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A Role for IFN-γ in Primary and Secondary Immunity Generated by NK Cell-Sensitive Tumor-Expressing CD80 In Vivo[1]

Janice M. Kelly,* Kazuyoshi Takeda,† Phillip K. Darcy,* Hideo Yagita,† and Mark J. Smyth[2]*

We have investigated the primary and secondary immunity generated in vivo by a MHC class I-deficient tumor cell line that expressed CD80 (B7-1). CD80 expression enhanced primary NK cell-mediated tumor rejection in vivo and T cell immunity against secondary tumor challenge. CD80 expression enhanced primary NK cell-mediated tumor rejection, and both NK cell perforin and IFN-γ activity were critical for the rejection of MHC class I-deficient RMA-S-CD80 tumor cells. This primary rejection process stimulated the subsequent development of specific CTL and Th1 responses against Ags expressed by the MHC class I-deficient RMA-S tumor cells. The development of effective secondary T cell immunity could be elicited by irradiated RMA-S-CD80 tumor cells and was dependent upon NK cells and IFN-γ in the priming response. Our findings demonstrate a key role for IFN-γ in innate and adaptive immunity triggered by CD80 expression on tumor cells. ***The Journal of Immunology,*** **2002, 168: 4472–4479.**

Natural killer cells are critical effector cells of the innate immune system (1). Recent studies have indicated that NK cells may be affected directly by costimulatory molecules that have the potential to activate their effector function (2–5). Previous studies of costimulatory molecules such as CD28 (6–9) have largely focused on the function of these receptors in stimulating NK cell cytotoxicity against target cells. In contrast, the role NK cell IFN-γ secretion plays in immune regulation has been given far less attention (10–12). As NK cells produce a number of inflammatory cytokines, it has been suggested that these cells may modulate the development of adaptive immune responses (13). Nevertheless, acceptance of this hypothesis has been limited, and a molecular demonstration that NK cell cytokine secretion shapes the adaptive immune response has not been provided.

Cells with impaired TAP function express low levels of cell surface MHC class I molecules and are generally sensitive to lysis by NK cells. We have investigated the primary and secondary immunity elicited by TAP-deficient RMA-S tumors expressing CD80 that interact with CD28, CD152, or a related molecule expressed on NK cells (12). We demonstrate that the primary rejection of MHC class I-deficient RMA-S-CD80 tumor cells was mediated by NK cells and perforin (pfp)[3]- and IFN-γ-dependent mechanisms. This NK cell-mediated process also very efficiently evoked the subsequent development of tumor-specific CTL and Th1 responses against Ags expressed by the parental tumor, and priming of the secondary response was also IFN-γ dependent.

Materials and Methods

Mice

Inbred wild-type C57BL/6 (WT) mice were purchased from The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The following gene-targeted mice were bred at The Peter MacCallum Cancer Institute: C57BL/6 IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice (provided by Genentech, South San Francisco, CA), C57BL/6 pfp-deficient (pfp$^{-/-}$) mice, and C57BL/6 RAG-1-deficient (RAG-1$^{-/-}$) mice (from Dr. L. Corcoran, The Walter and Eliza Hall Institute of Medical Research). C57BL/6 mice doubly deficient for pfp and RAG-1 (pfp$^{-/-}$RAG-1$^{-/-}$) were produced and bred at The Peter MacCallum Cancer Institute. Mice 6–12 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), mouse CD8 (53-6.7), mouse CD16/32 (2.4G2), mouse NK1.1 (PK136), and mouse IFN-γ (R4-6A2) were all purified from hybridomas. Isotype control for rat IgG1 (R3-34), rat IgG2a (R35-95; BD PharMingen, San Diego, CA), and PE-labeled goat anti-rat IgG (Caltag Laboratories, Burlingame, CA) were purchased. Rabbit asialoGM1 Ab was purchased from WAKO (Richmond, VA). Human IL-2 was provided by Chiron (Emeryville, CA).

Cell lines

All cell lines used in this study were derived from C57BL/6 (B6, H-2^b) mice. RMA and RMA-S cell lines were T cell lymphomas derived from the Rauscher murine leukemia virus (MuLV)-induced RBL-5 cell line (14). MBL-2 was isolated from a Moloney MuLV-inoculated B6 mouse (15). EL-4 is a dimethylbenzanthracene-induced thymoma cell line (16). B16F10 mouse melanoma cells have been described previously (17). All cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD) as previously described (18, 19). RMA-S-m and RMA-S-CD80 infectants were prepared using a previously described method (20). Briefly, mouse CD80 cDNA was cloned into the retroviral vector plasmid MSCV. The plasmid was cotransfected with an amphotropic packaging plasmid into 293T cells by calcium phosphate precipitation. After 48 h, the supernatant was harvested and added to tumor cells every 8 h for 3 days. The cells were allowed to recover and then analyzed for green fluorescence protein (GFP) expression by flow cytometry. The highest 10% of cells were sterilely sorted, expanded, resorted, and subsequently expanded in oligoclonal pools. Cells were subsequently screened for expression of CD80 and sorted by flow cytometry

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[3] Abbreviations used in this paper: pfp, perforin; DC, dendritic cell; env, envelope; gagL, gag leader; GFP, green fluorescence protein; -m, vector alone-infected controls; MuLV, murine leukemia virus; WT, wild type.

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using the 1G10 anti-mCD80 mAb. Parental tumor cells were also infected with the empty MSCV vector alone, and GFP-expressing cells were similarly selected (RMA-S-m). GFP expression had no effect on the K^b or D^b expression on tumor cells.

Flow cytometry

Mononuclear cells were prepared from the spleen as previously described (21). To avoid the nonspecific binding of Abs to FcγR, the cells were preincubated with anti-mouse CD16/32 (2.4G2) before staining. Flow cytometric sorting of cells was performed following staining with FITC -conjugated anti-$\alpha\beta$TCR (clone H57-597) and PE-conjugated anti-NK1.1 (clone PK-136). All flow cytometry reagents were purchased from BD PharMingen, unless otherwise indicated. Expression of CD80 was determined on infectants using the anti-mCD80 mAb (IG10) and a secondary PE-labeled goat anti-rat IgG Ab.

