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Inducible Costimulator Costimulates Cytotoxic Activity and IFN-γ Production in Activated Murine NK Cells[1]

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The functions of NK cells are regulated by the balance of activating and inhibitory signals. The inhibitory NK cell receptors are well understood; however, less is known about the activating signaling pathways. To explore whether a costimulatory receptor, inducible costimulator (ICOS), is involved in NK cell function, we assessed the role of ICOS in NK cell-mediated cytotoxicity and cytokine production. In addition, to determine whether ICOS contributes to the elimination of tumors in vivo, we examined the tumor growth survival of mice injected with a tumor expressing the ICOS ligand, B7RP-1. We found that ICOS was up-regulated by cytokine stimulation in murine NK cells. Consistent with ICOS expression on activated NK cells, ICOS-dependent cytotoxicity and IFN-γ production were observed, and appeared to require signaling through the phosphoinositide 3-kinase pathway. Interestingly, ICOS-mediated stimulation allowed activated NK cells to kill more efficiently tumor cells expressing MHC class I. Furthermore, fewer metastases appeared in the liver and spleen of mice injected with the ICOS ligand-expressing tumor compared with mice bearing the parental tumor. These results indicate that NK cell functions are regulated by ICOS. ***The Journal of Immunology,*** **2002, 169: 3676–3685.**

Natural killer cells play a critical role in eliminating tumors and virally infected cells (1, 2). This is accomplished by their ability to kill the target cells directly or to produce cytokines, such as IFN-γ and TNF-α, which mediate the response (1). The induction of cytotoxicity and cytokine production in NK cells is controlled by opposing signals from activating and inhibitory receptors. Cells expressing MHC class I can turn off NK cells by delivering an inhibitory signal through mouse Ly49 or human killer cell Ig-like receptors (3–5). Therefore, tumors or virus-infected cells that express either an aberrant or no class I may be killed by NK cells if activating receptors are engaged to initiate the response. While the inhibitory NK cell receptors are well understood, less is known about the activating signaling pathways. In particular, it remains unclear which activating receptors permit NK cells to kill cells that retain MHC class I expression.

For the optimal activation of T cells, they must be stimulated by the TCR together with a costimulatory receptor (6). Costimulatory receptors also function to activate NK cells (7–10). For example, mouse NK cells, which express CD28, efficiently kill tumor cells expressing the CD28 ligand, CD80 (B7.1), even if the tumors express MHC class I (8). Recently, inducible costimulator (ICOS),[3] a costimulatory molecule related to CD28, was identified on T cells and was found to be induced by TCR activation (11). Studies have revealed that ICOS is important for T cell activation, proliferation, and cytokine production, which lead to the generation of Th2 cells in human and mouse models (12–15). In addition, it has been reported that ICOS controls Th1 responses in cardiac transplantation (16). A ligand of ICOS was identified as a B7 (CD80/CD86) family member and is termed the B7 homologue (B7-H) in humans and the B7-related protein-1 (B7RP-1/B7H) in mice (17–20). The ICOS ligand is expressed as a cell surface glycoprotein by B cells, APCs, and certain nonhemopoietic tissues. ICOS does not bind to other B7 family members, e.g., CD80, CD86, B7H1, B7H3, or PD1 ligand (19–23).

Whether ICOS is also involved in NK cell function has not been explored. In this study we assessed the role of ICOS in NK cell-mediated cytotoxicity and cytokine production. To determine whether ICOS contributes to the elimination of tumors in vivo, we examined tumor growth and the survival of mice injected with a tumor expressing the ICOS ligand.

Materials and Methods

Mice

Six- to 8-wk-old C57BL/6 mice were purchased from Charles River (Wilmington, MA). All mice were maintained under specific pathogen-free conditions in the animal facility of University of California, San Francisco. All experiments were performed according to the guidelines of the University of California, San Francisco committee on animal research.

Reagents and cytokines

Human rIL-2 was provided by the National Cancer Instititute BRB Preclinical Repository. Mouse rIL-12 and human rIL-15 were provided by Dr. J. P. Houchins (R&D Systems, Minneapolis, MN). The phosphoinositide 3-kinase (PI3 kinase) inhibitor, LY294002, and the Syk family kinase inhibitor, piceatannol, were purchased from Calbiochem (La Jolla, CA). An anti-mouse ICOS mAb (IgG1 isotype) was generated by immunizing rats with a mouse ICOS-Fc fusion protein, produced as described previously (20). The anti-mouse ICOS mAb blocks the binding of B7RP-1 Ig fusion protein to ICOS-bearing cells (data not shown).

Cytokine production

Tissue culture plates were coated with DOTAP (1 mg/ml; Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature (to enhance Ab binding to the plates), washed with PBS, incubated with mAbs diluted in 0.1 M bicarbonate buffer (pH 9.0) for 16 h at 4°C, and then washed with PBS. To

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[3] Abbreviations used in this paper: ICOS, inducible costimulator; PI3 kinase, phosphoinositide 3-kinase; RAE-1γ, retinoic acid early inducible-1γ.

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block Fc receptor-dependent activation, NK cells were pretreated with soluble anti-CD16/32 mAb 2.4G2 (10 μg/ml) for 30 min. NK cells (2×10^5) were placed in RPMI 1640 containing 10% FCS and recombinant human IL-2 (2000 U/ml) and were cultured for 18 h in plates containing the immobilized Abs. The amount of IFN-γ in the culture supernatants was determined with a mouse IFN-γ-specific ELISA kit (OptEIA mouse IFN-γ set; BD PharMingen, San Diego, CA) used according to the manufacturer's instructions.

