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CUTTING EDGE

Cutting Edge: Novel Priming of Tumor-Specific Immunity by NKG2D-Triggered NK Cell-Mediated Tumor Rejection and Th1-Independent CD4$^+$ T Cell Pathway[1]

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NKG2D is an activation receptor on NK cells and has been demonstrated as a primary cytotoxicity receptor for mouse NK cells. Primary rejection of class I-deficient RMA-S lymphoma cells expressing the NKG2D ligand, retinoic acid early inducible-1β, was critically dependent upon NK cell perforin and occurred independently of T cells. NKG2D-triggered NK cell rejection of RMA-S-retinoic acid early inducible-1β tumor primed a secondary tumor-specific T cell response mediated by both CD4$^+$ and CD8$^+$ T cells in the effector phase. Surprisingly, during the priming phase, CD4$^+$ T cells, but not CD8$^+$ T cells, were also required to generate this secondary T cell immunity; however, T cell priming was independent of Th1 cytokines, such as IFN-γ and IL-12. These data imply a novel pathway for priming T cell immunity, that is, stimulated upon NK cell-mediated cytotoxicity of NKG2D ligand-expressing tumor cells, dependent upon CD4$^+$ T cells in the primary phase, and independent of conventional Th1-type immunity. The Journal of Immunology,* 2003, 171: 757–761.

Communication between cells of the innate and adaptive immune systems is of considerable interest in current investigations of host defense mechanisms against certain virus infections and cancer. NK cells, one of the most primitive cells of the innate system, have been shown to play an important role in tumor immunosurveillance and viral clearance (1–4) as well as forming an important link between innate and adaptive immunity. Recent studies have indicated that the functions of NK cells are tightly regulated by the balance of signals integrated from inhibitory and activating receptors expressed on their surface (5–9). The NKG2D receptor is a typical NK cell-activating receptor that is also expressed on effector cells of the adaptive immune system such as CD8$^+$ T and γδ$^+$ T cells where it regulates activation of those cells through their TCR (10–12).

NKG2D is triggered by ligands that are structurally related to MHC class I molecules and expressed either by normal tissues early in ontogeny or by stressed, infected, and transformed tumor cells in adult life (13, 14). NKG2D plays a key role in immune responses, including those against tumor (15, 16). It has been shown recently that the function of NKG2D on NK cells and CD8$^+$ T cells might be mediated by two distinct pathways of signaling through its association with two distinct adapter proteins, DAP10 and DAP12 (17, 18). DAP10 has a YxxM motif in its cytoplasmic tail, similar to CD28 that binds to p85 subunit of phosphatidylinositol 3-kinase (10). DAP12 has a typical immunoreceptor tyrosine-based activation motif that recruits Syk family protein/tyrosine kinases. Two splice variants of NKG2D exist in mouse NK cells: a long variant (NKG2D-L), which is constitutively expressed on NK cells and can associate only with DAP10, and a short variant (NKG2D-S), which is expressed only on activated NK cells and can associate with either DAP10 or DAP12 (17). In addition, it has been demonstrated recently that NKG2D ligation triggers cytotoxicity, but not cytokine production, in mouse NK cells that even lack DAP12 or Syk family kinases, suggesting the NKG2D/DAP10 receptor complex acts as a primary cytotoxicity receptor for mouse NK cells (19, 20). Consistent with these molecular studies, our previous work has shown that NKG2D ligand-expressing, TAP-deficient RMA-S-retinoic acid early inducible-1β (Rae-1β)[4] cells, are rejected in vivo by NK cell perforin-mediated cytotoxicity, but not IFN-γ production (21), suggesting the importance of NKG2D as a primary cytotoxicity receptor for NK cells in surveillance of stressed cells. Alternatively, we appreciate that NK cell IFN-γ secretion plays a regulatory role in antitumor immune responses and potentially following NK cell costimulation by stimulating adaptive immunity (22, 23). In contrast to NK cell cytokine production, it has not been possible to assess the

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[4] Abbreviations used in this paper: Rae-1β, retinoic acid early inducible-1β; WT, wild type.

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role of NK cell-mediated target lysis in provoking Ag-specific antitumor immune responses.