Peptides

The following purified peptides purchased from Auspep (Parkville, Australia) were used to determine CTL and Th1 functions: the MuLV gag leader (gagL) 75–83 (CCLCLTVFL) epitope presented by H-$2D^b$, the MuLV envelop (env) 189–196 (SSWDFITV) epitope presented by H-$2K^b$ (22), the RMA/EL-4 common tumor (NKGENAQL) epitope presented by H-$2D^b$ (23), the control OVA (SIINFEKL) epitope presented by H-$2K^b$, the env-H19 (EPLTSLTPRCNTAWNRLKL) epitope presented by I-A^b, and the control OVA-H (ISQAVHAAHAEINEAGR) epitope presented by I-A^b.

Cell culture

Spleen cells from RAG-$1^{-/-}$ and pfp$^{-/-}$RAG-$1^{-/-}$ mice were incubated in tissue culture dishes at 37°C for 1 h to deplete adherent cells. Harvested cells were routinely >95% NK1.1^+TCR$\alpha\beta^-$ as estimated by flow cytometry. Purified NK cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 25 mM sodium bicarbonate ($NaHCO_3$) in humidified 5% CO_2 at 37°C as previously reported (24). In coculture experiments, purified NK cells (3×10^5/well) were cocultured with irradiated (15,000 rad) RMA-S-m or RMA-S-CD80 cells (3×10^4/well) in 96-well, round-bottom culture plates in the presence of 25 U/ml IL-2. Before incubation, some NK cell cultures were depleted of NK1.1^+ cells by anti-NK1.1 (10 μg/ml) and complement (C′). CTL, and Th1 responses were analyzed in splenocytes from mice that had been primed 5 wk earlier with 5×10^5 live RMA-S-CD80 tumor cells, 10^7 irradiated (15,000 rad) RMA-S-CD80 tumor cells, or 10^7 irradiated (15,000 rad) RMA-S-m tumor cells. For CTL assays, splenocytes from each group of mice were pooled, and cell cultures were incubated overnight to delete adherent cells. Responder cells were treated with anti-CD4 (GK1.5) and complement as previously described (25), and quadruplicate cultures for each group were then incubated for 7 days with irradiated RMA tumor cells in RPMI containing 5% FCS before assay for cytotoxic activity (see below). For Th1 assays, splenocytes from each group of immunized mice were pooled, and cell cultures (3×10^6/well of 24-well plates) were stimulated with an equal number of irradiated B6 spleen cells (3,000 rad) and irradiated RMA (15,000) tumor cells. Seven days later, bulk cultures (by flow cytometry, 70–80% $CD4^+$) were assessed for proliferative response and IFN-γ production as described below.

Proliferation assays

Proliferation assays were conducted in 96-well U-bottom plates. The NK cell cultures were incubated for 2 days and pulsed with 0.5 μCi/well [^{3}H]thymidine (Amersham, Little Chalfont, U.K.) for the last 16 h. The bulk $CD4^+$ cultures at 1×10^5, 5×10^4, and 10^4 cells/well were stimulated with irradiated spleen cells (10^5/well) with or without a Th peptide (5 μg/ml) or irradiated RMA tumor cells (5×10^4/well). No exogenous IL-2 was added, and after 3 days, the cultures were pulsed with 0.5 μCi/well [^{3}H]thymidine (Amersham) for the last 16 h. Incorporation of radioactivity was measured in a Tri-Carb 2100TR liquid scintillation counter (Packard Instrument, Downers Grove, IL). Cell-free supernatants from both NK and $CD4^+$ cultures were harvested after incubation for 3 days and subjected to IFN-γ ELISA.

ELISA

IFN-γ levels in the culture supernatants were evaluated using a mouse IFN-γ-specific ELISA kit (BD PharMingen) according to the manufacturer's instructions.

Cytotoxicity assay

Cytotoxic activity of NK cells and CTL cultures was assessed against ^{51}Cr-labeled tumor target cells in a standard 4-h ^{51}Cr release assay (18). RMA-S-m target cells were cultured for 24 h at 25°C and then pulsed with 100 μM peptide for 2–4 h at 33°C. All cells were then labeled with 50 μCi ^{51}Cr for 1 h at 37°C and washed three times. B16F10 target cells were pulsed with 100 μM peptide for 1 h at 37°C before the assay, and peptides remained present during the assay. Effectors and targets were coincubated at various E:T cell ratios for 4 h as previously described (18). Spontaneous release of ^{51}Cr was determined by incubating the target cells with medium alone and was always <15%. Maximum release was determined by adding SDS to a final concentration of 5%. The percent ^{51}Cr release was calculated as follows: 100 × ((experimental release − spontaneous release)/(maximum release − spontaneous release)). Each experiment was performed twice using triplicate samples.

Tumor growth assays

Groups of five untreated (WT, RAG-$1^{-/-}$, IFN-$\gamma^{-/-}$, pfp$^{-/-}$, or Ab-treated WT (see *Lymphocyte subset depletion*) mice were injected s.c. with vector alone-infected or CD80-infected tumor cells (range, 10^5–10^7 cells) in 0.2 ml PBS as indicated. On occasion some mice initially received a nonlethal dose of live parental tumor or irradiated (20,000 rad) parental tumor cells. Mice were observed every 2 days for tumor growth using a caliper square measuring along the perpendicular axes of the tumors (the product of two diameters ± SE). Mice were sacrificed when tumors reached >12 mm in diameter, and no signs of rejection were observed. Following the primary rejection of CD80-expressing tumor cells, the same mice were secondarily challenged (4 or 12 wk from the first challenge) in the opposite flank with either parental tumor cells or irrelevant tumor cells as indicated. Tumors were measured as described in this section. Mice without any signs of tumor growth were kept under observation for at least 40 days after secondary tumor inoculation.

Lymphocyte subset depletion

In some experiments in vivo, to deplete T cells and NK cells, mice were treated with Ab on days −1, 0, and 7 (where day 0 is the day of primary or secondary tumor inoculation). The following doses of Abs were used: anti-CD4 (GK1.5), 100 μg i.p.; anti-CD8 (53-6.7), 100 μg i.p.; anti-NK1.1 (PK136), 100 μg i.p.; and rabbit anti-asGM1, 200 μg i.p. These protocols have previously been shown to effectively deplete T and NK cell subsets in C57BL/6 mice (18, 26). Alternatively, some groups of mice were treated as described above with 200 μg control rabbit Ig or twice weekly with 0.5 mg control IgG or anti-mIFN-γ mAb.

