400 μg/ml in RPMI media was begun 48 h after cells were exposed to precipitate. More than 100 colonies were pooled for each transfection and expanded in culture. Single MHC locus transfectants were stained with mAb 28-14-8 (anti-$H\text{-}2D^b$) followed by goat anti–mouse IgGγ2aFITC (Southern Biotechnology Associates, Birmingham, AL) or with mAb B8-24-3 (anti-K^b) followed by goat anti–mouse IgG1FITC (Southern Biotechnology Associates). Double transfectants were stained with mAb B8-24-3 followed by goat anti–mouse IgGPE (Southern Biotechnology Associates), unlabeled mouse Ig, and then 28-14-8 FITC. Positive cells were collected on a FACS® (Becton Dickinson & Co., Mountain View, CA) recovering >50,000 cells per sort. Conformation of purity was performed after in vitro expansion, and as many as five sorts were required to obtain populations with homogeneous expression. GM-CSF production was achieved via retroviral transduction of the MHC variants with MFG GM-CSF as described (29). All cellular constructs were expanded in vitro and aliquots frozen in liquid nitrogen before in vivo studies, enabling newly thawed reagents to be used in each experiment.

GM-CSF Assays. GM-CSF production was quantified by collection of tumor supernatant (2×10^5 cells in 5 ml for 24 h) and assaying for support of the GM-CSF–dependent cell line NFS-60. Dilutions of supernatant were transferred to 96-well microtiter plates containing 10,000 NFS-60 cells per well. After 48 h, [^{3}H]thymidine (1 μCi/well) was added for 12 h after which incorporation was assessed with a cell harvester. Units of GM-CSF were calculated by comparison to a standard curve generated with recombinant GM-CSF.

CTL Assays. Spleens were removed from BALB/c and C57BL/6 mice and single cell suspensions were created by mechanical dissociation and passage over nylon mesh. Red cells were removed by Ficoll density centrifugation, and lymphocytes were washed and counted. BALB/c responder cells were plated in 24-well plates at 5×10^6 cells/well. $B78H1D^bK^b$ cells or C57BL/6 splenocytes were added as stimulators after incubation with mitomycin C (50 μg/ml for 1 h at 37°C, then three washes in RPMI-10% FCS). 10 U/ml of recombinant murine IL-2 was added per well. After 5 d, ^{51}Cr-release assays were performed as described (30) using B78H1 or $B78H1D^bK^b$ as targets.

In Vivo Protection Assays. Cells for injection were harvested from in vitro culture by trypsinization after limited expansion and washed three times in serum-free HBSS. GM-CSF–producing vaccine cells were irradiated (5,000 rad) before injection. All injections were in 0.1 ml given subcutaneously in the left (vaccine) or right (challenge) flank. All experiments included 10 mice per group, and each was repeated at least once. Mice were monitored twice weekly, and euthanized after the development of tumor.

In Vivo Antibody Depletions. In vivo antibody depletions were started 1 wk before vaccination. mAb GK1.5 (31) was used for CD4 depletions, mAb 2.43 (32) was used for CD8 depletions, and mAb PK136 (33) was used for NK depletion. Ammonium sulfate–purified ascites fluid (titered at >1:2,000 by staining of splenocytes on the FACS®) was injected intraperitoneally (0.1 ml per mouse) every other day for the first week and once per week afterward. Depletion of lymphocyte subsets was assessed on the day of vaccination, the day of live tumor challenge, and weekly thereafter by flow cytometric analysis of spleen cells stained with 2.43 or GK1.5 followed by FITC-labeled goat antibody to rat IgG (Kirkegaard & Perry Labortories, Inc., Gaithersburg, MD) or PK136 followed by FITC-labeled goat antibody to mouse IgG. For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of other subsets.

Results

B78H1 Does not Express MHC Class I Molecules. The murine melanoma B78H1 is an amelanotic clone of the C57BL/6-derived tumor B16 (34). B16 expresses low levels of MHC class I molecules, it is not truly MHC class I^-, and expres-

sion can be significantly increased after exposure to IFN-γ. In contrast, B78H1 has been shown to express no detectable MHC class I antigen in vitro or after in vivo passage, and such expression is not inducible with IFN-γ (35). The absence of surface expression of MHC class I is due to an absence of mRNA transcripts. RNA was isolated from B78H1 and B16F10 cultured with and without IFN-γ. cDNA was prepared by reverse transcriptase reaction, and PCR amplification was performed using H-2D^b, H-2K^b, or dihydrofolate reductase (DHFR)[1]–specific oligonucleotide primers. H-2D^b and H-K^b transcripts were evident from B16F10 but could not be detected from B78H1, whereas DHFR transcripts were detected in all samples (Fig. 1).