Cell lines and transfectants

The Ba/F3 pro-B cell line was provided by Dr. T. Kitamura (University of Tokyo, Tokyo, Japan), and the RMA T leukemia cell line was provided by Dr. J. Ryan (University of California, San Francisco). These cells were cultured in RPMI 1640 medium containing 10% FCS, 5×10^{-5} M 2-ME, and 2 mM glutamine. Since Ba/F3 cells are dependent on IL-3 for their proliferation, Ba/F3 cells were transfected with a mouse IL-3 cDNA plasmid to provide for autocrine growth. All transfectants (Ba/F3 B7RP-1, Ba/F3 mock, Ba/F3 retinoic acid early inducible-1γ (RAE-1γ), Ba/F3 B7RP-1 RAE-1γ, P815 mock, P815 B7RP-1, RMA B7RP-1, RMA mock, RMA RAE-1γ, and RMA B7RP-1 RAE-1γ) were established by retroviral transduction (24, 25). RMA cells were transduced using retroviruses generated with the pMX-puro vector (24, 25). Other stable transfectants were established using retroviruses generated with the pMX-pie vector. The murine B7RP-1 cDNA was cloned by PCR using oligonucleotide primers (sense, 5′-ATG CAG CTA AAG TGT CCC TG-3′; antisense, 5′-TCA GGC GTG GTC TGT AAG TTC-3′), and the cDNA was ligated into the pMX-pie and pMX-puro vectors. This construct was transfected into Phoenix-A packaging cells (provided by Dr. G. Nolan, Stanford, CA) using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD) (24). Two days later, the supernatants containing viruses were collected and used to infect 5×10^4 Ba/F3, RMA, and P815 cells in the presence of polybrene (8 μg/ml). Transduced cells were cloned by limiting dilution.

Preparation of NK cells

Liver mononuclear cells were prepared as described previously (26). Splenic mononuclear cells from 6- to 8-wk-old C57BL/6 mice were prepared by passing spleens through a steel mesh, and erythrocytes were lysed in an ammonium chloride solution (Sigma). NK cells were enriched by T and B cell depletion. Briefly, spleen cells were incubated with anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 53-6.7); thereafter these cells were mixed with magnetic microbeads coated with goat anti-mouse Ig Ab and goat anti-rat Ig Ab (Advanced Magnetic, Cambridge, MA). CD4, CD8, and surface Ig-positive cells were removed by magnetic cell sorting. The $CD4^-$, $CD8^-$, and Ig-depleted splenocytes were stained with a PE-conjugated pan-NK cell mAb DX5 (BD PharMingen), followed by incubation with magnetic microbeads coated with anti-PE-Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Thereafter, $DX5^+$ cells were isolated by magnetic cell sorting using a MACS (Miltenyi Biotec). The purity of the $DX5^+$ cells was >95%, as determined by flow cytometric analysis. The purified NK cells were cultured in RPMI 1640 supplemented with 10% FCS and 5×10^{-5} M 2-ME in the presence of 4000 U/ml human rIL-2 for 7–10 days.

Flow cytometric analysis

Nonspecific staining was blocked with 5% normal mouse serum and 5% normal rat serum (Sigma). Cells were stained with anti-NK1.1-FITC, anti-DX5-FITC, anti-CD4-FITC, anti-CD8-FITC, and anti-CD3-FITC (BD PharMingen). To detect ICOS, we used the extracellular domain of mouse B7RP-1 fused to human IgG1 Fc (mB7RP1-Ig) (20). A PE-conjugated goat anti-human Fcγ fragment (Jackson ImmunoResearch, West Grove, PA) was used as a second-step reagent. The cells (1×10^6) were stained with 0.5 μg mB7RP1-Ig and 0.25 μg of the other mAbs. Incubation was conducted for 20 min, after which cells were washed with PBS containing 0.01% NaN_3. Cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA) or a small desktop Guava personal cytometer with ViaCount and Express software (Guava, Hayward, CA). Viable lymphocyte populations were identified based on characteristic forward and side light scatters and by exclusion of propidium iodide staining.

Cytotoxic assay

Target cells were labeled with 100 μCi $Na_2[^{51}Cr]O_4$ for 60 min at 37°C in RPMI 1640 medium containing 10% FCS, washed three times with medium, and used in cytotoxicity assays. ^{51}Cr-labeled target cells (5×10^3) and effector cells were mixed in U-bottom wells of a 96-well microtiter plate at the indicated E/T cell ratios in triplicate. After a 4-h incubation, cell-free supernatants were collected, and radioactivity was measured in a Microbeta counter (Wallac, Turku, Finland). The spontaneous release was <15% of the maximum release. The percentage of specific ^{51}Cr release was calculated according to the following formula: % specific lysis = (experimental − spontaneous) release/(maximal − spontaneous) release × 100.

In vivo tumor experiments

Groups of eight C57BL/6 mice were injected in the tail vein with 1×10^4 cells of RMA mock transfectants or RMA B7RP-1 transfectants. Mice were monitored daily and were sacrificed when the tumor burden became excessive, to avoid pain and suffering. To evaluate the tumor growth in the liver and spleen, mice were sacrificed, and livers and spleens were weighed on day 14 after tumor injection. To determine the contribution of NK cells to ICOS-mediated tumor immunity, mice were injected i.p. with anti-NK1.1 Ab (200 μg) or PBS (control) on days −2 and 1; tumors were injected i.v. on day 0. Statistical analysis was performed using a two-sample *t* test.

