Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor *in vivo*

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Edited by Max D. Cooper, University of Alabama, Birmingham, AL, and approved August 3, 2001 (received for review May 14, 2001)

In 1986, Kärre and colleagues reported that natural killer (NK) cells rejected an MHC class I-deficient tumor cell line (RMA-S) but they did not reject the same cell line if it expressed MHC class I (RMA). Based on this observation, they proposed the concept that NK cells provide immune surveillance for "missing self," e.g., they eliminate cells that have lost class I MHC antigens. This seminal observation predicted the existence of inhibitory NK cell receptors for MHC class I. Here, we present evidence that NK cells are able to reject tumors expressing MHC class I if the tumor expresses a ligand for NKG2D. Mock-transfected RMA cells resulted in tumor formation. In contrast, when RMA cells were transfected with the retinoic acid early inducible gene-1 γ or δ (RAE-1), ligands for the activating receptor NKG2D, the tumors were rejected. The tumor rejection was mediated by NK cells, and not by CD1-restricted NK1.1$^+$ T cells. No T cell-mediated immunological memory against the parental tumor was generated in the animals that had rejected the RAE-1 transfected tumors, which succumbed to rechallenge with the parental RMA tumor. Therefore, NK cells are able to reject a tumor expressing RAE-1 molecules, despite expression of self MHC class I on the tumor, demonstrating the potential for NK cells to participate in immunity against class I-bearing malignancies.

tumor rejection | NKG2D ligands | H60

Natural killer (NK) cells are innate effector cells serving as a first line of defense against certain viral infections and tumors (1, 2). They have also been implicated in the rejection of allogeneic bone marrow transplants (3, 4). Innate effector cells recognize and eliminate their targets with fast kinetics, without prior sensitization. Therefore, NK cells need to sense if cells are transformed, infected, or "stressed" to discriminate between abnormal and healthy tissues. According to the "missing self" hypothesis proposed by Kärre and colleagues (5), NK cells might accomplish this by looking for and eliminating cells with aberrant MHC class I expression. This concept was validated by showing that NK cells are responsible for the rejection of the MHC class I-deficient lymphoma cell line RMA-S, but not its parental MHC class I-positive line RMA.

Inhibitory receptors specific for MHC class I molecules have been identified in mice and humans. The human killer cell Ig-like receptors (KIR) recognize HLA-A, -B, or -C; the murine Ly49 receptors recognize H-2K or H-2D; and the mouse and human CD94/NKG2 receptors are specific for Qa1^b or HLA-E, respectively (reviewed in refs. 6–8). However, the structures on class I-negative cells that activate NK cells to initiate their effector function have not been identified. Moreover, there are many situations in which activated NK cells can kill tumors expressing normal levels of MHC class I (9–12).

Recently, activating NK cell receptors specific for classic MHC class I molecules, nonclassic MHC class I molecules or MHC class I-related molecules have been described (reviewed in ref. 13). Of particular interest is NKG2D, a membrane receptor expressed on NK cells, $\gamma\delta$-TcR$^+$ T cells, and CD8$^+$ $\alpha\beta$-TcR$^+$ T cells (14). NKG2D is associated with the transmembrane adapter protein DAP10 (15), whose cytoplasmic domain contains a YXXM motif, which binds to the p85 subunit of the PI-3 kinase.

In humans, two families of ligands for NKG2D have been described (reviewed in refs. 16 and 17). NKG2D binds to the polymorphic MHC class I chain-related molecules (MIC)-A and MICB (14). These are expressed on many human tumor cell lines, on several freshly isolated tumor specimens, and at low levels on gut epithelium (18). MIC proteins can be induced by "heat shock" of epithelial cell lines (19). Recently, Cosman and colleagues (20, 21) identified another family of ligands for human NKG2D, designated the UL binding proteins (ULBP)-1, -2, and -3 molecules. Although similar to class I MHC molecules in their α1 and α2 domains, the genes encoding these proteins are not localized within the MHC. Like MIC (19), the ULBP molecules do not associate with β_2-microglobulin or bind peptides. Although no murine MIC molecules have been found, the mouse orthologs of ULBP are likely to be the retinoic acid early inducible-1 gene products (RAE-1) and the related H60 minor histocompatibility antigen (22, 23). RAE-1 and H60 were identified as ligands for mouse NKG2D by expression cloning these cDNA from a mouse transformed lung cell line (22). Transcripts of RAE-1 are rare in adult tissues but abundant in the embryo and on many mouse tumor cell lines, indicating that these are oncofetal antigens. Although ectopic expression of human MICA (14) or ULBP (20), or mouse RAE-1 (23) renders MHC class I-bearing targets susceptible to the attack of *in vitro*-cultured NK cell lines, the *in vivo* relevance of these molecules as potential tumor rejection antigens has not been evaluated. Here we have addressed this issue in the same experimental model originally used to postulate the "missing self" hypothesis.