In this study, we have investigated the secondary T cell immunity elicited by NK cell perforin-mediated rejection of TAP-deficient RMA-S tumors expressing NKG2D ligand, Rae-1β. NKG2D-triggered NK cell-mediated cytotoxicity very efficiently evoked the subsequent development of secondary tumor-specific immunity that was effected by both CD4$^+$ and CD8$^+$ T cells. Surprisingly, however, CD4$^+$ T cells were also important for optimal induction of T cell immunity during the priming phase. Unexpectedly, this priming of secondary T cell immunity was not dependent upon IFN-γ, IL-12, or invariant Vα14 NKT cells (including CD4$^+$), suggesting that NK cell perforin-mediated tumor rejection primed adaptive T cell immunity in a manner dependent upon conventional CD4$^+$ Th cells, but independent of a typical Th1-type immune response.

Materials and Methods

Mice

Inbred wild-type C57BL/6 (WT) mice were purchased from Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The following gene-targeted mice were bred at the Peter MacCallum Cancer Centre: C57BL/6 IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice, C57BL/6 IL-12-deficient (IL-12$^{-/-}$) mice, and C57BL/6 Jα18-deficient (Jα18$^{-/-}$) mice. Mice over 6 wk of age were used in all experiments, which were performed according to animal experimental ethics committee guidelines.

Abs and reagents

Purified mAbs reactive with mouse CD4 (GK1.5), CD8 (53-6.7), and NK1.1 (PK136) were all affinity purified from hybridomas. Depletion by mAbs was assessed by flow cytometry using FITC-conjugated anti-CD3, PE-conjugated anti-CD8, and PE-conjugated anti-CD4 (BD PharMingen, San Diego, CA).

Tumor cell lines

The RMA and RMA-S cell lines used in this study were derived from C57BL/6 (B6, H-2^b) mice. RMA and RMA-S are T cell lymphomas derived from the Rauscher murine leukemia virus-induced RBL-5 cell line (24). RMA-S-Rae-1β infectants were prepared and selected by flow cytometry as previously described (21). Single-cell cloning of the RMA-S-Rae-1β sorted cells was undertaken to select a clone expressing high levels of Rae-1β, and negligible levels of CD70, CD80, and CD86.

Tumor growth assays

Tumor cells were injected s.c. into groups of 5–20 mice (WT, IFN-$\gamma^{-/-}$, IL-12$^{-/-}$, or Jα18$^{-/-}$) or mAb-treated WT mice (anti-CD4, anti-CD8, or anti-NK1.1). Mice were first injected s.c. with a primary challenge of 5×10^5 RMA-S-Rae-1β tumor cells in 0.2 ml of RPMI 1640 medium. After 5–8 wk, mice were injected s.c. with a secondary challenge of either RMA-S, RMA, or B16F10 tumor cells (5×10^5 unless otherwise shown). Mice were monitored every 2–3 days for tumor growth, and s.c. tumors were measured with a caliper meter along the perpendicular axes of the tumors. Mice were sacrificed when tumors reached a size >12 mm in diameter or the mice became moribund. Mice with no tumor growth were kept under observation for at least 100 days. In various groups of mice, NK cells were depleted by injecting anti-NK1.1 mAb (200 μg, i.p.), CD4$^+$ cells were depleted by injecting anti-CD4 mAb (200 μg, i.p.) or CD8$^+$ cells were depleted by injecting anti-CD8 mAb (200 μg, i.p.). Depletion at the time of priming was done on days -1, 0, 1, 7, and 14 (where 0 is the day of primary tumor challenge), or at the time of RMA challenge on days -1, 0, 1, 7, and 14 (where 0 is the day of secondary tumor challenge). These protocols have previously been shown to completely deplete the appropriate lymphocyte subsets (22).

Statistical analysis

Fisher's exact test was used for statistical analysis of data. Values of $p < 0.05$ were considered significant.