Results

Tumor rejection promoted by CD80 expression

RMA-S, a MHC class I-deficient variant of RMA, is the prototypic NK cell-sensitive target for in vitro and in vivo studies (18, 27, 28). To examine the effect of CD80 on NK cell-mediated tumor rejection, we prepared by retroviral infection, a series of RMA-S cell lines that expressed increasing levels of CD80. Infectant populations with high CD80 expression and vector alone-infected controls (-m) were chosen for further studies (Fig. 1). The control and CD80-expressing cell lines had similar growth kinetics in vitro, as determined by several different assays of proliferation (data not shown).

Tumor escape variants that have lost MHC class I expression, such as RMA-S, are efficiently controlled in vivo by NK cells (14). Consistent with these previous data, RMA-S-m tumor cells grew equivalently over a 25-day period in WT B6 and B6 RAG-$1^{-/-}$ mice (deficient in T, B, and NKT cells), but at an enhanced rate in NK cell-depleted (using asialoGM1 Ab) WT mice (Fig. 2*a*). Similar results were obtained at higher (10^6 cells, Fig. 2*b*; 10^7 cells, Fig. 2*c*) doses and using anti-NK1.1 mAb to deplete NK cells (data not shown). In contrast, RMA-S-CD80 tumor cells were rejected, and this process was mediated mostly by NK cells at the lowest tumor challenge doses (Fig. 2*a*). Despite the limited ability of NK cells to control larger tumor cell numbers, RMA-S-CD80 tumor cell growth was even inhibited at higher doses of RMA-S-CD80 tumor cells (10^7, Fig. 2*c*), but rejection in this case required both

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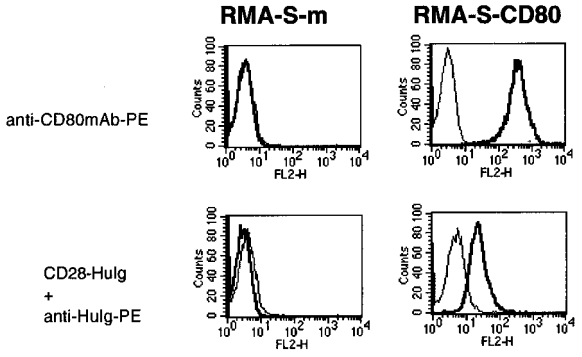

FIGURE 1. Expression of CD80 on tumor cells. RMA-S tumor cells were retrovirally infected with MSCV vector alone (-m) or MSCV containing mouse CD80 cDNA (-CD80) and stained with rat anti-mouse CD80 mAb (IG10) (*top panels*), or CD28-human Ig followed by PE-anti-human Ig (*bottom panels*; thin lines). Control staining of cells with an irrelevant PE-labeled mAb or secondary PE-anti-human Ig is shown (*thick lines*).

T cells and NK cells (Fig. 2*c*). Overall, there was a >98% reduction in the growth of RMA-S-CD80 tumor in WT mice compared with RMA-S-m tumor. These data indicated that CD80 expression could enhance both primary NK cell- and/or T cell-mediated tumor rejection depending upon the cell number inoculated.

CD80-stimulated NK cell-mediated protection requires pfp and IFN-γ

We then demonstrated that CD80 expression on RMA-S cells could induce the proliferation and IFN-γ production of NK cells (Fig. 3, *a* and *b*). Increased proliferation (Fig. 3*a*) and IFN-γ production (Fig. 3*b*) were abrogated by anti-CD80 or the depletion of NK cells using anti-NK1.1 (data not shown). CD80-stimulated NK cell proliferation was not inhibited by an anti-IFN-γ or control mAb (Fig. 3*b*). While NK cells from pfp$^{-/-}$RAG-1$^{-/-}$ mice could produce IFN-γ following interaction with RMA-S-CD80 cells (Fig. 3*b*), these NK cells were not cytotoxic toward either RMA-S-m or RMA-S-CD80 target cells (Fig. 3*c*). Notably, RMA-S-CD80 were considerably more sensitive than RMA-S-m target cells to NK cell-mediated cytolysis in 4-h cytotoxicity assays in vitro (Fig. 3*c*). IFN-γ production, proliferation, and cytotoxicity of NK cells derived from CD28-deficient mice were not augmented when cocultured with RMA-S-CD80 tumor cells (data not shown). Collectively, these data indicated that ligation of CD28 on NK cells by CD80 on tumor cells induced IFN-γ production, proliferation, and cytotoxicity mediated by NK cells. We and others have previously shown that NK cell-mediated rejection of RMA-S tumor cells is pfp-dependent (18, 29). The growth of RMA-S-m tumor cells was pfp and, to a lesser extent, IFN-γ-dependent (Fig. 3*d*). Importantly, NK cell-mediated rejection of RMA-S-CD80 in WT mice was not observed in either pfp$^{-/-}$ or IFN-γ$^{-/-}$ mice, indicating the critical requirement of these molecules in CD80-stimulated tumor rejection by NK cells (Fig. 3*d*).

CD80-induced primary rejection stimulates tumor-specific T cell immunity

Given primary rejection of RMA-S tumor cells that express CD80, we next determined whether the mice could resist secondary tumor challenge. WT mice initially rejecting RMA-S-CD80 were rechallenged in the opposite flank with RMA (1×10^6, 12 wk after primary inoculation) and coincidently depleted of NK cells, CD8$^+$ T cells, or CD4$^+$ T cells. The growth of RMA cells was similar in all untreated and lymphocyte-depleted naive WT mice (Fig. 4*a*). As demonstrated in Fig. 4*a* (*right panel*), WT mice previously