Materials and Methods

Mice. Inbred C57BL/6 (B6) mice were purchased from Charles River Laboratories. CD1-deficient mice were originally kindly provided by Luc Van Kaer (Vanderbilt University, Nashville, TN) and backcrossed for at least seven generations onto the B6 background. For tumor experiments, 6-week-old female mice were used. All experiments were performed according to animal experimental ethics committee guidelines.

Cell Lines and Transfectants. The RMA (H-2^b positive) lymphoma cell line and the mutant RMA-S (H-2^b deficient) cell line (kindly provided by Jay Ryan, University of California, San Francisco) were grown in RPMI medium 1640 supplemented with 10% FCS, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml strepto-

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This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: NK, natural killer; RAE-1, retinoic acid early inducible-1; ULBP, UL16-binding protein; KIR, killer cell Ig-like receptor; MIC, MHC class I-related chain; NKG2D-Ig-FP, NKG2D-Ig Fc-fusion protein; PE, phycoerythrin.

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mycin, and 50 μM 2-mercaptoethanol. Stable transfectants of RMA-RAE-1δ, RMA-RAE-1γ or mock-transfected RMA cells were established by electroporation: 30 μg of RAE-1γ-pCDEF3 plasmid (RAE-1γ transfectant), RAE-1δ-pCDEF3 plasmid (RAE-1δ transfectant) or pCDEF3 plasmid (mock-transfectant) was added to 1×10^7 cells in RPMI-1640 medium in a 4-mm cuvette (Bio-Rad), respectively. The pCDEF3 vector was kindly provided by Art Weiss (Univ. of California, San Francisco). Electroporation was performed by using a Bio-Rad Gene Pulser (250 V, 960 μF). Forty-eight hours after electroporation, RAE-1γ and mock-transfected cells were cultured in complete RPMI medium 1640 supplemented with 1 mg/ml G418 (GIBCO/BRL). RMA-RAE-1 transfectants were subsequently sorted for high expression of mNKG2D-ligands by flow cytometry.

Flow Cytometry. The mouse NKG2D-Ig Fc-fusion protein (mNKG2D-Ig-FP) was prepared as described (22). For flow cytometry, 2×10^5 cells were stained for 15 min on ice with 0.5 μg of mNKG2D-Ig-FP or control human IgG. A phycoerythrin (PE)-conjugated goat anti-human Fcγ fragment (Jackson ImmunoResearch) was used as a second step reagent. H-2D^b was detected by using a FITC-conjugated anti-H-2D^b mAb (PharMingen). Flow cytometry was performed by using a FACScan (Becton Dickinson Immunocytometry Systems).

^{51}Cr Release Assay. NK cells used as effectors were prepared from B6 splenocytes. B cells were removed by negative depletion using anti-Ig-coated magnetic beads (Dynal, Oslo). Subsequently, NK cells were positively selected by using DX5 mAb-coupled magnetic beads with the corresponding column system (Miltenyi Biotech, Bergisch Gladbach, Germany). Polyclonal mouse NK cell lines were cultured in 5000 units/ml mouse IL-2 (generously provided by S. Menon, DNAX Research Institute, Palo Alto, CA) for at least 10 days before being used as effectors in a cytotoxicity assay, as described (22).