Results

Primary NKG2D-stimulated NK cell-mediated tumor rejection induces tumor-specific secondary immunity

Our previous work has shown that RMA-S-Rae-1β was rejected in vivo by NK cell perforin-mediated cytotoxicity (21). To test whether an immunological memory to tumor was established following NK cell-mediated tumor rejection, the mice that rejected a primary RMA-S-Rae-1β challenge (5×10^5 cells) were rechallenged on the opposite flank 6 wk later with a lethal dose of RMA (parental tumor expressing MHC class I; Fig 1*A*), RMA-S (TAP-deficient variant; *B*), or B16F10 (syngeneic tumor; *C*). All s.c. inoculated tumors grew avidly in naive B6 mice at the dose chosen. As shown in Fig. 1, the numbers of the mice tumor free upon the secondary challenge with RMA cells was largely increased (57%) compared with naive B6 mice (0%). There was also a slight, but significant, increase in tumor-free mice (14%) that received a secondary challenge with RMA-S cells. Secondary challenge with RMA (after priming with RMA-S-Rae-1β) was repeated in several independent experiments, and collectively overall survival was 77% ($n = 48$) in primed mice, compared with 0% survival ($n = 25$) in naive mice. Groups of mice receiving B16F10 cells developed tumors regardless of whether they had been primed or were naive, suggesting that primary NKG2D-triggered NK cell-mediated cytotoxicity primes specific secondary tumor immunity. Similar results have been obtained when challenging mice with a range of tumor cell doses (data not shown).

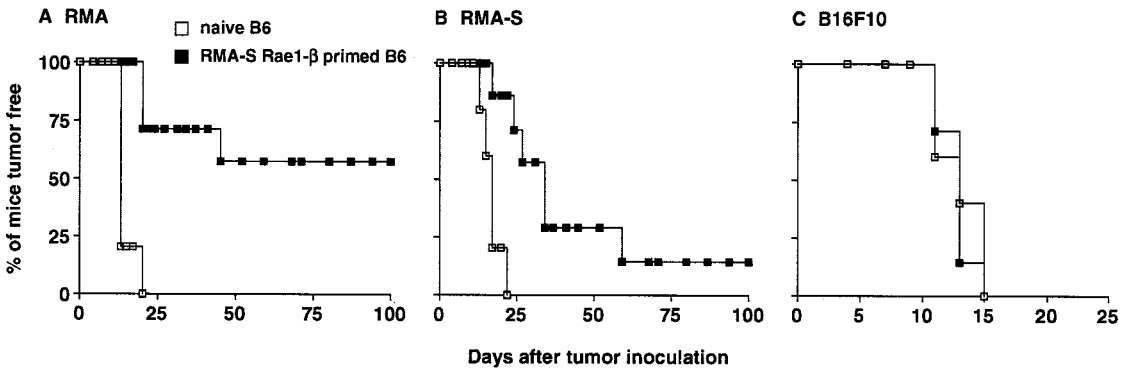

FIGURE 1. NKG2D-mediated primary NK cell tumor rejection induces immunological memory against the ligand-negative parental tumor. Secondary rejection responses were monitored in WT mice that had previously rejected primary RMA-S-Rae-1β cells (5×10^5). Mice were challenged 6 wk later with RMA (*A*), RMA-S (*B*), or B16F10 cells (*C*) (5×10^5). Tumors were monitored every 2–3 days, and mice were not considered tumor free when their tumors reached a size >12 mm in diameter or mice with tumors became moribund.