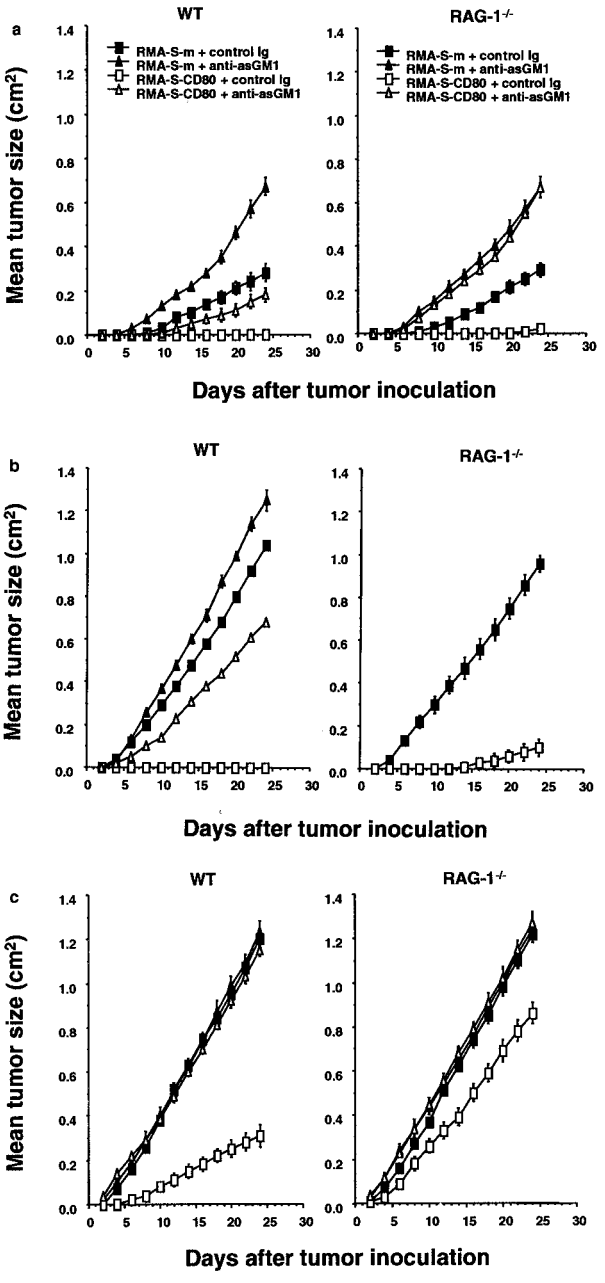

FIGURE 2. Tumor rejection promoted by CD80 expression. Subcutaneous inoculation of mock-infected or CD80-expressing tumor cells as indicated into WT or RAG-1$^{-/-}$ mice: *a*, RMA-S-m and RMA-S-CD80, 10^5 cells; *b*, 10^6 cells; and *c*, 10^7 cells. WT (*left panels*) and RAG-1$^{-/-}$ (*right panels*) mice were treated with anti-asGM1 (triangles) or control rabbit Ig (squares) from the day of tumor inoculation. Tumor growth was followed every second day, and data are expressed as the mean ± SE of five mice in each group.

inoculated with RMA-S-CD80 cells were able to effectively reject 1×10^6 RMA tumor cells. Similarly, WT mice depleted of NK cells were also able to effectively reject secondary challenge with RMA tumor cells, suggesting that NK cells were not playing a key role in the secondary RMA tumor rejection. Depletion of either CD8$^+$ or CD4$^+$ T cells prevented the secondary rejection of RMA tumor cells, although depletion of CD4$^+$ T cells was not as effective, suggesting that CD8$^+$ T cells could mount some response independently of CD4$^+$ T cells (Fig. 4*a*).

The specificity of T cell immunity primed by prior NK cell- and CD80-mediated tumor rejection was then assessed. The mice that

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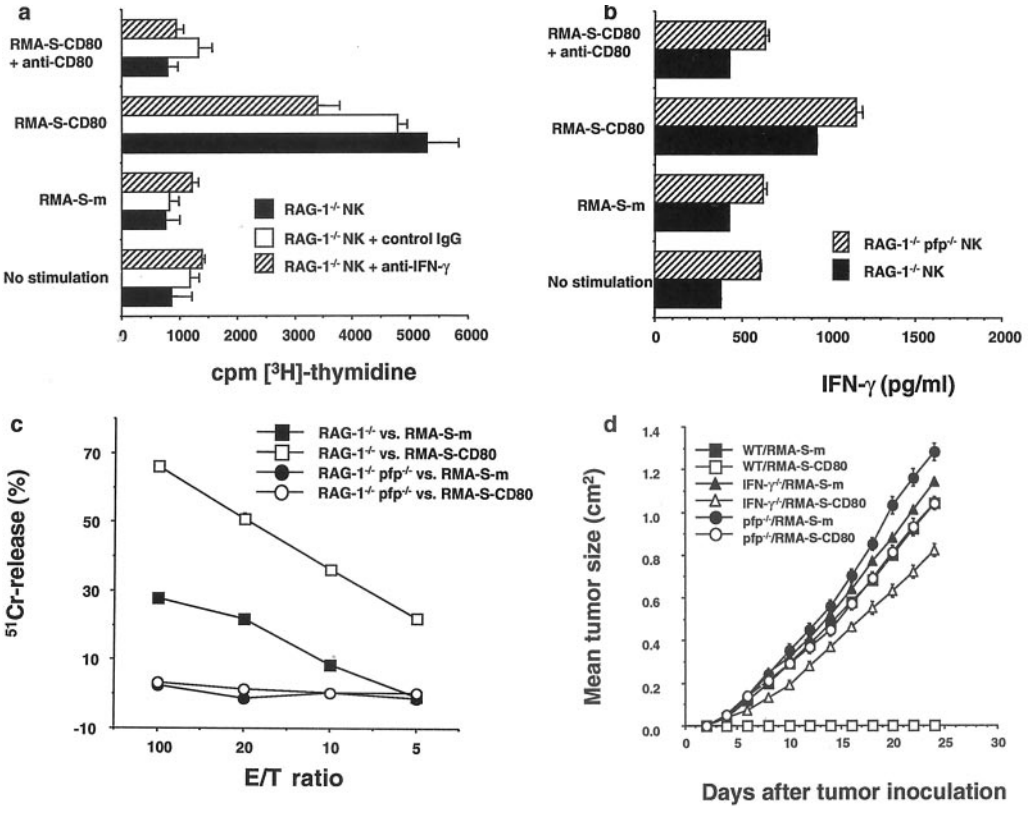