Tumor Experiments. Groups of six animals per experiment were injected i.p. with 1×10^4 RMA cells, which were mock-transfected or transfected with RAE-1γ or RAE-1δ. Preliminary experiments titrating the number of tumor cells injected indicated that 1×10^4 RMA cells resulted in tumor formation in 100% of animals. Mice were monitored daily for tumor ascites development, indicated by swelling of the abdomen, and were killed when tumor burden became excessive to avoid pain and suffering. Animals were regarded as tumor free when surviving longer than 8 weeks. For depletion of NK1.1$^+$ cells, animals were injected i.p. on days -4 and -2 before tumor inoculation and weekly thereafter with the anti-NK1.1 depleting mAb (PK136, 200 μg per mouse, a kind gift from Anne O'Garra and Bob Coffman, DNAX Research Institute). The effectiveness of the depletion was confirmed by the absence of DX5$^+$ cells in spleens, lymph nodes, and thymus of depleted animals. For the rechallenge experiments, animals were inoculated with 1×10^4 RMA-RAE-γ cells and after the indicated time rechallenged with 1×10^4 RMA-mock cells.

Results

Ectopic Expression of RAE-1 Renders the MHC Class I-Positive Cell Line RMA Susceptible to NK Cell Attack *in Vitro*. To evaluate the *in vivo* significance of the NKG2D–RAE-1 interaction, we established stable RAE-1γ and RAE-1δ transfectants in the MHC class I-positive lymphoma cell line RMA (Fig. 1*A Left*). In these experiments, expression of RAE-1 was detected by using a soluble protein consisting of the extracellular domain of mouse NKG2D fused onto the Fc part of human Ig (mNKG2D-Ig-FP), which was detected by using a PE-conjugated anti-human Fc second-step reagent in flow cytometry (22). RMA cells or RMA cells transfected with the vector only did not stain with the mNKG2D-Ig-FP, indicating that RMA cells do not express endogenous ligands for mNKG2D (Fig. 1*A Left*). We confirmed that both mock-transfected and RAE-1-transfected cells were positive for H-2D^b (Fig. 1*A Right*) and H-2K^b (data not shown).

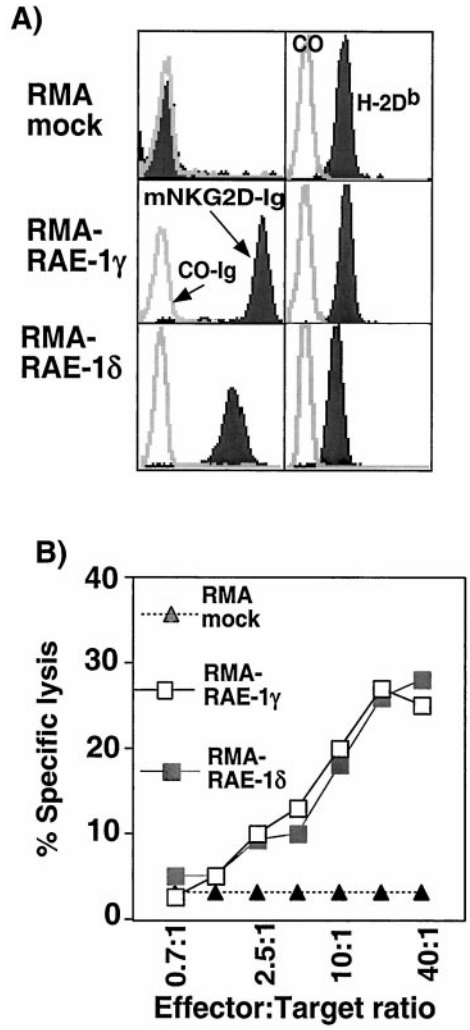

Fig. 1. Ectopic expression of RAE-1 renders the MHC class I-expressing lymphoma cell line RMA susceptible to NK cell attack. (*A*) Mock-transfected RMA cells (*Top*) or RMA cells transfected with RAE-1γ or RAE-1δ (*Middle* and *Bottom*) were stained with the mNKG2D-Ig-FP (filled histograms, *Left*) or a control Ig fusion protein (CO-Ig-FP; open histograms, *Left*) and a PE-conjugated goat anti-human IgG second-step antibody. Transfectants were also stained with an H-2D^b mAb (filled histograms, *Right*) or an isotype-matched control Ig (open histograms, *Right*). Cells were analyzed by flow cytometry. Data are displayed as histograms (*x* axis, fluorescence, 4-decade log scale; *y* axis, relative number of cells). (*B*) IL-2 activated NK cells from B6 mice were used as effectors in 4-h ^{51}Cr-release assays at the indicated effector-to-target ratios. Targets were mock-transfected RMA cells (▲) or RMA cells transfected with RAE-1γ (□) or RAE-1δ (■). Results from a representative experiment of three independent experiments are shown.