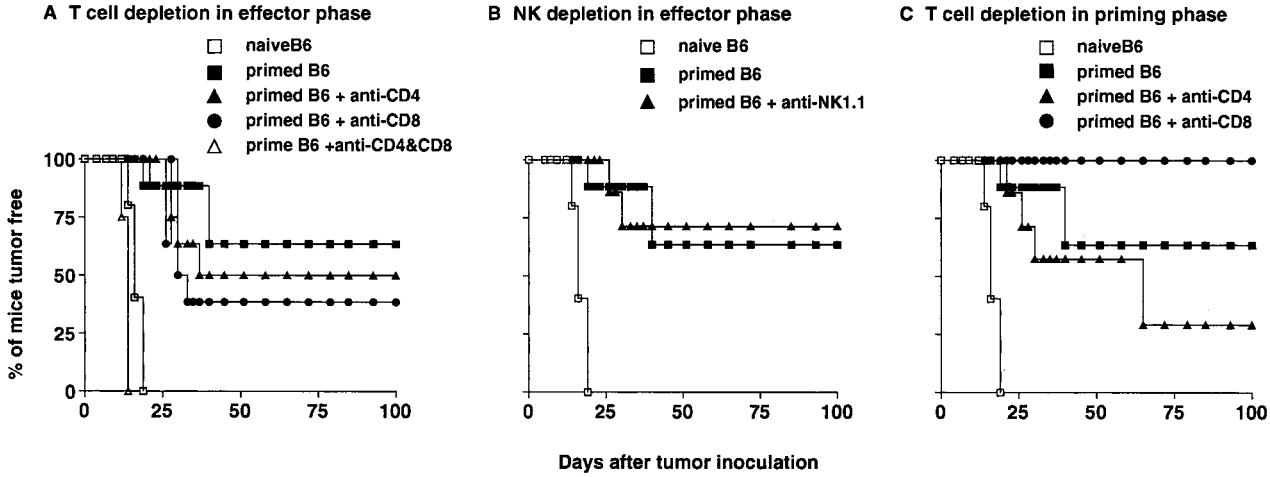

FIGURE 2. Differential contribution of $CD4^+$ and $CD8^+$ T cells in the priming and effector phases of a secondary antitumor response following NK cell-mediated tumor rejection. Secondary rejection responses were monitored in WT mice that had previously rejected primary RMA-S-Rae-1β tumor cells (5×10^5). Six to 7 wk later, some mice were depleted of $CD4^+$ and/or $CD8^+$ T cells (*A*), or NK cells (*B*), and then given a secondary RMA tumor challenge (5×10^5 cells). *C*, Some mice were depleted of $CD4^+$ or $CD8^+$ T cells in the RMA-S-Rae-1β tumor cell-priming phase as described in *Materials and Methods*. Mice were then challenged 7 wk later with RMA tumor cells (5×10^5). Tumors were monitored every 2–3 days, and mice were not considered tumor free when their tumors reached a size >12 mm in diameter or mice with tumors became moribund.

Differential contribution of $CD4^+$ and $CD8^+$ T cells as effectors or primers of secondary immunity following NK cell-mediated tumor rejection

To further determine which cells mediated secondary immunity following NKG2D-triggered NK cell-mediated tumor rejection, we examined the depletion of $CD4^+$, $CD8^+$, or both $CD4^+$ and $CD8^+$ T cells at the time of secondary challenge with RMA (Fig. 2*A*). Depleting both $CD4^+$ and $CD8^+$ T cells clearly resulted in mice failing to respond to secondary RMA tumor challenge, whereas single depletion of either $CD4^+$ or $CD8^+$ T cells resulted in only a minor reduction in the secondary response to RMA tumor (50 or 38% of tumor-free mice in CD4- or CD8-depleted mice, respectively, compared with 63% of control mice). In contrast to the critical importance of NK cells in mediating primary RMA-S-Rae-1β tumor rejection (21), NK cells were not required for secondary responses against RMA tumor cells (Fig. 2*B*).

Next, we explored whether T cell subsets played any role in priming a secondary immune response to tumor in the context of NK cell-mediated tumor rejection. $CD4^+$ or $CD8^+$ T cells were depleted at the time of priming with RMA-S-Rae-1β cells (5×10^5). Depletion over the first 2 wk and repopulation of each subset of T cells during all tumor experiments were confirmed by flow cytometry (data not shown). Depletion of $CD8^+$ T cells during the priming phase did not reduce, but if anything somewhat enhanced (not significant, $p = 0.3$), the subsequent secondary response to challenge with RMA tumor cells, whereas depletion of $CD4^+$ T cells during this phase significantly reduced the efficacy of the subsequent secondary response to RMA tumor compared with an untreated control group (Fig. 2*C*). This result has been confirmed with three independent experiments, and collectively, 16 of 34 mice were tumor free in the CD4-depleted group, whereas 24 of 33 mice were tumor free in the control group ($p = 0.048$). Neither $CD4^+$ T cell nor $CD8^+$ T cell depletion affected primary RMA-S-Rae-1β tumor rejection (data not shown and Ref. 21). Thus, these results indicated that, although both $CD4^+$ and $CD8^+$ T cells were required in the secondary effector phase,