Creation of MHC Class I Variants. The C57BL/6 strain of mice from which these tumors originated expresses only two classical MHC class I loci, H-2D^b and H-2K^b. Three MHC class I$^+$ variants of B78H1 were created via transfection of the tumor with either the plasmid vector pMo/D^b (containing a neomycin resistance gene and an H-2D^b genomic segment), plasmid pK^{b}T.8 (containing a neomycin-resistance gene and an H-2K^b cDNA), or both. Control transfections with the vectors minus the MHC genes were also performed. Transfectants were selected in the neomycin analog G418, and G418 resistant lines were prepared from more than 100 pooled clones of approximately equal size. Expanded polyclonal populations were stained with the mAb 28-14-8 (anti-D^b), B8-24-3 (anti-K^b), or both, and sorted by flow cytometry. Analysis of the sorted transfectants revealed heterogeneously positive populations for the appropriate MHC antigens (Fig. 2). Murine GM-CSF–producing vaccine was created by retroviral transduction of the parental class I$^-$ B78H1 and the H-2D^b, H-2K^b, and H-2D^{b}K^b variants using MFG-murine GM-CSF (10,000–20,000 U/2 × 10^5 cells/24 h), as determined by in vitro bioassay on the GM-CSF–dependent cell line NFS-60 (data not shown).

MHC Class I Expression In Vivo. To determine the fidelity of MHC class I expression in vivo, 10^6 cells of each MHC variants was injected subcutaneously in C57BL/6 mice. Animals were killed on day 11, and tumor samples were frozen in liquid nitrogen and prepared for immunohistochemical staining. Representative samples are shown (Fig. 3) and reveal no detectable MHC class I expression on B78H1, whereas the D^b and K^b variants are positive for the correct locus in situ. This result not only confirms the expression of the transfected MHC molecules in vivo, but also suggests that unidentified host factors are not inducing MHC class I expression in the B78H1 wild-type tumor.

H-2b–specific Allogeneic CTL Recognize B78H1 Class I$^+$ Variants but not B78H1 Wild Type. Although mAb staining confirms that the B78H1 MHC class I transfectants express the class I epitope to which the antibody binds, the relevant function that the MHC molecule must serve is the ability to present antigen to T cells. As a measure of this function, an allogeneic CTL assay was performed. BALB/c splenocytes (H-2d) were stimulated in vitro with mitomycin C–treated C57BL/6 (H-2b) splenocytes, or with B78H1D^{b}K^b. After 5 d in culture, the cells were harvested and tested for their ability to lyse ^{51}chromium-labeled B78H1D^{b}K^b targets or B78H1 wild-type targets. Significant anti-B78H1D^{b}K^b CTL activity was detectable after incubation with either H-2b splenocytes or with B78H1D^{b}K^b itself, suggesting that allogeneic T cells were capable of recognizing the transfected MHC class I molecules on B78H1D^{b}K^b (Fig. 4 *A*). The majority of this in vitro activity was blocked by incubation with antibody to CD8. In contrast, the same allogeneic CTL had very little activity against the MHC class I$^-$ B78H1 wild-type targets, and none of this was blocked with anti-CD8 antibody (Fig. 4 *B*), indicating that CD8$^+$ T cells with specificity for H-2D^b and H-2K^b do not recognize B78H1. Similarly, B78H1 wild type did not generate a class I–specific allogeneic CTL when used as a stimulator cell in vitro (data not shown).

In Vivo Immune Response to MHC Class I$^-$ Tumor. Previous studies of the systemic antitumor immune response induced by cytokine-producing tumor vaccines have revealed the dependence on MHC class I–restricted, CD8$^+$ CTL (29, 30, 36). Given that these effector cells are incapable of recognizing B78H1 in vitro, we tested the in vivo response against

[1] *Abbreviation used in this paper:* DHFR, dihydrofolate reductase.

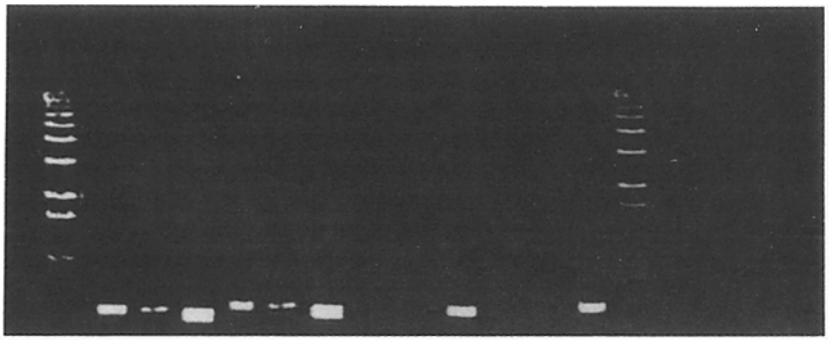

Figure 1. Detection of class I MHC mRNA by RT-PCR. B78H1 and B16F10 cells were grown in the presence and absence of IFN-γ (100 U/ml) after which total cellular RNA was isolated and cDNA created by reverse transcriptase reaction. PCR amplification was performed on each sample using oligonucleotide primers specific for H-2D^b, H-2K^b, and dihydrofolate reductase. Reaction products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Lanes: (*1*) B16F10, (*2*) B16F10 IFN-γ–treated, (*3*) B78H1, (*4*) B78H1 IFN-γ–treated. Lanes: (*A*) H-2D^b primers, (*B*) H-2K^b primers, (*C*) DHFR primers.