Prior studies have established that RMA tumor cells are not efficiently killed by syngeneic NK cells when they are tested by using *in vitro* cytotoxicity assays. In contrast, the MHC class I-deficient variant RMA-S is sensitive to NK cell-mediated cytotoxicity (5). Although the NK cell receptors responsible for recognition and lysis of RMA-S have not been identified, these results imply that the inhibitory NK cell receptors for MHC class I prevent killing of the parental RMA cell line. Mock-transfected RMA and RMA expressing RAE-1γ or RAE-1δ were analyzed

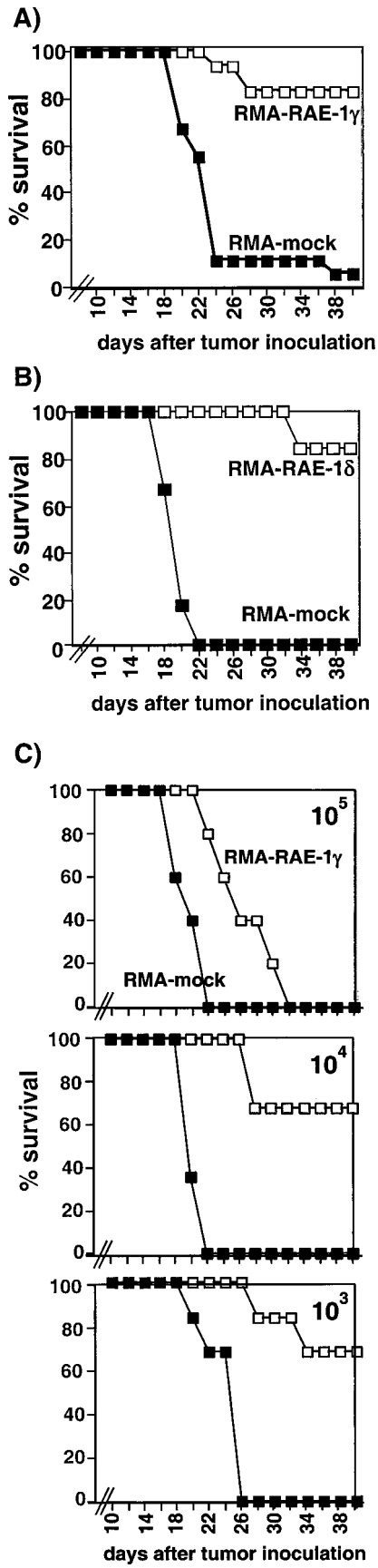

Fig. 2. RAE-1 expression on the RMA lymphoma causes tumor rejection *in vivo*. (*A*) B6 mice were injected i.p. with 1×10^4 mock-transfected RMA cells (■) or RAE-1γ transfected RMA cells (□). Data were compiled from three independently performed experiments using six mice in each group. Significance was determined by the log rank test to compare survival curves; $P \leq 0.001$, $n = 18$. (*B*) B6 mice were injected i.p. with 1×10^4 mock-transfected RMA cells (■) or RAE-1δ transfected RMA cells (□). Survival data for six animals per group are shown. (*C*) B6 mice were injected i. p. with 1×10^5 (*Top*), 1×10^4 (*Middle*), or 1×10^3 (*Bottom*) mock-transfected RMA cells (■) or RAE-1γ transfected RMA cells (□). Survival data for five animals per group (*Top*) or six animals per group (*Middle* and *Bottom*) are shown.

as targets for *in vitro* IL-2-expanded NK cells from B6 mice (Fig. 1*B*). Consistent with prior results, mock-transfected RMA cells were killed only at low levels by mouse NK cells. The RAE-1γ and RAE-1δ-transfected RMA cells, however, were efficiently lysed. Because prior studies have established that expression of H-2 on RMA protects these cells from lysis by NK cells, these data indicated that *in vitro* the ectopic RAE-1 expression enables NK cells cultured in IL-2 to kill these class I-bearing tumors. We also have evidence that freshly isolated splenic NK cells can efficiently kill RAE-1γ-expressing tumor cells (unpublished observation).