Results

ICOS expression is increased on cytokine-activated NK cells

To examine the expression of ICOS on NK cells, we stained NK cells from the spleen or liver of C57BL/6 mice using a mouse

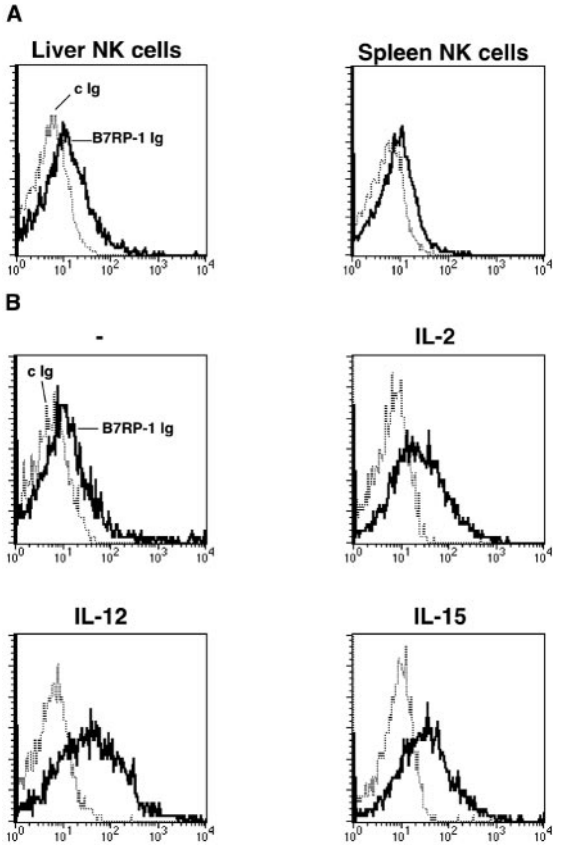

FIGURE 1. ICOS expression on NK cells. *A*, ICOS expression on freshly isolated NK cells. Hepatic and splenic mononuclear cells from C57BL/6 mice were stained with anti-NK1.1 FITC and B7RP-1 human Ig Fc fusion protein (B7RP-1 Ig) or control human Ig (cIg). To detect the binding of B7RP-1 Ig, a PE-conjugated anti-human Ig Ab (anti-human Ig PE) was used as a second-step Ab. The dotted line represents control human Ig staining on $NK1.1^+$ NK cells. The thick line shows ICOS expression on NK cells. Representative data are shown. *B*, ICOS induction on activated NK cells. NK cells were enriched from the spleen by depletion of $CD4^+$, $CD8^+$, and surface Ig^+ cells, and the cells were cultured with IL-2 (4000 U/ml), IL-12 (1 ng/ml), or IL-15 (100 ng/ml) for 24 h. Cells were stained as described in *A*. The dotted line is control human Ig staining, and the thick line represents ICOS expression on $NK1.1^+$ NK cells. Similar results were observed by gating on $DX5^+$ NK cells (data not shown). Representative data are shown.