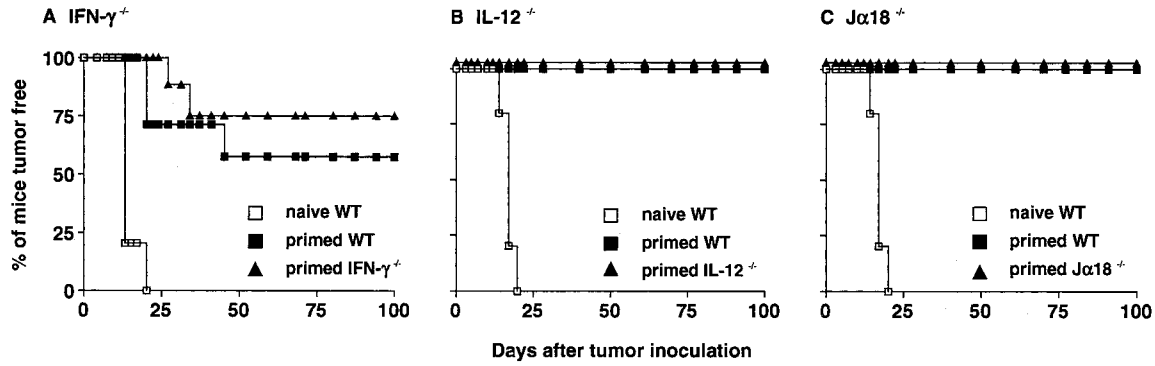

FIGURE 3. Priming secondary T cell immunity does not require IFN-γ, IL-12, or invariant NKT cells. Secondary RMA tumor rejection responses were monitored in IFN-$\gamma^{-/-}$ (*A*), IL-$12^{-/-}$ (*B*), or J$\alpha 18^{-/-}$ (*C*) mice that had previously rejected primary RMA-S-Rae-1β cells (5×10^5). Mice were challenged 6 wk later with RMA cells (5×10^5). Tumors were monitored every 2–3 days, and mice were not considered tumor free when their tumors reached a size >12 mm in diameter or mice with tumors became moribund.

only CD4$^+$ T cells were required in priming phase to generate optimal secondary tumor-specific immune responses following NKG2D-triggered NK cell-mediated tumor rejection.

Priming of T cell immunity does not require a typical Th1 immune response

We further characterized the factors that were required for priming of the T cell-mediated secondary response to tumor. Surprisingly, both Th1 cytokines, IFN-γ and IL-12, were not important for either primary rejection of RMA-S-Rae-1β tumors by NK cells (Ref. 21 and data not shown) or for priming or mediating a secondary T cell response against RMA cells (Fig. 3, *A* and *B*). In addition, we excluded the possibility that the priming CD4$^+$ T cells included the CD4$^+$ subset of innate invariant Vα14 NKT cells that have been shown to influence both innate and adaptive antitumor immune responses (25). Jα18$^{-/-}$ mice effectively rejected RMA-S-Rae-1β tumors (data not shown) and generated secondary T cell immunity against RMA cells (Fig. 3*C*). These results clearly indicated that the optimal priming of T cell immunity triggered by NK cell-mediated cytotoxicity did require CD4$^+$ T cell help, but independently of a typical Th1 response or invariant NKT cells.

Discussion

Recent findings have clearly indicated that the NKG2D-activating receptor is a primary cytotoxicity receptor for murine NK cells (12, 19). Consistent with this idea, our previous study demonstrated that tumor rejection following NKG2D receptor-ligand interaction is critically dependent upon NK cell perforin-mediated cytotoxicity (21). The data presented in this study now indicates the ability of NK cell-mediated tumor rejection to stimulate adaptive T cell-mediated immunity to the parental tumor. Both CD4$^+$ and CD8$^+$ T cells were critical for mediating this T cell memory response to tumor, and, most interestingly, there was a key role for CD4$^+$ T cells in the priming of such immunity.