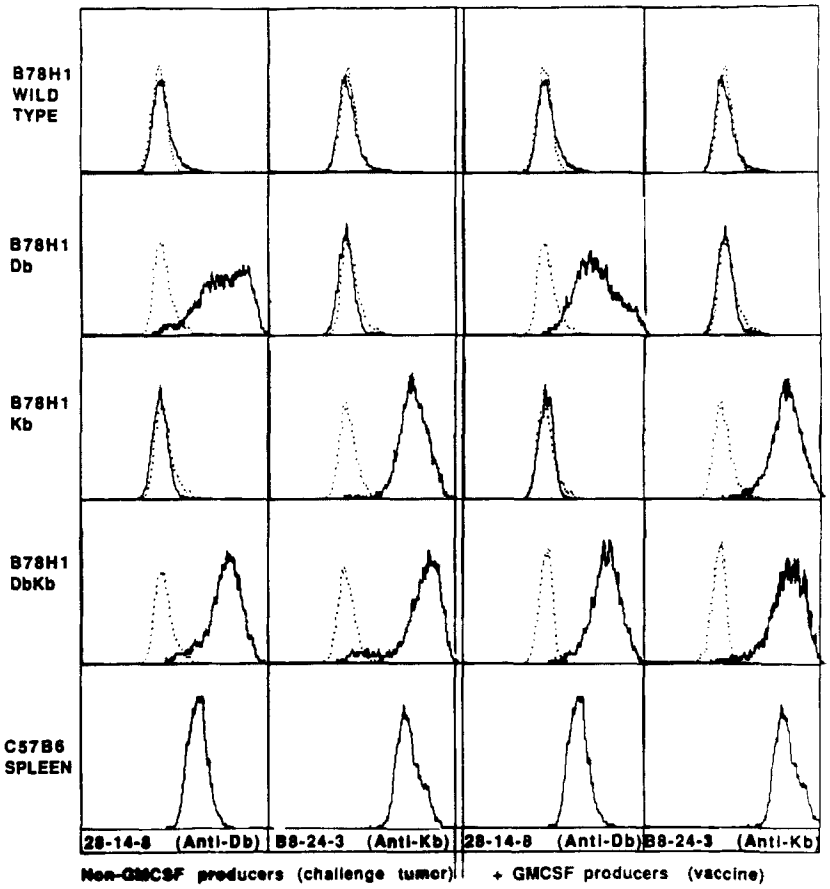

Figure 2. Class I MHC expression of B78H1 variants. Each of the four GM-CSF-producing vaccines and four non-GM-CSF-producing challenge tumors was stained for class I MHC expression with antibody to H-2D^b (mAb 28-14-8 followed by goat anti-mouse IgG2aFITC) and to H-2K^b (mAb B8-24-3 followed by goat anti-mouse IgG1FITC) and analyzed by flow cytometry. Also shown is the staining of normal C57BL/6 splenocytes for comparison.

a B78H1 challenge (Fig. 5 *A*). Remarkably, mice immunized with irradiated B78H1GM-CSF were largely protected against the MHC class I$^-$ tumor challenge at a distant site. Immunization with irradiated nontransduced B78H1 cells also produced some systemic protection against live B78H1 challenge but at a much lower level than GM-CSF transduced vaccine. To identify which cell populations are involved in this response, in vivo lymphocyte subset depletion was performed with injections of mAb against CD4$^+$, CD8$^+$, or NK1.1$^+$ cells initiated before the immunization with irradiated B78H1GM-CSF and maintained throughout the experiment (Fig. 5 *B*). Subsequent challenge with B78H1 resulted in tumor formation in mice depleted of CD4$^+$ T cells and NK1.1$^+$ cells comparable to unimmunized controls, whereas the CD8$^+$ T cell–depleted group was still significantly protected. The effect of CD8$^+$ T cell depletion was small but was seen consistently over three experiments and may represent the elimination of a subset of NK cells that expresses CD8. Given the requirement for CD4$^+$ T cells in the response to a MHC class I$^-$ tumor challenge, explanted tumor was stained for expression of MHC class II molecules (Fig. 6). B78H1 grown in vitro has no detectable MHC class II expression by FACS® analysis, although expression is induced with exposure to IFN-γ. Explanted B78H1 fails to demonstrate detectable levels of MHC class II expression. It is therefore unlikely that CD4$^+$ T cells are acting directly as cytolytic effector cells. Taken together, these results suggest that NK1$^+$ cells are a major effector cell in the response to a MHC class I$^-$ tumor challenge, and that these cells require the presence of CD4$^+$ Th cells that have encountered antigen on host MHC class II$^+$ APCs.

In Vivo Immune Response to MHC Class I$^+$ Tumor. We next sought to characterize the in vivo response against a MHC class I$^+$ tumor challenge. Mice were immunized with irradiated B78H1D^{b}K^{b}GM-CSF and subsequently challenged with either B78H1D^b, B78H1K^b, or B78H1D^{b}K^b (Fig. 7 *A*). There was a high level of protection against the MHC class I$^+$ variants that expressed H-2K^b (B78H1K^b and B78 H1D^{b}K^b), but not H-2D^b alone, suggesting that in this tumor system, an immunodominant antigen(s) recognized by class I MHC–restricted T cells is presented on H-2K^b. Selective in vivo lymphocyte subset depletion demonstrated that rejection of the MHC class I$^+$ tumor B78H1D^{b}K^b also required CD4$^+$ T cells, but in contrast to the MHC class I$^-$ response, depletion of CD8$^+$ T cells resulted in complete loss of protection against B78H1D^{b}K^b (Fig. 7 *B*). It is interesting to note that NK1$^+$ cell depletion also resulted in the development of tumor outgrowth. Mice from this group were killed at the time of tumor detection and the tumors were explanted and stained for MHC class I expression (Fig. 8). In all cases, tumors that developed in mice depleted of NK1$^+$ cells lost expression of the dominant T cell restric-