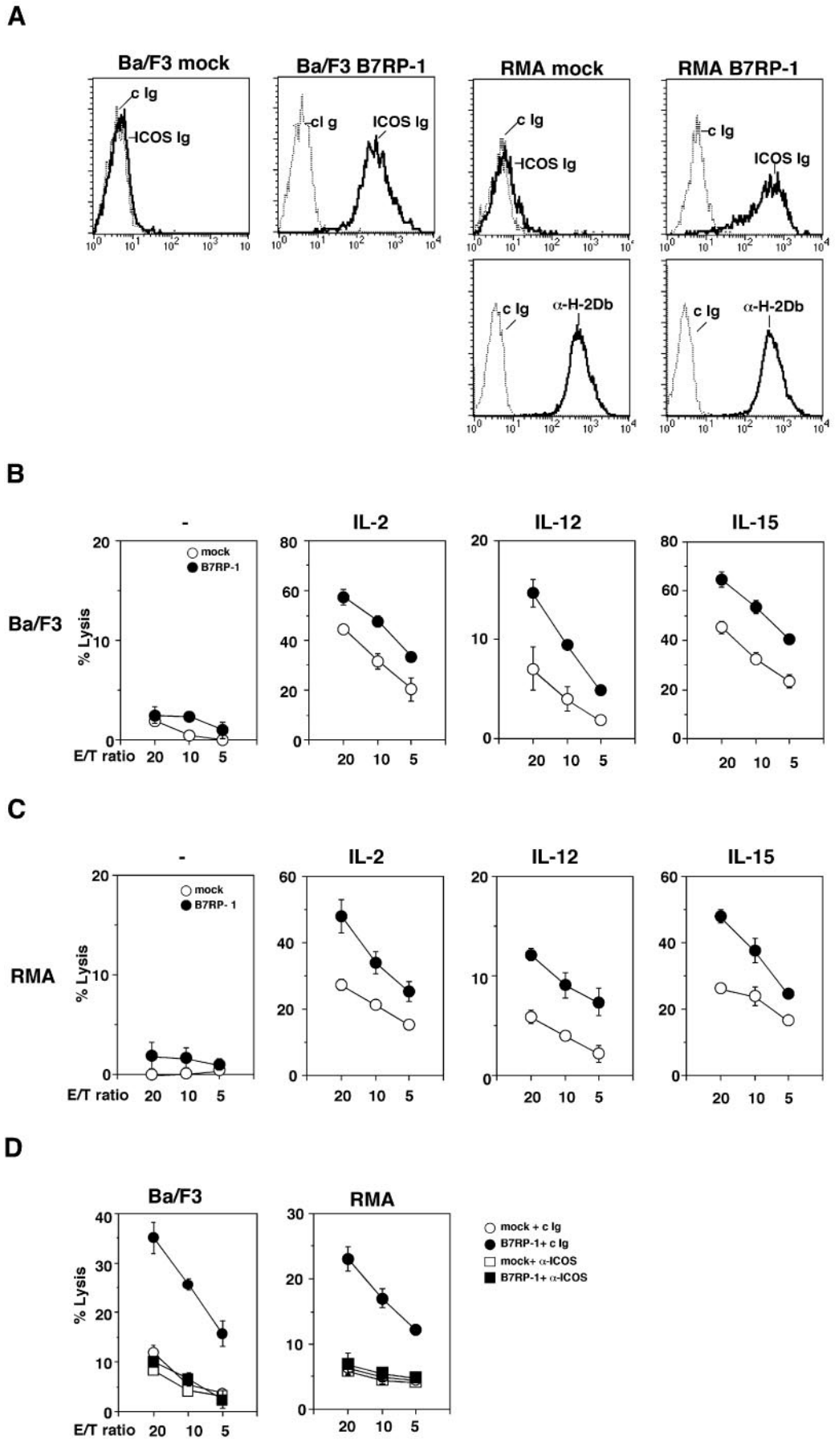

Table I. *ICOS induction on NK cells*

Stimulation	Mean Fluorescence Intensity	
	cIg staining	B7RP-1 staining
Ab cross-linking		
None	5.0	16.1
Anti-NK1.1	5.1	17.0
Anti-CD16	5.5	11.1
Anti-CD28	6.1	18.2
Anti-NK1.1 + anti-CD28	5.0	16.9
Anti-CD16 + anti-CD28	4.8	17.6
Cytokines		
IFN-γ	5.8	13.9
IL-2	6.2	49.0
IL-12	7.4	71.4
IL-15	7.1	51.4
Cells and transfectants		
Mock transfectants	3.8	10.4
B7RP-1 transfectants	4.9	15.4
RAE-1γ transfectants	3.8	11.8
B7RP-1 and RAE-1γ transfectants	5.1	14.4

B7RP-1 human Ig Fc fusion protein. Although ICOS was expressed on fresh NK cells from the spleen and liver, the level was very low (Fig. 1*A*). Since ICOS expression is induced on T cells by TCR stimulation, we investigated whether the activation of NK cells induces ICOS expression. For this purpose, NK cells were stimulated using cytokines or mAbs directed against NK1.1 and CD16, which have been shown to activate NK cells. As expected, ICOS expression was induced on T cells by stimulation with both anti-CD3 mAb and anti-CD28 mAb for 24 h (data not shown). However, ICOS was not induced on NK cells by stimulation with anti-NK1.1 mAb, anti-CD16 mAb, or anti-CD28 mAb. In addition, coculture of NK cells with cells expressing RAE-1γ, a ligand of the NKG2D receptor, also did not induce ICOS on NK cells in vitro (Table I). On the other hand, IL-2, IL-12, and IL-15 stimulation up-regulated ICOS expression on NK cells (Table I and Fig. 1*B*), but not on $CD4^+$ T cells cultured under the same conditions (data not shown). These results indicate that ICOS is induced by cytokine stimulation in NK cells and that the mechanism of ICOS induction in NK cells may be different from that in T cells.

ICOS on activated NK cells enhances their cytolytic activity

To investigate whether the ICOS on NK cells is functional, we tested whether ICOS ligand expression affected the NK cell-mediated killing of tumor targets. To this end, we generated stable transfectants of Ba/F3 (mouse pro-B) cells and RMA (mouse T leukemia) cells expressing B7RP-1 (Fig. 2*A*). H-$2D^b$ is also expressed on RMA cells (Fig. 2*A*), but not on Ba/F3 cells (data not shown). To perform the cytotoxicity assay, we enriched NK cells from spleen by depletion of $CD4^+$, $CD8^+$, and surface Ig^+ cells. The population was >70% NK cells and contained <0.5% $CD8^+$ T cells (data not shown). The cytotoxicity of freshly isolated NK cells against B7RP-1^+ RMA and Ba/F3 transfectants was low, similar to the killing of the parental tumors (Fig. 3*B* and data not shown). Thus, the low expression of ICOS on freshly isolated NK cells apparently did not enhance the cytotoxicity against these tumor targets.

To examine whether ICOS on cytokine-activated NK cells enhances cytotoxicity, we prepared NK cells and induced ICOS up-regulation by culturing them in vitro with IL-2 (4000 U/ml), IL-12 (1 ng/ml), or IL-15 (100 ng/ml). After IL-12 stimulation, the ICOS-dependent cytotoxicity was substantially increased (Fig. 2, *B* and *C*), consistent with the ICOS up-regulation on the effector cells. NK cells activated by IL-2 or IL-15 also showed ~60–70% more cytotoxicity against B7RP-1^+ transfectants than against mock transfectants (Fig. 2, *B* and *C*). Interestingly, the killing of B7RP-1-transfected RMA, which expressed MHC class I, was also elevated when NK cells activated by IL-2, IL-12, and IL-15 were used as effectors (Fig. 2*C*). Similar results were observed against B7RP-1^+ RMA-S (RMA-S is a MHC class I-deficient variant of RMA) transfectants (data not shown).

To confirm that ICOS was responsible for the enhanced cytotoxicity, we examined whether the augmented cytotoxicity was inhibited by an anti-ICOS mAb. To obtain NK cells expressing high levels of ICOS, we purified NK cells and then cultured them with IL-2 (4000 U/ml) for 7 days. ICOS expression on the activated NK cells was increased ~20-fold compared with that on freshly isolated NK cells (data not shown). The anti-ICOS mAb completely inhibited ICOS-dependent cytotoxicity against the Ba/F3 and RMA transfectants (Fig. 2*D*). Therefore, these data indicated that ICOS is involved in the enhancement of cytotoxicity by activated NK cells and suggested that the ICOS-ICOS ligand interactions can permit more efficient killing of MHC class I-bearing tumors, such as RMA.