RAE-1-Transfected RMA Cells Are Rejected *in Vivo*. In accordance with prior findings (5), we observed that mice injected with 1×10^4 RMA tumor cells all developed tumors and died, whereas those injected with the MHC class I-loss variant, RMA-S, rejected the tumor and showed prolonged survival (data not shown). To investigate the *in vivo* consequence of RAE-1 expression on an aggressive class I-bearing tumor, B6 mice were inoculated i.p. with 1×10^4 mock-transfected RMA cells (Fig. 2*A* and *B*, filled squares), or RMA cells transfected with RAE-1γ (Fig. 2*A*, open squares) or RAE-1δ (Fig. 2*B*). One week after tumor cell implantation, recipients of mock-transfected tumor cells showed the first signs of tumor growth and became moribund between days 19 and 23. In contrast, the majority of animals given the RAE-1γ- and RAE-1δ-transfected RMA cells did not develop tumors. Experiments titrating the tumor cell dose revealed that mice receiving 1×10^5 RAE-1γ-transfected RMA cells demonstrated prolonged survival, compared with mice inoculated with the same number of mock-transfected RMA cells, but ultimately developed tumors (Fig. 2*C*). Similar findings were obtained when tumor cells were injected s.c. (data not shown). In parallel experiments, the MHC class I-deficient cell line RMA-S was rejected, as were RMA-S cells transfected with RAE-1γ (data not shown). Together, these data indicate that ectopic expression of a single molecule—RAE-1—can result in rejection of a MHC class I-positive tumor.

NK Cells Cause the Rejection of RAE-1-Transfected RMA Cells. The receptor for RAE-1 molecules, NKG2D, is expressed on all NK cells, and we investigated whether NK cells were responsible for the rejection of RAE-1-transfected RMA tumors. Mice were treated *in vivo* with a depleting monoclonal antibody specific for NK1.1 (mAb PK136) before and after tumor cell inoculation (Fig. 3*A*). Depletion of NK cells was confirmed by analyzing splenocytes and thymocytes from a group of mice treated in parallel with anti-NK1.1 mAb (data not shown). Recipients of mock-transfected RMA cells became moribund between days 19 and 23 (Fig. 3*A*, filled squares). Similarly, injection of the RAE-1γ-transfected RMA cells (Fig. 3*A*, open squares) into mice treated with anti-NK1.1 mAb resulted in tumor growth, and these animals became moribund at the same time as recipients of the mock-transfected tumor. In contrast, rejection of the RAE-1γ-transfected RMA tumor was observed in mice with an intact NK cell compartment (Fig. 2*B*).

The anti-NK1.1 mAb not only depletes NK cells *in vivo* but might also deplete $NK1.1^+$ T (NKT) cells. NKT cells express NKG2D (unpublished observations) and might participate in the

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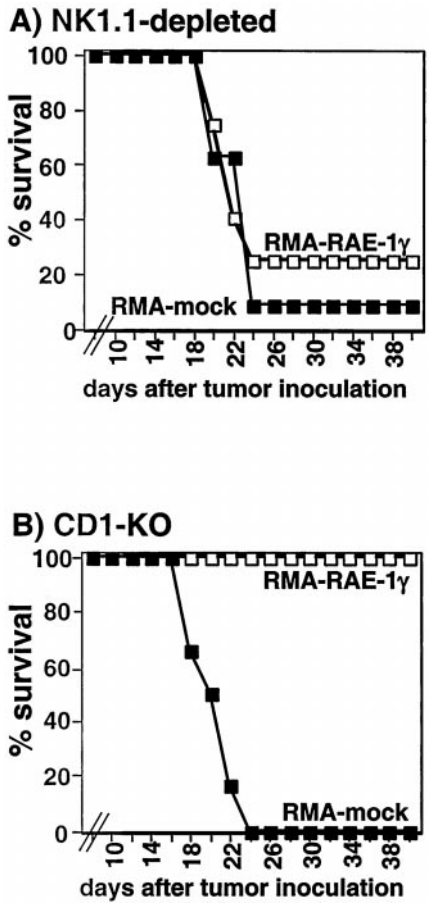

Fig. 3. NK cells cause the rejection of RAE-1γ-transfected RMA tumors. (*A*) About 1×10^4 mock-transfected RMA cells (■) or RMA cells transfected with RAE-1γ (□) were injected i.p. into B6 animals. On days −4 and −2 before tumor inoculation and weekly thereafter, recipient mice were treated with anti-NK1.1 mAb i.p. (200 μg per mouse). Data were compiled from two independent experiments using six mice in each group. Survival curves were not significantly different as determined by the log rank test to compare survival curves, $P \leq 0.442$, $n = 12$. (*B*) CD1-deficient mice on a C57BL/6 background were injected i.p. with 1×10^4 mock-transfected RMA cells (■) or RMA cells transfected with RAE-1γ (□). Survival data for six animals per group are shown.