FIGURE 3. CD80 stimulates NK cell-mediated protection that requires perforin and IFN-γ. Induction of NK cell proliferation (*a*) and IFN-γ (*b*) production by CD28/CD80 interaction in vitro. Freshly isolated RAG-$1^{-/-}$ splenic NK cells were cocultured with irradiated RMA-S-m or RMA-S-CD80 in the presence or absence of anti-CD80 mAb (5 μg/ml), anti-IFN-γ mAb (10 μg/ml), or control IgG (10 μg/ml) and 25 U/ml IL-2. After 2 days, the proliferative response was assessed by pulsing the culture with [^{3}H]TdR for the last 16 h (*a*). Cell-free culture supernatants were collected after 3 days, and IFN-γ was measured by ELISA (*b*). Data are expressed as the mean ± SE of triplicate cultures. *c*, The cytotoxicity of freshly isolated splenic NK cells from RAG-$1^{-/-}$ and RAG-$1^{-/-}$ pfp$^{-/-}$ mice was tested against ^{51}Cr-labeled RMA-S-m or RMA-S-CD80 tumor cells at the indicated E:T cell ratios. Data are expressed as the mean ± SE of triplicate wells of two independent experiments. *d*, Subcutaneous inoculation of 10^6 RMA-S-m (filled symbols) and RMA-S-CD80 (open symbols) tumor cells into WT (squares), IFN-$\gamma^{-/-}$ (triangles), or pfp$^{-/-}$ (circles, solid lines) mice (*d*). Tumor growth was followed every second day, and data are expressed as the mean ± SE of five mice in each group.

rejected RMA-S-CD80 tumor challenge (5×10^5 cells) by a NK cell-dependent mechanism were rechallenged on the opposite flank 4 wk later with a lethal dose of RMA-S-m, RMA (noninfected parental tumor expressing MHC class I), or EL-4 and B16F10 (syngeneic tumors; Fig. 5). All tumors inoculated s.c. grew avidly in naive B6 mice at the doses chosen. Strikingly, of the mice that had undergone a primary NK cell-mediated rejection of RMA-S-CD80, only the group receiving B16F10 (1×10^6 s.c.) developed tumors. By contrast, the growth of RMA-S-m, RMA, and EL-4 were not detected in these mice. These data indicated that the initial NK cell-mediated rejection triggered by tumor CD80 expression had not simply caused prolonged NK cell activity, but had promoted strong immunity to parental-related tumors that lacked CD80 expression.

CTL and Th1 responses to defined endogenous Ags in RMA tumor cells were also monitored in immunized mice that had rejected RMA-S-CD80 tumors (Fig. 6). The CTL response against leukemias induced by MuLV of the Moloney (MBL-2), and Rauscher (RMA, RMA-S) types in B6 mice is predominantly H-2D^b restricted (22, 30). A peptide derived from the gag leader sequence was identified as the major target epitope (murine leukemia virus gagL aa 75–83, $gagL_{75-83}$) (31). A subdominant H-2K^b-restricted response to an envelope-derived peptide (murine leukemia virus env aa 189–196, $env_{189-196}$) (22) and one potent MuLV env-derived Th epitope (32) have been described. Although EL-4 is a dimethylbenzanthracene-induced thymoma (and not MuLV-induced), EL-4 and MBL-2 also share a recently described nonviral Ag with RMA, and indeed RMA and EL-4 may share a common origin (23). Therefore, we monitored responses to each of these Ags in the spleens of mice challenged 6 wk earlier with 5×10^5 live RMA-S-CD80 tumor cells, 10^7 irradiated RMA-S-CD80 tumor cells, or 10^7 irradiated RMA-S-m tumor cells. Initially, CD8$^+$ T cells from these mice were isolated and stimulated in vitro as bulk cultures with irradiated RMA tumor cells. The CTL assay suggested that most specificity and activity generated by RMA-S-CD80 priming in vivo were directed at the $gagL_{75-83}$ epitope (Fig. 6, *a* and *b*). Notably, irradiated RMA-S-CD80 tumor cells could also stimulate the CTL response, albeit to a lesser extent (Fig. 6, *c* and *d*), whereas irradiated RMA-S-m tumor cells did not stimulate detectable CTL activity (Fig. 6, *e* and *f*). The CTL activity against the $gagL_{75-83}$ epitope (Fig. 6) was consistent with the ability of RMA-S-CD80-primed mice to successfully reject RMA, but not B16F10 tumors that do not express the MuLV Ags (Fig. 5). The ability of immunized mice to reject EL-4 and RMA-S tumors (Fig. 5) was supported by the CTL response to EL-4 or RMA-S alone or to B16F10 pulsed with a common nonviral Ag shared between EL-4 and RMA (Fig. 6).

In the same immunized mice, we monitored Th responses to the I-A^b-binding env-H19 peptide (Fig. 7). CD4$^+$ T cells from mice primed with live RMA-S-CD80 tumor cells displayed proliferative capacity in response to env-H19, but not control Th peptide OVA-H (Fig. 7*a*). These cultures also produced high levels of

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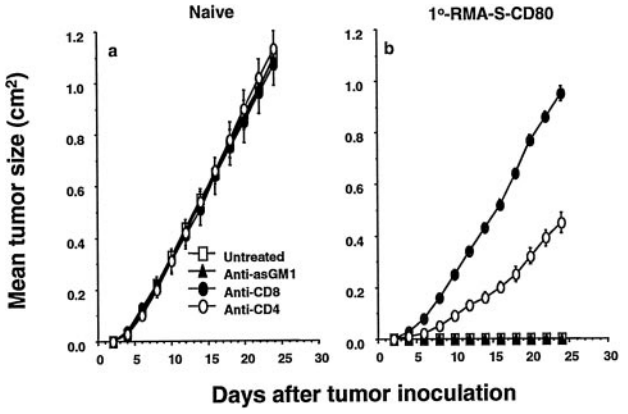

FIGURE 4. Tumor CD80 expression evokes tumor-specific T cell memory. Secondary rejection responses were monitored in WT mice that previously rejected primary RMA-S-CD80 tumors (5×10^5). Mice were challenged 6 wk later with 10^6 RMA tumor cells. Coincidently upon secondary challenge with parental tumor cells, some mice were depleted of NK cells (anti-asGM1, ▲), $CD8^+$ cells (●), or $CD4^+$ cells (○). Naive mice (naive) were treated similarly to control mice. Tumor growth was followed every second day, and data are expressed as the mean ± SE of five mice in each group.