ICOS augments NKG2D-dependent cytotoxicity

Although the above data revealed that ICOS enhanced the cytotoxicity mediated by activated NK cells, the receptors responsible for the lysis of RMA and Ba/F3 have not been defined. Therefore, we devised a model to determine whether ICOS can function as a costimulatory molecule, working with a known NK receptor. The activating NKG2D receptor is expressed on all NK cells, γδ T cells, and activated $CD8^+$ T cells and augments cytotoxicity

FIGURE 2. ICOS-dependent cytotoxicity mediated by activated NK cells. *A*, Generation of B7RP-1-expressing cell lines. RMA and Ba/F3 cells were transduced with retroviruses containing no cDNA insert or B7RP-1, as described in *Materials and Methods*. Surface expression was detected by staining with mouse ICOS-Ig Fc fusion protein, and cells were analyzed using flow cytometry. The dotted line is control human Ig staining, and the thick line shows B7RP-1 expression. Stable expression of the transgenes by these transfectants was observed. To confirm MHC class I expression on RMA transfectants, RMA and RMA B7RP-1 were stained with PE-conjugated anti-H-$2D^b$ mAb or control mouse Ig. The dotted line shows isotype-control Ig staining, and the thick line is H-$2D^b$-specific staining. *B* and *C*, ICOS-mediated cytotoxicity of activated NK cells against Ba/F3 transfectants (*B*) and RMA transfectants (*C*). NK cells were enriched and cultured with IL-2 (4000 U/ml), IL-12 (1 ng/ml), or IL-15 (100 ng/ml) for 24 h as described in Fig. 1*B*. Cytotoxic activity against B7RP-1-bearing target cells (●) or mock transfectants (○) was tested using a 4-h ^{51}Cr release assay. Data are presented as the mean percent cytotoxicity ± SD (determined in triplicate). Similar results were obtained in two independent experiments. *D*, ICOS-dependent cytotoxicity was blocked by an anti-ICOS mAb. Purified NK cells were cultured for 7 days in IL-2 and then preincubated with an anti-ICOS mAb (rat IgG1 isotype) or an isotype-control rat IgG1 for 30 min before addition of radiolabeled target cells. The anti-ICOS mAb (■ and □) or the rat control IgG1 (● and ○) was present throughout the cytotoxic assay. The final Ig concentration was 10 μg/ml. Cytotoxicity was measured by ^{51}Cr release after 4 h. Target cells were as follows: Ba/F3 mock with rat control IgG1 (○), Ba/F3 mock with anti-ICOS mAb (□), Ba/F3 B7RP-1 with rat control IgG1 (●) and Ba/F3 B7RP-1 with anti-ICOS mAb (■), RMA mock with rat control IgG1 (○), RMA mock with anti-ICOS mAb (□), RMA B7RP-1 with rat control IgG1 (●), and RMA B7RP-1 with anti-ICOS mAb (■). Data are presented as the mean percent cytotoxicity ± SD (triplicate determinations). Similar results were obtained in two independent experiments.