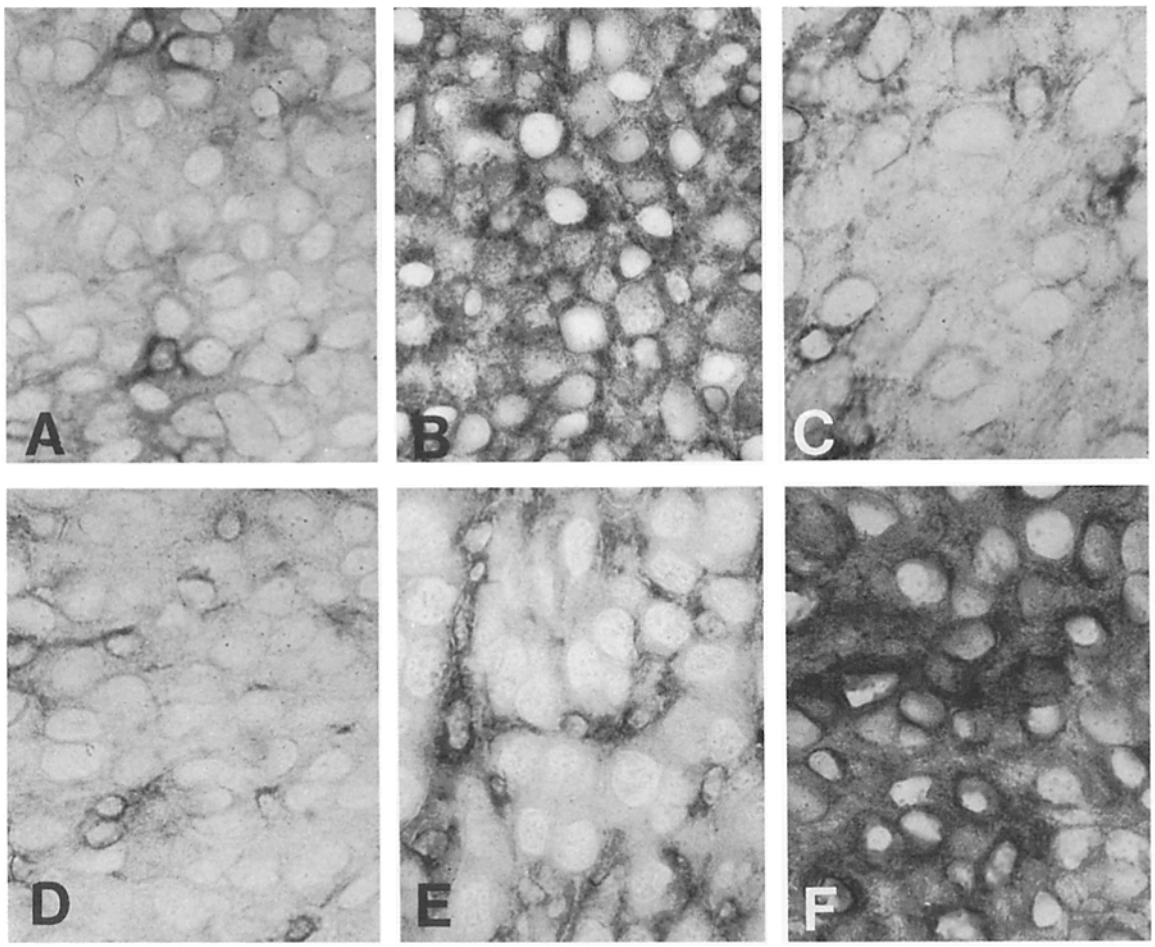

Figure 3. MHC Class I expression of B78H1 variants in vivo. Immunohistochemical staining for H-2D^b and H-2K^b was performed on tumor explained 11 d after subcutaneous injection into C57BL/6 mice. Shown are the patterns with antibody to (*top*) H-2D^b (mAb 28-14-8); (*bottom*) H-2K^b (mAb B8-24-3) using an avidin-biotin-peroxidase methodology (Vectastain). (*A* and *D*) Wild-type B78H1; (*B* and *E*) B78H1D^b; and (*C* and *F*) B78H1K^b. (*B* and *F*) Strong staining of the tumor indicating MHC class I expression in situ. The remaining panels show no staining of the tumors, although positive staining is present on smaller mononuclear cells or endothelial cells that have infiltrated the tumor injection site. ×400.