rejection of RAE-1-transfected tumor cells. CD1-restricted NKT cells are absent in CD1-deficient mice (24, 25). To investigate the potential involvement of CD1-restricted NKT cells in the rejection of RAE-1-positive tumor cells, CD1-deficient mice were inoculated with mock-transfected or RAE-1γ-transfected RMA cells. In CD1-deficient animals, RAE-1γ-transfected RMA cells were rejected (Fig. 3*B*, open squares), whereas the mock-transfected RMA cells formed tumors and killed these animals (Fig. 3*B*, filled squares). These data indicate that CD1-restricted NKT cells are not required for the rejection of RAE-1γ-transfected RMA tumors. We cannot exclude, however, that other populations of NKT cells, which are not restricted by CD1, are involved in the rejection of RAE-1-transfected tumors.

Simultaneous Inoculation of Mock-Transfected RMA Cells and RAE-1-Transfected RMA Cells Results in Tumor Formation. To investigate whether rejection of the RAE-1 transfected RMA tumors elicits effector cells that are able to attack the parental RMA tumor, mice were inoculated i.p. with a 1:1 mixture of the RAE-1-transfected and mock-transfected RMA tumors and were monitored for tumor development (Fig. 4). As observed previously (Fig. 2), most animals rejected the RAE-1γ-transfected RMA

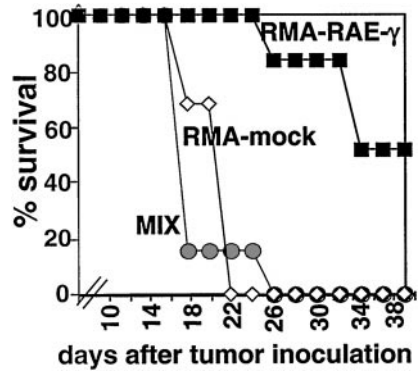

Fig. 4. Simultaneous inoculation of RMA-mock and RMA-RAE-1γ results in tumor formation. Mock-transfected RMA cells (1×10^4, ◇), RMA cells transfected with RAE-1γ (1×10^4, ■) or a 1:1 mixture of mock-transfected RMA cells and RMA cells transfected with RAE-1γ (●) were injected i.p. into B6 animals. Survival data for six animals per group are shown.

tumor cells, whereas all animals inoculated with mock-transfected RMA cells died between days 19 and 22 after tumor inoculation. In mice receiving the 1:1 mixture of tumor cells, all recipient animals developed tumors with growth kinetics comparable to recipients receiving only the mock-transfected tumors. In experiments where mice were inoculated s.c. with mock-transfected RMA cells on one flank and RAE-1-transfected RMA cells on the other flank, the RAE-1-transfected RMA tumor failed to grow or was substantially retarded in growth, whereas the parental RMA tumor grew with kinetics similar to control animals receiving only a s.c. injection of mock-transfected RMA cells (data not shown). These data indicate that effector cells likely kill the RAE-1-bearing tumors by a process that requires a direct effector-to-target cell interaction involving RAE-1.

Animals That Had Rejected RAE-1-Transfected RMA Cells Succumb to Parental Tumors on Rechallenge. Efficient tumor vaccination protocols are based on the ability of the host to reject tumors on rechallenge after immunization. Therefore, we investigated whether T cell-mediated immunological memory against tumor-associated antigens present on the parental RMA tumor was generated in recipients of RAE-1-transfected RMA tumors. Recipients of RAE-1γ-RMA cells were rechallenged with mock-transfected RMA cells 3 months after the primary tumor inoculation (Fig. 5, open diamonds). In parallel, mock-transfected RMA cells were also inoculated into naïve unprimed animals as controls (filled squares). Both groups of mice, i.e., naïve mice or mice that had previously rejected RAE-1γ-RMA tumors, succumbed to challenge with the parental RMA tumor with similar tumor growth kinetics. These data suggested that NK cell-mediated rejection of the RAE-1γ-transfected RMA tumor did not result in the generation of tumor-specific memory T cells against antigens on the parental RMA tumor.