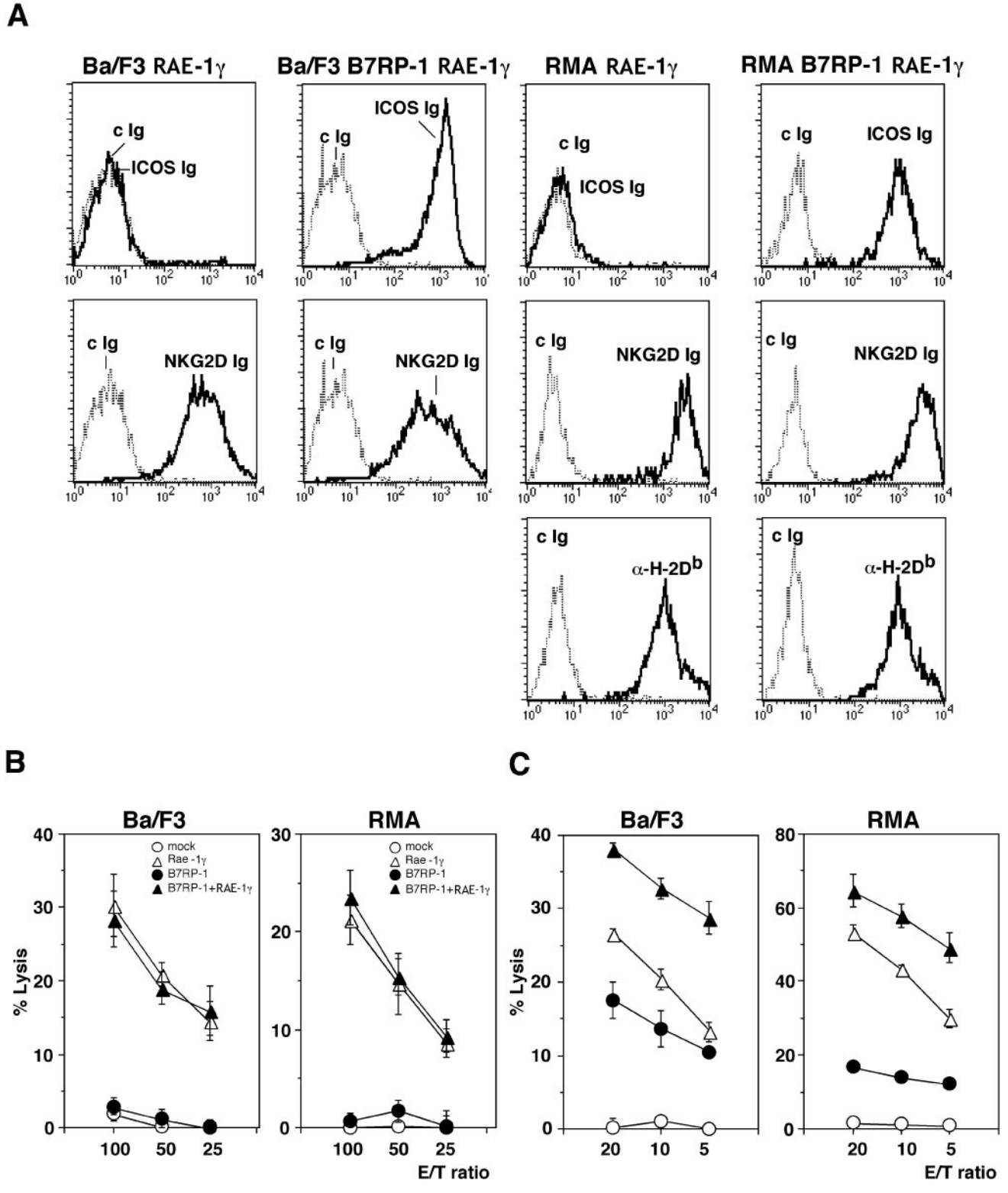

FIGURE 3. ICOS on activated NK cells augments NKG2D-dependent cytotoxicity. *A*, Generation of B7RP-1- and RAE-1γ-expressing target cells. RMA and Ba/F3 transfectants were generated as described in *Materials and Methods*. B7RP-1 and H-2D^b expression were detected as described in Fig. 2*A*. Stable expression of B7RP-1 and RAE-1γ was observed on these transfectants. *B*, ICOS did not augment the NKG2D-dependent cytotoxicity mediated by freshly isolated, resting NK cells. NK cells were enriched from the spleen by depletion of CD4$^+$, CD8$^+$ surface Ig$^+$ B cells, as described previously. The cytotoxicity of enriched NK cells against B7RP-1-expressing target cells (●), mock transfectants (○), RAE-1γ transfectants (△), and B7RP-1- and RAE-1γ-expressing cells (▲) was evaluated using a ^{51}Cr release assay. Representative data are shown. These experiments were repeated twice with similar results against Ba/F3 and RMA transfectants, respectively. *C*, Cooperative effect of ICOS-and NKG2D-dependent cytotoxicity mediated by activated NK cells. NK cells were purified from spleen and were cultured with IL-2 (4000 U/ml) for 7–10 days, as described in *Materials and Methods*. The purity of NK1.1$^+$ cells was >95% in all experiments. Cytotoxic activity was measured using a ^{51}Cr release assay. Target cells are as follows: B7RP-1 transfectants (●), mock transfectants (○), RAE-1γ transfectants (△), and B7RP-1- and RAE-1-expressing cells (▲). The results were reproducible, and representative data are shown.