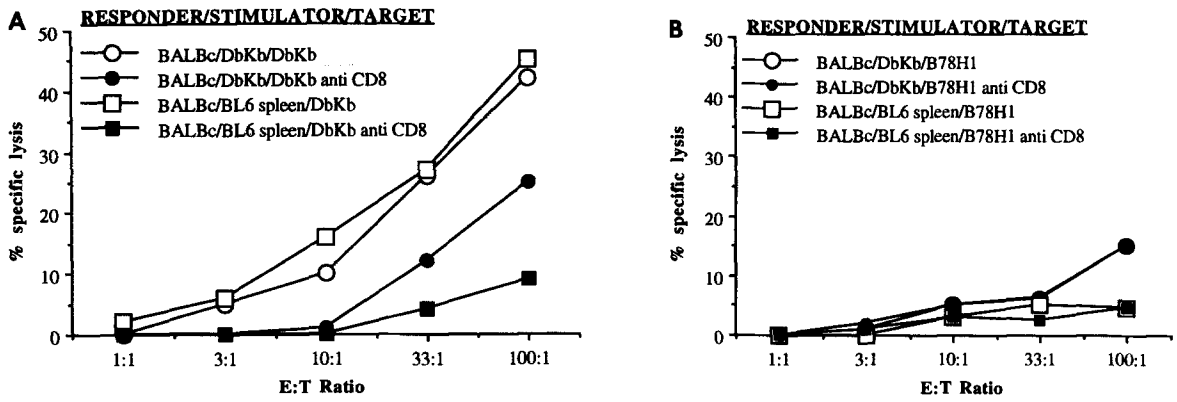

Figure 4. Recognition of class I MHC expression by allogeneic T cells. BALB/c splenocytes were cultured for 5 d with mitomycin C–treated C57BL/6 splenocytes or with B78H1D^{b}K^b cells in the presence of IL-2. At the end of the culture, live cells were mixed with ^{51}Cr-labeled targets at different E/T ratios with or without the addition of anti-CD8 antibody in a 4-h ^{51}Cr-release assay. (*A*) Lysis of B78H1D^{b}K^b targets. (*B*) Lysis of B78H1 wild-type targets.

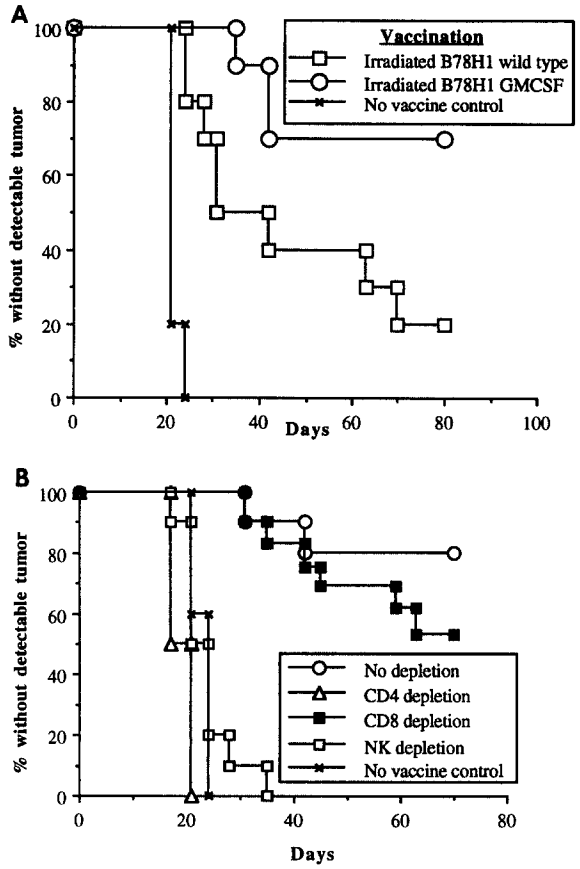

Figure 5. (*A*) Ability of immunized mice to reject a class I MHC$^-$ tumor challenge. C57BL/6 mice were injected subcutaneously in the left flank with 10^6 irradiated (5,000 rad) B78H1 wild type, or B78H1GM-CSF cells, followed 2 wk later by subcutaneous challenge in the right flank with 10^5 live B78H1 cells. Also shown is B78H1 growth without preimmunization. Tumor growth was assessed twice a week by inspection and palpation. 10 mice are included per group. Data are presented as a Kaplan-Meier plot. (*B*) Effect of lymphocyte subset depletion on the protective in vivo response to class I MHC$^-$ B78H1GM-CSF cells against a live B78H1 challenge. C57BL/6 mice were depleted of CD4$^+$ T cells, CD8$^+$ T cells, or NK1.1$^+$ cells by intraperitoneal injections of purified mAb GK1.5, 2.43, or PK136 respectively. They were then injected subcutaneously with 10^6 irradiated (5,000 rad) B78H1GM-CSF cells. 2 wk later, they were challenged in the opposite flank with 10^5 live B78H1 cells. Also shown is the growth of B78H1 cells without subset depletion or preimmunization.

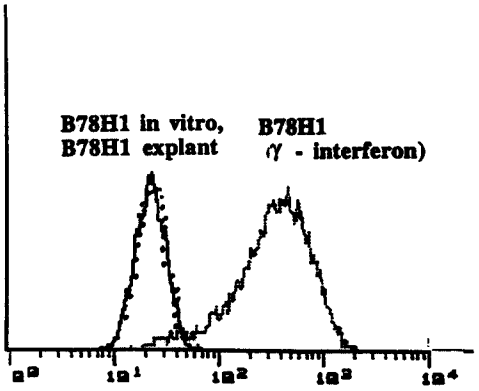

Figure 6. Expression of MHC class II antigen on explanted B78H1 cells. B78H1 was excised from the right flank of C57BL/6 mice after reaching 1.0 cm. The tumor tissue was minced in media to <1-mm fragments with scalpel blades and then passed over nylon mesh. Plastic adherent cells were collected after a 4-h incubation at 37°C, 5% CO_2. Cells were stained for expression of I-A^b using the biotinylated mAb Y3P followed by streptavidin-FITC and analyzed by flow cytometry. Also shown is I-A^b expression of B78H1 grown in vitro in the presence or absence of IFN-γ (100 U/ml for 72 h).

tion element H-2K^b, and often, H-2D^b as well. Tumor loss of K^b expression was also seen in some of the few undepleted mice that failed to be protected. The relative rarity of tumor outgrowth in this group compared to those depleted of NK1$^+$ cells, underscores the significance of NK cell–mediated rejection of tumor that has escaped the CD8$^+$ T cell response through the loss of MHC class I expression.