CD4$^+$ T cells are known to be important in the rejection of certain types of tumors by providing help in generating and maintaining tumor-specific CD8$^+$ T cells or by releasing cytokines to recruit and activate other inflammatory cells (26). It has been well established that the induction of Th1-biased responses is concomitant with CTL generation in the presence of CD4$^+$ T cell help, and that IFN-γ and IL-12 are key cytokines in promoting this Th1 bias (27, 28). Recently, a novel and pivotal role of CD4$^+$ T cell help has been demonstrated in the maintenance of protective CD8$^+$ T cell memory development (29–31). Alternatively, NK cells have also been shown to promote Ag-specific CTL responses through either their cytokine production (22, 23) or perforin-mediated cytotoxicity (32, 33). Unexpectedly, from experiments in IFN-$\gamma^{-/-}$ and IL-12$^{-/-}$ mice, our present data clearly indicate that NK cell NKG2D-triggered immunity to tumor optimally requires CD4$^+$ T cell help, but not via a conventional Th1 pathway.

It remains unclear exactly how CD4$^+$ T cells and NK cells might together promote the development of adaptive tumor immunity. There are two possibilities whereby NK cell NKG2D-triggered tumor lysis and CD4$^+$ T cells could prime tumor-specific T cell responses. The first might be that NK cell-mediated lysis of tumor cells might produce Ag for cross-presentation by professional APCs, such as dendritic cells to prime CD4$^+$ T cells. Importantly, in contrast to challenge with live tumor cells, gamma-irradiated RMA-S-Rae-1β tumor cells did not offer strong protective immunity that was attributed to tumor cell Rae-1β expression (data not shown). Thus, challenge with gamma-irradiated tumor cells (rather than live tumor cells) was not a useful means by which to assess the role of NK cells or perforin in the priming of secondary T cell immunity. Rather, the data suggest that the specific death mediated by NK cells may impact on the mechanism by which secondary immunity is generated. The second possibility might be that NKG2D-activated NK cells directly aid early CD4$^+$ T cell priming through an alternative route that is distinct from a conventional Th1 pathway. It has been described previously that NK cells regulate CD4$^+$ T cell responses before Ag presentation through their IL-10 production (34). However, NK cells may produce IL-10 only in very specific circumstances, and there is no existing evidence that NKG2D-Rae-1β interactions trigger NK cell IL-10 production. Alternatively, a recent study has shown that human NK:DC interactions provide help for generating Th1 and CTL responses. In particular, NK cells were shown to be capable of inducing stable type 1 polarized DC, and that both their production of type I IFN and ability to kill could contribute to the development of Th1 responses (35). In this context, it has been shown that memory and effector CD4$^+$ T cells require help from DC to control Ag-specific antitumor immune responses (36), and thus NK cells might facilitate the development of DC help. Of note, it has been shown recently in patients with rheumatoid arthritis that disease correlated with large numbers of unusual CD4$^+$CD28$^-$ T cells (37). Interestingly, this unique subset of CD4$^+$ T cells expressed NKG2D. It is unknown whether these NKG2D-expressing CD4$^+$ T cells exist in mice and difficult to envisage how they might engage MHC class II-deficient tumors directly or prime tumor-specific T cell responses by virtue of their NKG2D expression, but their potential involvement in NKG2D-mediated immune responses will be of future interest. Additionally, it remains possible that this form of tumor rejection and priming may be triggered by receptors other than NKG2D. There are many other cytokines produced by NK cells and/or CD4$^+$ T cells that might be critical for priming antitumor T cell responses in the context of NKG2D-mediated tumor rejection (such as IL-4, IL-10, IL-13, TNF-α, etc.), and these remain to be tested. Although we have not established whether alternative NK cell-derived cytokines might be required to induce T cell priming following a NKG2D receptor-ligand interaction, NK cells did generate adaptive tumor-specific T cell responses in an IFN-γ- and IL-12-independent manner. Our current data suggest that NK cell NKG2D-triggered immunity may provide an interesting biological system in which to evaluate the development of CTL responses independently of a conventional Th1 response.

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