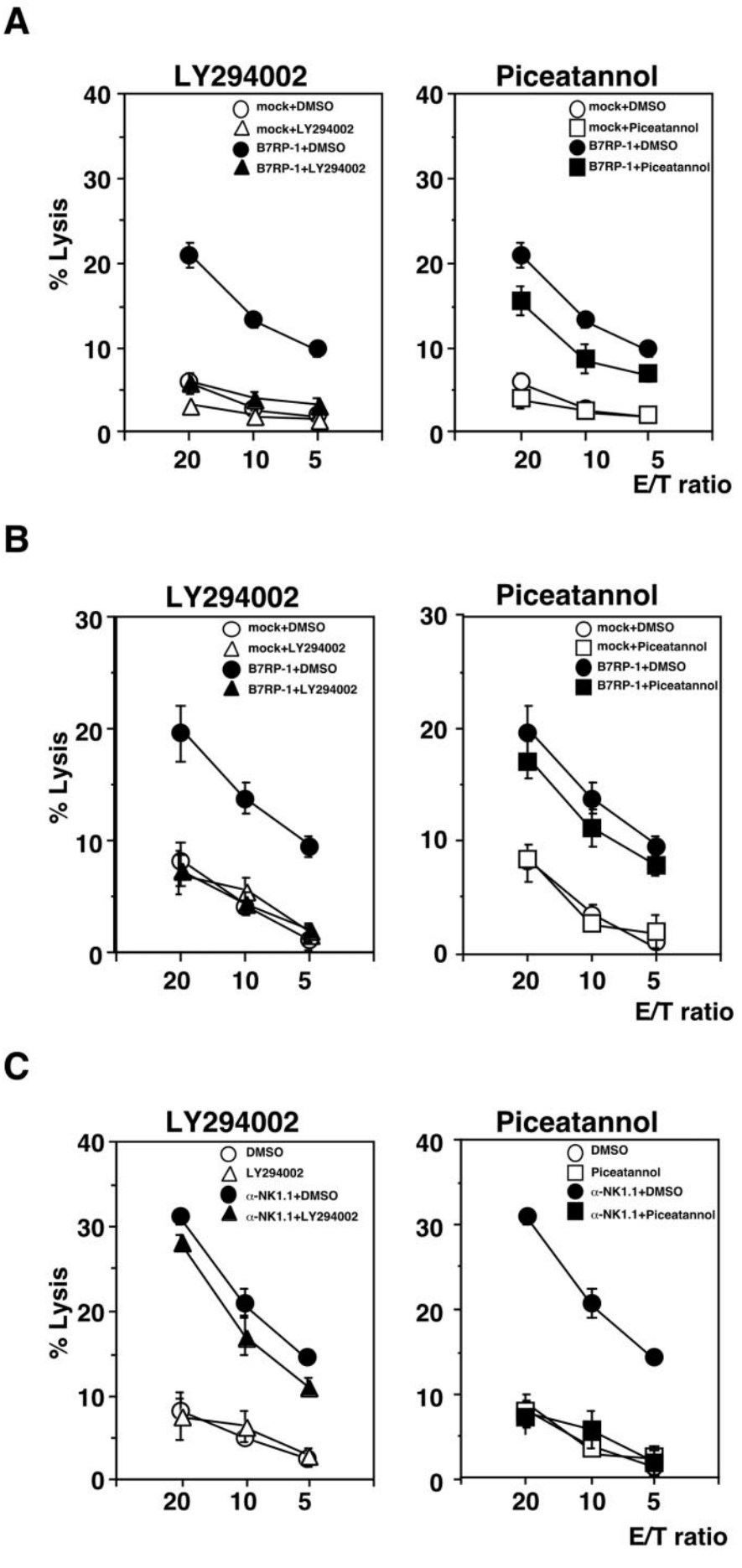

FIGURE 4. PI3 kinase activity is necessary for ICOS-dependent cytotoxicity. *A* and *B*, ICOS-dependent cytotoxicity of NK cells requires PI3 kinase activity. Purified, IL-2-activated NK cells were precultured with DMSO (control), LY294002 (20 or 50 μM), or piceatannol (20 or 50 μM) for 30 min and then were mixed with target cells. Cytotoxic assays were performed in the presence of kinase inhibitors (final concentration, 10 or 25 μM) or DMSO (vehicle control). *A*, Target cells are as follows: Ba/F3 mock with DMSO (○), Ba/F3 mock with LY294002 (△), Ba/F3 B7RP-1 with DMSO (●), Ba/F3 B7RP-1 with LY294002 (▲), Ba/F3 mock with DMSO (○), Ba/F3 mock with piceatannol (□), Ba/F3 B7RP-1 with DMSO (●), and Ba/F3 B7RP-1 with piceatannol (▲). *B*, Target cells are as follows: P815 mock with DMSO (○), P815 mock with LY294002 (△), P815 B7RP-1 with DMSO (●), P815 B7RP-1 with LY294002 (▲), P815 mock with DMSO (○), P815 mock with piceatannol (□), P815 B7RP-1 with DMSO (●), and P815 B7RP-1 with piceatannol (▲). *C*, Syk family kinase activity is essential for anti-NK1.1 mAb-dependent cell cytotoxicity. Purified, IL-2-activated NK cells were precultured with anti-NK1.1 mAb for 30 min in the presence of PI3 or Syk kinase inhibitors. The kinase inhibitor treatment was performed as described in *A* and *B*. Cytotoxic assays were performed in the presence of anti-NK1.1 mAb and kinase inhibitors. Target cells are as follows: P815 mock with DMSO (○), P815 mock with LY294002 (△), P815 mock with anti-NK1.1 mAb and DMSO (●), P815 mock with anti-NK1.1 mAb and LY294002 (▲), P815 mock with DMSO (○), P815 mock with piceatannol (□), P815 mock with anti NK1.1 mAb and DMSO (●), and P815 mock with anti-NK1.1 mAb and piceatannol (■). These data are presented as the mean percent cytotoxicity ± SD (triplicate determinations). Data from the cytotoxicity assays using cells treated with the kinase inhibitors at 10 μM (final concentration) are shown. Similar results were obtained using the kinase inhibitors at 25 μM and in two independent experiments.

against tumors expressing NKG2D ligands: MICA/B, ULBP in humans, and RAE-1α, -β, -γ, -δ, and -ϵ and H-60 in mice (27–30). To address whether ICOS augments NKG2D-dependent cytotoxicity, we established transfectants of RMA cells or Ba/F3 cells expressing both RAE-1γ and B7RP-1. RAE-1γ and B7RP-1 were highly expressed on these transfectants (Fig. 3*A*). As shown in Fig. 3*B*, freshly isolated NK cells efficiently killed RAE-1γ-expressing RMA cells, although the RMA cells expressed MHC class I. However, B7RP-1 on Ba/F3 and RMA transfectants expressing RAE-1γ did not enhance the cytotoxicity (Fig. 3*B*), possibly because resting NK cells express only minimal levels of ICOS. In contrast, cytokine-activated NK cells, which express ICOS, demonstrated remarkably higher lysis of targets with both the ICOS ligand and RAE-1γ (Fig. 3C). These cooperative effects of ICOS and NKG2D were observed using both Ba/F3 and RMA transfectants. These results suggest that ICOS functions as a costimulator of the NKG2D-dependent cytotoxicity mediated by activated NK cells.

PI3 kinase activity is essential for ICOS-dependent cytotoxicity

PI3 kinase and the Syk family kinases have been implicated in different NK cell functions (31). Since ICOS possesses a YxxM motif (12) in its cytoplasmic region, which recruits the p85 subunit of PI3 kinase, it is likely that the ICOS-dependent cytotoxicity mediated by NK cells uses the PI3 kinase signaling pathway. We conducted cytotoxicity assays using a specific inhibitor of PI3 kinase, LY294002, and as a control the Syk family kinase inhibitor, piceatannol. As expected, ICOS-dependent cytotoxicity by activated NK cells was completely abrogated by LY294002 (Fig. 4*A*). In contrast, piceatannol had only a minimal effect on ICOS-dependent cytotoxicity (Fig. 4*A*). Similar results were observed using another target cell, B7RP-1$^+$ P815 transfectants (Fig. 4*B*). In contrast, using P815 transfectants as targets, the anti-NK1.1 Ab-dependent, cell-mediated cytotoxicity was inhibited by piceatannol, but not by LY294002 (Fig. 4*C*). These results indicated that ICOS-dependent cytotoxicity employed the PI3 kinase pathway, whereas the signaling induced by anti-NK1.1 mAb used the Syk family kinase pathway. Therefore, PI3 kinase activity is essential for ICOS-dependent cytotoxicity.