MHC Class I Expression on the GM-CSF–producing Vaccine Impairs the Response to a MHC Class I$^-$ Tumor Challenge. Given the potential for a successful protective response against the MHC class I$^-$ tumor challenge, we sought to determine how this response is effected by the MHC class I status of the immunizing cells. Mice were vaccinated with each of the GM-CSF–producing MHC class I variants and challenged 2 wk later with B78H1 wild type (Fig. 9). Significant protection against the MHC class I$^-$ tumor challenge was seen when the vaccine expressed no MHC class I antigen, whereas vaccination with the MHC class I$^+$ variants of GM-CSF–producing tumor generated a much less protective response. Although these differences appear to be related to the MHC class I status of the vaccinating cells, it is possible that this outcome occurred as a consequence of subclone selection resulting in loss of tumor antigen in the vaccine relative to the polyclonal challenge. To address this concern, B78H1GM-CSF was subcloned by limiting dilution. In addition to four of these clones, B78H1GM-CSF transfected with each of the control vectors were tested for their ability to protect against B78H1 wild type compared to polyclonal B78H1GM-CSF and to B78H1D^{b}K^{b}GM-CSF. The level of protection against B78H1 wild type achieved by the clones and control transfectants of B78H1GM-CSF was comparable to that of polyclonal B78H1GM-CSF, whereas B7H1D^{b}K^b GM-CSF protected poorly (data not shown). A mixing experiment further demonstrated that MHC class I$^+$ vaccine inhibits the generation of a protective response to a MHC class I$^-$ tumor challenge (Fig. 10). Mice immunized with MHC class I positive and negative vaccine at separate sites were protected against an MHC class I$^-$ challenge comparably to those receiving MHC class I$^-$ vaccine alone. However, vaccination with an equal mixture of class I MHC positive and negative cells in the same inoculum resulted in markedly diminished protection, suggesting that class I MHC expression by the vaccinating cells exerts a dominant negative effect on the priming of the anti-class I MHC$^-$ response.

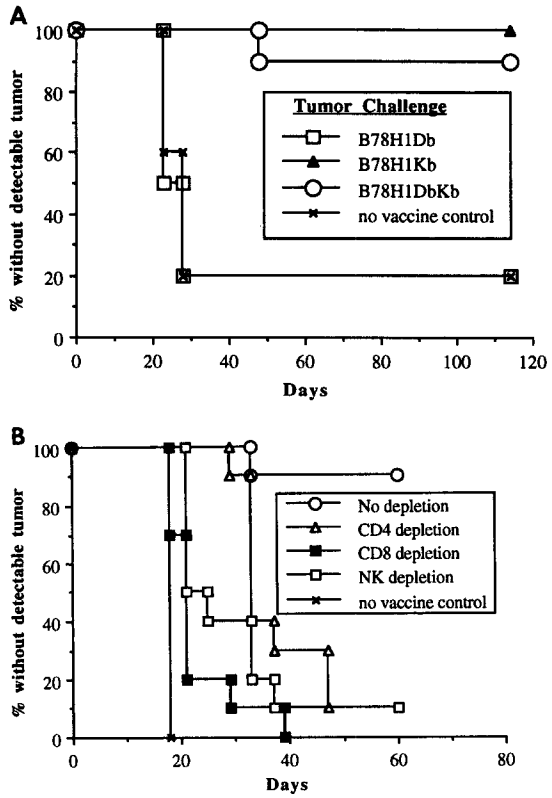

Figure 7. (*A*) Effect of injection with irradiated class I MHC$^+$ B78H1D1^{b}K^{b}GM-CSF cells on the response to challenge with live class I MHC$^+$ variants of B78H1 tumor. C57BL/6 mice were injected with 10^6 irradiated (5,000 rad) B78H1D^{b}K^{b}GM-CSF cells subcutaneously in the left flank. 2 wk later, mice were challenged on the right flank with 10^6 live B78H1D^b, B78H1K^b, or B78H1D^{b}K^b cells. Also shown is the growth of each of these challenge tumors without preimmunization. (*B*) Effect of lymphocyte subset depletion on the protective in vivo response to class I MHC$^+$ B78H1D^{b}K^{b}GM-CSF cells against a live B78H1D^{b}K^b challenge. C57BL/6 mice were depleted of CD4$^+$ T cells, CD8$^+$ T cells, or NK1.1$^+$ cells as in Fig. 6 *B*, followed by subcutaneous injection of 10^6 irradiated (5,000 rad) B78H1D^{b}K^b GM-CSF cells. 2 wk later they were injected on the opposite flank with 10^6 live B78H1D^{b}K^b cells. Also shown is the growth of B78H1D^{b}K^b cells without subset depletion or preimmunization.