Discussion

We show that ectopic expression of RAE-1γ or RAE-1δ, ligands for the activating NKG2D receptor, can cause rejection of a MHC class I-positive tumor mediated by NK cells. Our data are an *in vivo* demonstration that NK cells can eliminate a tumor with a defined ligand for an activating NK cell receptor, despite normal expression of self-MHC class I molecules on the tumor. In our studies, we have evaluated the RMA tumor because this lymphoma was initially used to propose the missing self hypothesis of NK cell recognition (5). This hypothesis provided an important stimulus for the discovery of the inhibitory NK cell receptors for MHC class I. The ability of NK cells to kill class

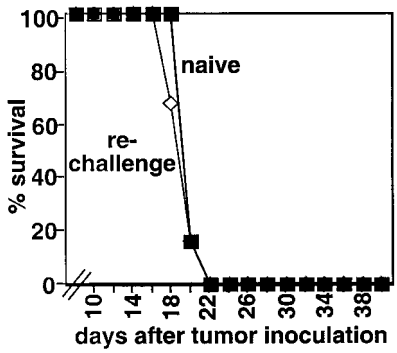

Fig. 5. No tumor-specific T cell immunological memory is observed in mice that have rejected RAE-1γ transfected tumors. B6 animals that had rejected 1×10^4 RMA cells transfected with RAE-1γ ($\diamond$) were injected with 1×10^4 mock-transfected RMA cells at 3 months after the primary tumor inoculation. In parallel, naïve, unprimed animals were injected with 1×10^4 mock-transfected RMA cells (■). Representative survival data for 12 animals per group are shown.

I-deficient cells has been validated in several experimental models. For example, activated mouse NK cells are able to kill lymphoblasts from β_2-microglobulin-deficient mice, but not wild-type mice (26), and NK cells in wild-type mice have been shown to reject bone marrow grafts from β_2-microglobulin-deficient donors (27). However, the ability of class I to inhibit NK cell responses has usually been demonstrated by using target cells that may not express ligands for NKG2D. In preliminary experiments, we have not detected NKG2D ligands on Con A-activated T lymphoblasts from normal mice (unpublished observation). There are situations in which NK cells have been shown to kill certain tumors *in vitro* even when these targets expressed significant levels of MHC class I on their cell surface (9–12). Thus, new concepts are required to explain how NK cells kill tumor cells expressing MHC class I, yet spare healthy cells expressing self-MHC class I molecules. Here we provide evidence that ectopic expression of RAE-1 on the RMA tumor permits *in vivo* rejection of a class I-bearing tumor. While prior *in vitro* experiments using cultured cytokine-activated human or mouse NK cell lines had suggested this possibility (14, 20, 23), our present findings validate this concept *in vivo*.

Although we assume that NKG2D, the known receptor for RAE-1 molecules, which is expressed on all NK cells, is required for the NK cell-mediated rejection of RAE-1-expressing tumors, we cannot exclude that other unknown receptors participate in this process. Blocking reagents against the mouse NKG2D receptor are currently not available to formally prove that NKG2D is necessary and sufficient for this activity. Because NKG2D is expressed constitutively on mouse and human NK cells *in vivo* (14, 23), regulation of NK cell activation by this pathway may occur by the availability of the ligands for this receptor. Expression of mouse RAE-1 apparently is rare in healthy adult tissues (22) and the human MIC proteins are present in low levels (19), whereas high levels of conventional MHC class I are present on most cells. NKG2D ligands are over-expressed by many of the examined tumors (22, 23). Whether the endogenous expression of NKG2D ligands on tumor cells is beneficial for antitumor immunity is unknown.

The stimuli that induce expression of NKG2D ligands are not well defined. Heat shock can induce MIC in certain human cell lines (19), and retinoic acid up-regulates transcription of the *RAE-1* genes in the mouse F9 carcinoma (22). It seems likely that transformation or viral infection (28) might regulate the expression of these NKG2D ligands, rendering these abnormal cells susceptible to NK cell attack.