IFN-γ (Fig. 7*b*). Similarly, primary challenge with irradiated RMA-S-CD80 tumor cells provoked detectable $CD4^+$ T cell proliferation (Fig. 7*c*) and IFN-γ production (Fig. 7*d*) in response to env-H19. We did not detect a Th1 response in the spleens of mice that had been primed with irradiated RMA-S-m tumor cells (Fig. 7, *e* and *f*). Thus, both specific CTL and Th1 responses to MuLV Ags were detected in the mice that had previously rejected RMA-S-CD80 tumor cells.

Priming of T cell immunity requires NK cells and IFN-γ

In agreement with CTL and Th1 responses, WT mice that had been initially primed with 10^7 irradiated RMA-S-CD80 tumor cells

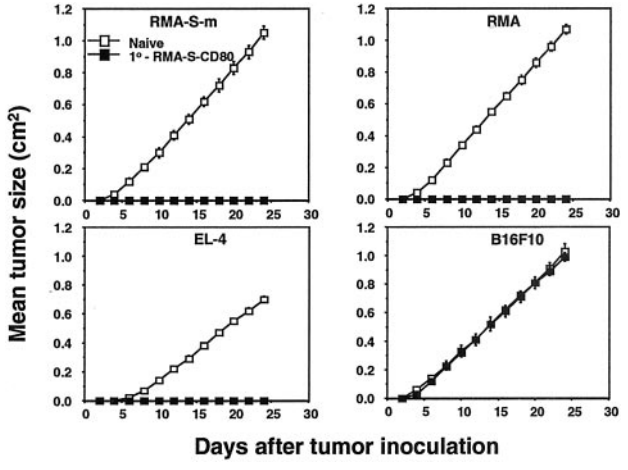

FIGURE 5. NK cell-mediated primary rejection evokes tumor-specific T cell immunity. Secondary rejection responses were monitored in WT mice that previously rejected RMA-S-CD80 tumor cells (1°-RMA-S-CD80). Specificity was examined by initially inoculating WT mice s.c. with 5×10^5 RMA-S-CD80 tumor cells and then challenging them in the opposite flank 4 wk later with RMA-S-m (1×10^6), RMA (1×10^6), EL-4 (5×10^6), or B16F10 (1×10^6) tumor cells. Tumor growth was compared with that in naive WT mice inoculated with the same tumor and doses. Tumor growth was followed every second day, and data are expressed as the mean ± SE of five mice in each group.

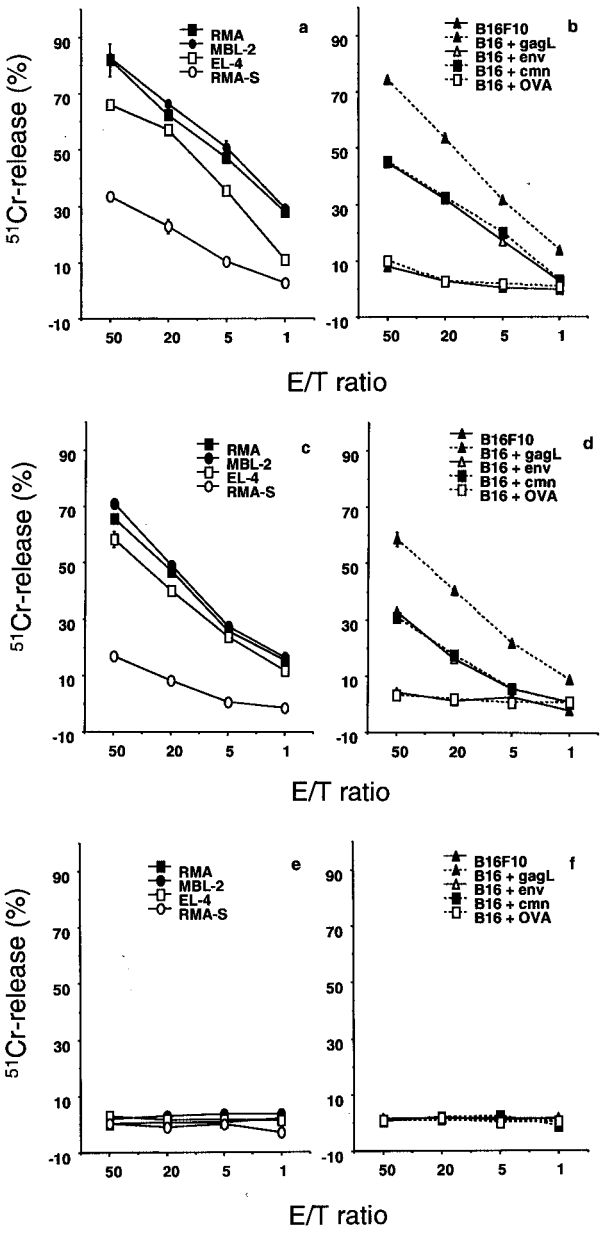

FIGURE 6. Specific CTL responses evoked by CD80-driven NK cell-mediated tumor rejection. Generation of CTL responses to MuLV Ags specific for RMA tumor cells was monitored in WT mice s.c. primed 6 wk previously with 5×10^5 live RMA-S-CD80 tumor cells (*a* and *b*), 10^7 irradiated RMA-S-CD80 tumor cells (*c* and *d*), and 10^7 irradiated RMA-S-m tumor cells (*e* and *f*). Effector CTL were induced as described in *Materials and Methods*, and their specificity was examined in a 4-h ^{51}Cr release assay. CTL activity was assayed against RMA, MBL-2, EL-4, RMA-S, and B16F10 or B16F10 pulsed with MuLV $gagL_{75-83}$ peptide, MuLV $env_{189-196}$ peptide, RMA/EL-4 common tumor peptide (cmn), or control $OVA_{257-263}$ peptide as indicated. Data are expressed as the mean ± SE of triplicate samples and are representative of four independent cultures from each group of immunized mice.