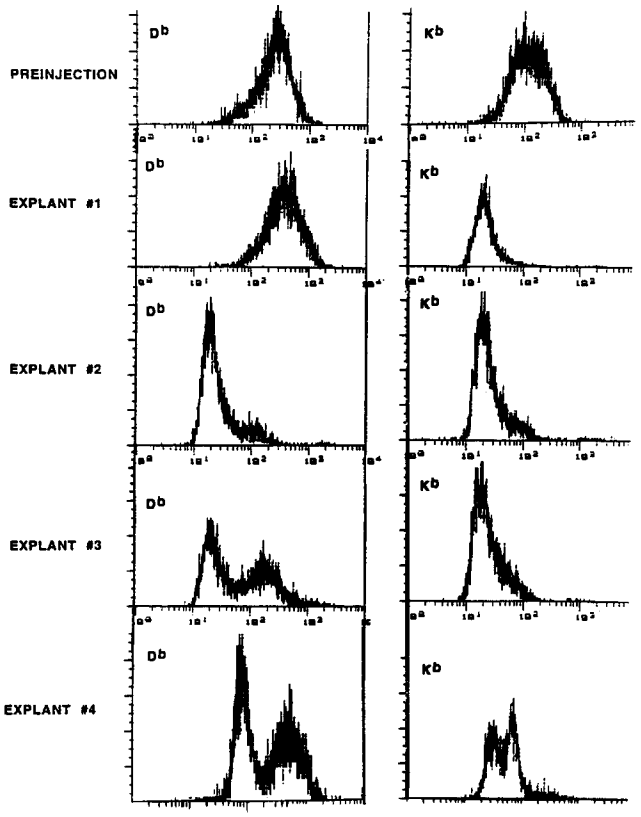

Figure 8. Class I MHC expression on tumors explanted from mice depleted of NK1$^+$ cells. C57BL/6 mice that had been depleted of NK1$^+$ cells and challenged with B78H1D^{b}K^b as in Fig. 7 *B* were killed after developing tumors of >1 cm in diameter. The tumor tissue was excised and processed as in Fig. 6. Cells were stained for expression of H-2D^b (mAb 28-14-8 followed by goat anti-mouse IgG2aFITC) and H-2K^b (mAb B8-24-3 followed by goat anti-mouse IgG1FITC), and analyzed by flow cytometry.

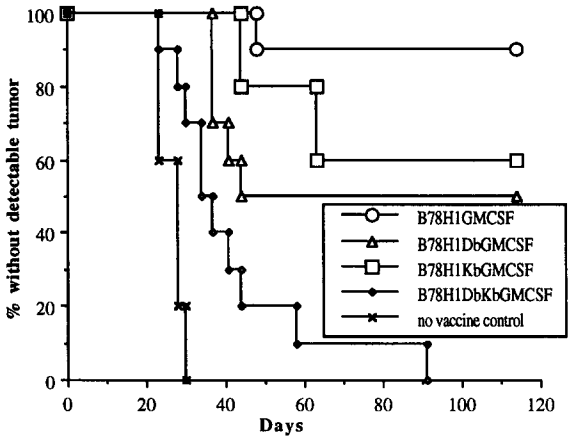

Figure 9. Effect of vaccine cell class I MHC expression on the response to a class I MHC$^-$ challenge. C57BL/6 mice were vaccinated subcutaneously on the left flank with 10^6 irradiated (5,000 rad) B78H1D^{b}K^{b}GM-CSF, B78H1D^{b}GM-CSF, B78H1K^{b}GM-CSF, or B78H1GM-CSF. 2 wk later, the mice were challenged on the right flank with 10^5 live B78H1 wild-type cells and monitored twice weekly for the development of tumor. Also shown is growth of B78H1 without preimmunization.

Discussion

These experiments identify two distinct in vivo effector responses to a tumor challenge and illustrate conditions under which each predominates. The host is capable of successfully responding to tumor that has lost MHC class I expression even though this event eliminates the potential for tumor-specific CD8$^+$T cell–mediated rejection. NK1$^+$ cells are capable of responding to such an event, and under certain circumstances, they may complement the T cell response. Their function may be mediated by direct tumor lysis and/or the release of cytokines influencing the activation of other cells involved in the response. Whereas it has long been appreciated that NK cells can lyse MHC class I$^-$ tumor in vitro, and are involved in the in vivo rejection of such tumor in unprimed recipients, this is the first study to examine the con-