Why might activation of NK cells through NKG2D permit lysis of class I-bearing cells, whereas other activating receptors are suppressed by the inhibitory MHC class I receptor? We propose that this difference in function may have evolved because of a fundamental difference in the nature of ligands recognized by different activating receptors. Although it appears that expression of NKG2D ligands is low or absent on most normal adult tissues, ligands for several other activating NK cells receptors are, in fact, broadly distributed. For example, CD48, a ligand for the activating receptor 2B4 (CD244), CD58, a ligand for CD2, intracellular adhesion molecule-1 (CD54), a ligand for lymphocyte function-associated antigen-1, and the MHC class I ligands for the activating isoforms of the KIR, Ly49, and CD94/NKG2C receptors are all abundantly expressed on many normal tissues (13, 29). For activating NK cell receptors recognizing these ubiquitously expressed ligands, the inhibitory receptors specific for self-MHC class I may provide a critical function, preventing attack against normal cells. Full NK cell activation is permitted only when many of these receptors are simultaneously engaged by the potential target cell or when the target cells have down-regulated expression of class I (30).

The biochemical basis for the resistance of the NKG2D-DAP10 signaling pathway to suppression by the inhibitory receptors is not yet defined. We cannot state that these inhibitory receptors have no influence on NKG2D-mediated activation. In fact, it has been shown that NK cells indeed kill target cells bearing NKG2D-ligands more efficiently if MHC class I is absent or blocked by anti-class I antibodies (14, 31). However, it is uncertain whether these results are because of a partial inhibitory effect of these MHC class I receptors on the NKG2D pathway or simply allowing other activating receptors to function and participate in the process when MHC class I is absent (31). In addition, it is unresolved whether NKG2D functions directly as an activating receptor in NK cells or serves as a costimulator with other undefined receptors. We have observed that transfection of RAE-1γ into class I-deficient RMA-S tumors renders these cells more sensitive to NK cell-mediated lysis *in vitro* (unpublished observation). This observation suggests that activation of NK cells by RAE-1γ-bearing tumors augments killing induced through an undefined receptor recognizing a ligand on RMA-S, a process that in the absence of RAE-1 molecules is apparently inhibited by the expression of class I on RMA cells.

Several effector molecules potentially involved in NK cell-mediated tumor immunity have been described. In addition to direct killing pathways involving perforin, granzymes, and fas ligand, cytokines, including tumor necrosis factor-α, IFN-γ and TNF-related apoptosis-inducing ligand, might also play a critical role (reviewed in ref. 32). Here, we show that when mice are simultaneously inoculated with RAE-1-transfected RMA cells and mock-transfected RMA cells, the result is tumor formation. These results suggest that a direct interaction between the NK cells and RAE-1-expressing RMA cells is involved in tumor elimination and that in this experimental model "bystander" tumor killing does not occur. In similar experiments, Ljunggren and colleagues (33) observed that MHC class I-bearing RMA cells did not prevent the rejection of class I-deficient RMA-S cells and the RMA-S cells did not induce rejection of the RMA tumors when mice were injected with a mixture of these tumors.

We show that the rejection of RAE-1-transfected RMA cells is caused by NK cells. Innate immunity is characterized by its ability to provide the first line of defense against pathogens, virus-infected cells, and possibly "stressed" or transformed cells. In contrast to adaptive immunity, innate immune cells cannot "remember" a prior encounter with a pathogen or tumor and immunological memory is not generated. In the case of the RAE-1γ-transfected RMA tumors, it appears that the innate immune system efficiently eliminated these tumors, but failed to

prime an adaptive immune response to provide protection and memory against rechallenge with the RAE-1-negative parental RMA cells. Whether this result is specific to the RMA tumor or more generally applies to other tumors expressing RAE-1 requires further investigation.

It is significant that RMA is one of only a few class I-bearing tumors that we have examined that completely lacks endogenous ligands for NKG2D. Based on our recent findings, we propose that immune surveillance mediated by NK cells is accomplished not only by constantly surveying tissues for defective expression of self-MHC class I molecules (missing self), but also by searching for cells that have acquired NKG2D ligands because of transformation, infection, or "stress."

We thank Luc van Kaer for the permission to use the CD1-deficient mice and Anne O'Garra and Bob Coffman for depleting antibodies. We are grateful to Dylan Daniel and Iris Ferber for advice, and to Bill Seaman for critically reading the manuscript. We also thank Thomas Pertel, Marina Abramova, and Leon Gardiner for expert technical assistance. The work is supported by National Institutes of Health Grant CA89189 and the Sandler Family Supporting Foundation. J.L.B. is supported by a Burroughs Wellcome Fund Career Award and a Pfizer Fellowship in infectious disease.

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