were resistant to a secondary RMA tumor challenge with up to 5×10^5 live tumor cells (Fig. 8*a*), whereas those primed with 10^7 irradiated RMA-S-m tumor cells were not (Fig. 8*a*). The RMA tumor rejection following irradiated RMA-S-CD80 tumor cell priming was as avid as that generated by immunization with live RMA-S-CD80 tumor cells (data not shown), as expected from the similar CTL and Th1 responses (Figs. 6 and 7). To

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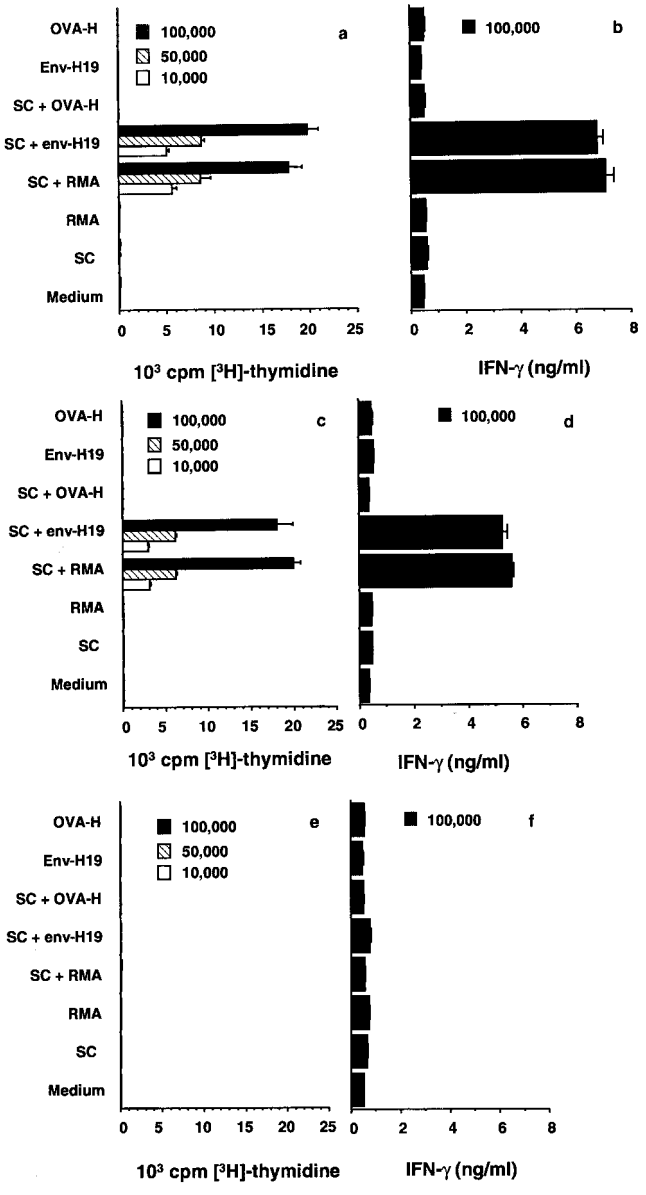

FIGURE 7. Specific Th1 responses evoked by CD80-driven NK cell-mediated tumor rejection. Generation of Th1 responses (proliferation and IFN-γ production) to a MuLV Ag specific for RMA tumor cells was monitored in WT mice primed 6 wk previously with 5×10^5 live RMA-S-CD80 tumor cells (*a* and *b*), 10^7 irradiated RMA-S-CD80 tumor cells (*c* and *d*), and 10^7 irradiated RMA-S-m tumor cells (*e* and *f*). Responder T cells were restimulated in vitro by the following Ags: medium alone, irradiated RMA, irradiated B6 spleen cells (SC), irradiated SC and irradiated RMA, irradiated SC and MuLV env-H19 peptide, irradiated SC and control OVA-H peptide, env-H19 peptide, or OVA-H peptide. Responders were used at 1×10^5, 5×10^4, and 10^4 cells/well. Proliferation was estimated by [^{3}H]TdR incorporation and IFN-γ production by ELISA. Data are expressed as the mean ± SE of triplicate samples and are representative of two independent cultures from each group of immunized mice.

determine the contribution of NK cells and IFN-γ to the priming of secondary rejection, WT mice were primed with 10^7 irradiated RMA-S-CD80 tumor cells and coincidently depleted of NK cells and/or IFN-γ for 2 wk after tumor inoculation. Five weeks after the primary tumor inoculation, the mice were challenged with 10^6 RMA tumor cells. Tumors were only rejected in untreated WT mice and WT mice receiving control Ig, whereas mice depleted of $CD4^+$ or $CD8^+$ cells displayed no

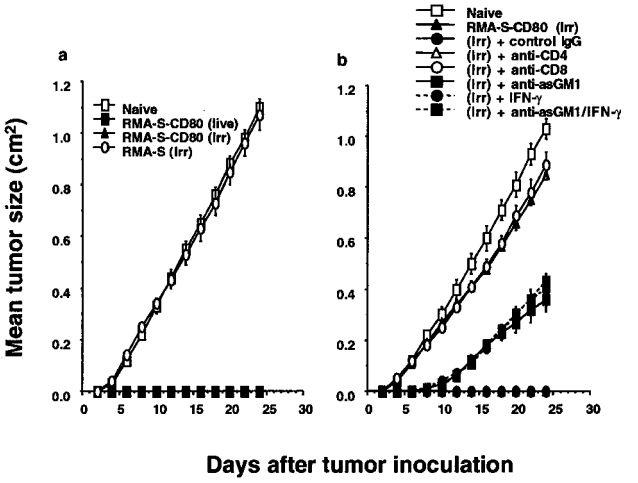

FIGURE 8. Priming of secondary T cell immunity requires NK cells and IFN-γ. Rejection of secondarily challenged RMA tumor cells (5×10^5 s.c.) was monitored in WT mice primed 5 wk previously with (*a*) 5×10^5 RMA-S-CD80 live tumor cells (■), 10^7 irradiated (irr) RMA-S-CD80 tumor cells (▲), or 10^7 irradiated RMA-S-m tumor cells (○) or (*b*) 10^7 irradiated RMA-S-CD80 tumor cells. Tumor growth was compared with that in naive WT mice inoculated with the same dose of RMA (□). *b*, Coincidently upon priming with irradiated RMA-S-CD80 tumor cells, some mice were depleted of $CD4^+$ cells (△, solid lines), $CD8^+$ cells (○, solid lines), NK cells (anti-asGM1; filled squares with solid lines), IFN-γ (●, dashed lines), or both (■, dashed lines) for 2 wk. Tumor growth was followed every second day, and data are expressed as the mean ± SE of five mice in each group.

resistance to tumor challenge. The mice depleted of NK cells and/or IFN-γ were unable to completely reject the challenge with an equivalent dose of RMA tumor cells (Fig. 8*b*). These results indicated that NK cells and IFN-γ were key factors in the development of adaptive T cell immunity following NK cell-mediated innate immune responses against tumors.