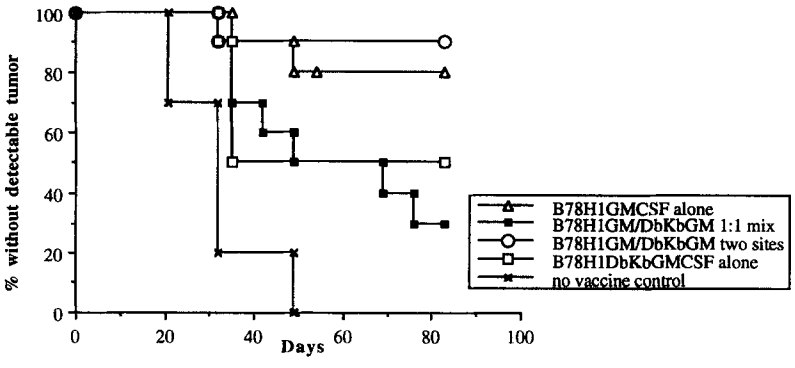

Figure 10. Effect of coinjecting class I MHC positive and negative tumor vaccine on the response to a class I MHC$^-$ tumor challenge. C57BL/6 mice were injected subcutaneously with irradiated (5,000 rad) tumor vaccine as follows: 10^6 B78H1GM-CSF alone, 10^6 B78H1GM-CSF at one site and 10^6 B78H1D^{b}K^{b}GM-CSF at a second site, a mixture of 10^6 each of B78H1GM-CSF and B78H1D^{b}K^b GM-CSF at the same site, or 10^6 B78H1D^{b}K^b GM-CSF alone. 2 wk later, the mice were challenged at a separate site with 10^5 B78H1 wild-type cells and monitored twice weekly for the development of tumor. Also shown is the growth of B78H1 without preimmunization.

sequences of tumor vaccine immunization on the activation of this cell type. The protective effect of a MHC class I$^-$ tumor vaccination against a MHC class I$^-$ challenge, as well as the impairment of this response when the vaccinating cells express MHC class I, are consistent with a durable alteration in NK1$^+$ cell function. This conclusion is somewhat at odds with the operational definition of NK cells as being "capable of lysing a variety of tumor cells in vitro without any known or deliberate sensitization," i.e., that resting cells are "naturally" functionally active. In vivo, however, viral infection or strong antigenic stimulation has been reported to lead to both activation of NK cells, and increase in NK cell number (37).

Alternatively, the priming of the host response to a MHC class I$^-$ challenge may be attributable to the level of CD4$^+$ T cell help generated. In the GM-CSF tumor vaccine system, the central role of CD4$^+$ Th cells may relate to the recruitment of "professional" APCs to the vaccine site which process and present tumor antigen on MHC class II molecules to CD4$^+$ Th cells (29). The activation of tumor-specific CD8$^+$ CTL whose TCRs have first been engaged by the appropriate antigen–MHC complex is dependent upon the local secretion of lymphokines such as IL-2 which is the hallmark of CD4$^+$ Th function. That most CTL responses require Th cells has long been appreciated (38–41). In contrast, this relationship to CD4$^+$ Th activation has not been previously reported for NK cell function, although both in vitro and in vivo data suggest that NK cell activation is enhanced by IL-2 and IFN-γ (42, 43). Our results suggest that CD4$^+$ Th cell/NK cell interdependence may result in an additional degree of target specificity, as primed antigen-specific CD4$^+$ Th cells would only release cytokines after recognition of the appropriate antigen presented on MHC class II of APCs in the region of a tumor deposit. It is also possible that CD4$^+$ T cells are functioning as cytotoxic effector cells, although in this system, the tumor has not been found to express MHC class II in vivo.

The in vivo protection experiments were also performed using irradiated, non-GM-CSF–secreting tumor cells as vaccine. These experiments resulted in a similar pattern of protection to that reported for the GM-CSF transduced tumor vaccine, although the magnitude of the responses was significantly diminished. Thus it appears that the influence of MHC class I expression on the nature of the ensuing antitumor response is not dependent on GM-CSF, although in both the response to MHC class I positive and negative tumor, the total tumor burden successfully rejected is increased by the paracrine expression of this cytokine.

The negative effect of tumor vaccine MHC class I expression on the subsequent response to class I$^-$ challenge suggests that such an encounter with tumor vaccine either fails to activate NK cells, or directly inhibits them. This observation is consistent with a pattern of NK cell recognition first described over 30 yr ago and termed hybrid resistance (for reviews see references 44 and 45). Lethally irradiated hybrid mice heterozygous at the MHC gene complex can reject bone marrow grafts from a homozygous parental donor. Host NK cells have been found to mediate this rejection (46). Immunogenetic analysis has revealed that the determinants for hybrid resistance map to the MHC between H-2S (complement) and H-2D (47). The nature of the NK cell recognition event remains controversial. The possibility of recessive inheritance of the antigens recognized by the F_1 recipient on the parental graft has led to a search for gene products encoded in the H-2 region that can explain the observed pattern of graft rejection (hybrid histocompatibility antigens) (48). An alternate explanation suggests that NK cells are inactivated upon encountering self-MHC (possibly plus peptide) on a potential target, the failure of this event leading to lysis of the target. This "missing self" hypothesis (49) was supported by the ability of mice transgenic for an allogeneic class I MHC molecule (H-2D^d) to reject an otherwise genetically identical nontransgenic bone marrow but not marrow expressing H-2D^d, formally demonstrating that expression of the class I MHC molecule itself can result in graft survival, whereas its absence can be recognized by the NK cells of the recipient leading to rejection (16). In our model, similar mechanisms may account for the inhibitory effect of MHC class I$^+$ tumor on the subsequent NK cell–mediated response to MHC class I$^-$ tumor.

One implication of these findings is that in a heterogeneous tumor burden where loss of MHC class I antigens has occurred in some fraction of the tumor, the CD8$^+$ T cell response is likely to be inadequate, and strategies of active immunotherapy must attempt to recruit alternate cytolytic effector cells such as NK cells which may facilitate tumor

rejection. The use of class I^- vaccinating cells as one component of the therapy may be effective in this setting. More generally, such an immunotherapeutic approach must include the consideration of a variety of responding cell populations and the conditions which affect the ability of each to contribute to a successful host response.

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