Discussion

The effector CD28-tumor CD80 interaction promoted potent NK cell- and T cell-mediated tumor rejection, although the relative involvement of NK cells and T cells was dose dependent. Only the lowest doses of MHC class I- and II-deficient RMA-S-CD80 tumor cells could be rejected exclusively by NK cells, whereas rejection of higher doses required T effector cells. Enhanced tumor rejection mediated by NK cells may reflect the ability of CD80 to stimulate NK cell IFN-γ secretion, proliferation, and cytotoxicity. Both pfp and IFN-γ were critical for NK cell-mediated rejection of RMA-S-CD80 tumors. RMA-S-CD80 tumor cells did not augment the IFN-γ secretion, proliferation, and cytotoxicity of NK cells from $CD28^{-/-}$ mice, and the growth of these tumor cells was not inhibited in $CD28^{-/-}$ mice, suggesting that CD80 mediated its effect exclusively via CD28 (data not shown). Cooperation existed between innate and adaptive immune responses against tumors, because NK cell activation by CD80 on MHC class I- and II-deficient RMA-S tumor cells was key in priming optimal T cell immunity against subsequent tumor challenge. Importantly, IFN-γ produced during the priming phase was also vital in the creation of secondary immunity to the parental tumors lacking CD80 expression.

Both CTL and Th1 immunity were induced against MuLV and other epitopes expressed by the RMA-S-CD80 tumor cells. The priming stimulus helped establish T cell memory that prevented the growth of parental RMA tumors at least 6 wk after the primary

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challenge. Interestingly, following RMA-S-CD80 vaccination, a previous study reported the generation of MHC class I-restricted $CD8^+$ CTLs that required target cell TAP deficiency for efficient recognition (33). We too detected such CTL following priming with RMA-S-CD80 tumor cells, because RMA-S tumor cells were rejected upon secondary challenge (Fig. 5), and CTL reactive with RMA-S were detected in bulk $CD8^+$ T cell cultures restimulated from immunized mice (Fig. 6*a*). In similar experiments with CD70, a weaker costimulator of NK cells, we did not detect CTL reactive with RMA-S (34).

NK cells, unlike T cells, are activated in the absence of self-MHC class I molecules, and they are particularly important in the earliest stages of immune responses, where they exert cytotoxicity and/or cytokine production. Several studies have shown that NK cells can regulate CTL responses, but only a few have suggested that IFN-γ produced by NK cells (10, 35) might induce Th1- and CTL-mediated immunity. Of note, IFN-γ production by activated NK cells has also been shown to promote Th1 responses and enhance B cell-mediated autoimmunity (36). Nevertheless, the idea that NK cell IFN-γ promotes T cell immunity has not been widely accepted. Another NK cell-activating receptor constitutively expressed by the majority of NK cells, CD27, also primes significant T cell memory (37). Unlike CD27, which only stimulates NK cell IFN-γ production and proliferation (38), CD28 also triggers NK cell cytolytic function (11, 12). In this study, RMA-S-CD80 tumor cells were more sensitive to NK cytotoxicity in vitro, and the potent in vivo rejection of RMA-S-CD80 tumor cells by NK cells was pfp dependent. However, priming of secondary immunity did not require live tumor cells, suggesting that ligation of CD28 on NK cells and IFN-γ production was sufficient. Recently, it was shown that STAT-1-deficient mice were incapable of rejecting RMA-S tumor doses that were rejected in IFN-γR-deficient mice (39). These data suggest that a STAT-1-dependent pathway, independent of IFN-γ, may also be critical for effective NK cell-mediated tumor rejection. It remains to be determined whether this STAT-1 pathway is also required in enhanced NK cell-mediated rejection primed by ligation of CD80.

In studies first demonstrating that CD80-expressing tumors were rejected in an NK cell-mediated fashion, CD80 facilitated rejection appeared to occur through a cross-presentation mechanism (40, 41). Given that RMA-S alone was not immunogenic in our study, it is possible that RMA-S-CD80 tumor cells triggered IFN-γ production and/or tumor cell death that was sufficient to enhance cross-priming. In this study, we demonstrated that IFN-γ aids the development of Th1 and CTL function, and thus the IFN-γ produced by CD28-stimulated NK cells may promote local APC activity and/or directly enhance CTL and Th1 development. Recent studies have indicated that activated NK cells can lyse target cells and surrounding immature dendritic cells (DCs) that have phagocytosed and processed foreign Ag, thereby providing antigenic cellular debris that can be internalized by maturing DCs that present to T cells in the lymph nodes (42). Costimulatory molecules such as CD80 are also typically expressed on APC, such as DC, and recently cell-to-cell contact between DC and resting NK cells was demonstrated to result in a substantial increase in both NK cell cytolytic activity and IFN-γ production (43). Lysis of autologous DC by NK cells in vitro has been shown to be dependent on granule exocytosis; however, induction of maturation of DC, including up-regulation of MHC class I and II molecules and costimulatory molecules, renders DC less susceptible to lysis by NK cells (44). Pfp-mediated cytotoxicity can eliminate tumor cells directly (29, 28, 45), but at the same time it may also limit the number of immature DC available for maturation by Ags, thus minimizing CTL activity and potential immunopathology (46, 47). Importantly, receptors mediating DC or target cell recognition by NK cells (including CD28) do not seem to be involved in DC-mediated NK cell priming (42). CD28 alone does not control NK cell-mediated lysis and IFN-γ production. Therefore, understanding the balance between various activation and inhibitory molecules on NK cells and whether they trigger cytotoxicity and/or IFN-γ production will be critical to elucidating the role of NK cells in innate and adaptive responses to tumors and thus in designing better immunotherapies.

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