ICOS cross-linking promotes IFN-γ production from activated NK cells

ICOS is known to promote the development of CD4$^+$ Th cells through the induction of IL-4, IL-10, and IL-13 (11, 12, 14, 15, 22). ICOS also induces IFN-γ production by activated T cells (20, 32–34). These findings prompted us to examine whether ICOS induces cytokine production by NK cells. These experiments showed that IFN-γ was produced by IL-2-activated NK cells after cross-linking with an anti-ICOS mAb (Fig. 5*A*). However, the amount of IFN-γ induced by anti-ICOS mAb stimulation was 5 times less than that induced by anti-NK1.1 mAb stimulation (Fig. 5*A*). To exclude the possibility that IFN-γ was induced through interactions between the anti-ICOS mAb and the Fc receptors on NK cells, we performed additional experiments using NK cells from FcRγ-deficient mice. The FcRγ-deficient NK cells, despite lacking CD16 expression, also produced IFN-γ after stimulation with the anti-ICOS mAb (data not shown). Thus, ICOS not only enhances cytotoxicity, but also induces IFN-γ production, in cytokine-activated NK cells.

To determine whether PI3 kinase activity is necessary for ICOS-dependent IFN-γ production from NK cells, experiments were performed in the presence of the PI3 kinase inhibitor, LY294002. As shown in Fig. 5*B*, LY294002 weakly inhibited IFN-γ production from activated NK cells induced by anti-NK1.1 mAb stimulation.

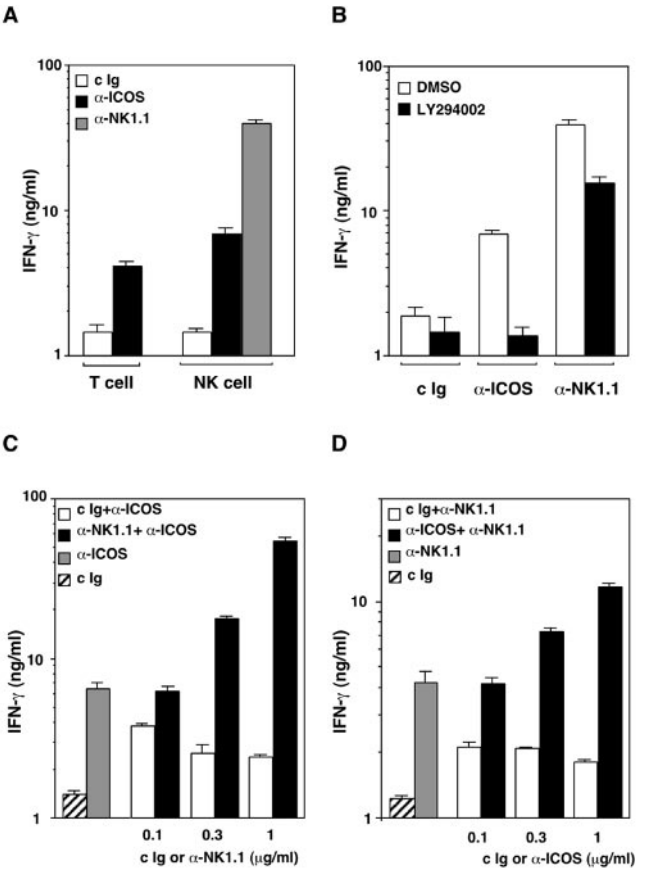

FIGURE 5. IFN-γ is produced by activated NK cells after cross-linking with anti-ICOS mAb. *A*, ICOS stimulation promotes IFN-γ production from activated NK cells. Activated T cells and NK cells were stimulated with immobilized mAb (1 μg/ml) for 18 h. Conditions were as follows: anti-ICOS mAb (■), rat control IgG1 (□), and anti-NK1.1 mAb (▨). Culture supernatants were collected for the measurement of IFN-γ by ELISA. *B*, PI3 kinase activity is essential for ICOS-dependent IFN-γ production. IL-2-activated NK cells were stimulated with immobilized mAb (1 μg/ml) and cultured with PI3 kinase inhibitor LY294002 (10 μM final concentration; ■) or DMSO (□) for 18 h. Culture supernatants were collected for measurement of IFN-γ by ELISA. *C* and D, Anti-ICOS mAb and anti-NK1.1 stimulation cooperate in the production of IFN-γ from IL-2-activated NK cells. Purified, IL-2-activated NK cells were stimulated with immobilized anti-ICOS and/or anti-NK1.1 mAbs, as indicated, for 18 h. *C*: ▨, The amount of IFN-γ produced by NK cells cultured only with plates coated with control rat IgG; ▨, the amount of IFN-γ induced by stimulation with anti-ICOS mAb (1 μg/ml); □, the amount of IFN-γ induced by stimulation with anti-ICOS mAb (1 μg/ml) together with rat control IgG at the indicated concentrations (0.1, 0.3, and 1 μg/ml); ■, the amount of IFN-γ produced by stimulation with anti-NK1.1 mAb at the indicated concentration (0.1, 0.3, and 1 μg/ml) together with anti-ICOS mAb (1 μg/ml). *D*: □, the amount of IFN-γ produced by stimulation with rat control IgG1 (the concentrations are indicated) and anti-NK1.1 mAb (0.3 μg/ml); ■, the amount of IFN-γ produced by the stimulation with anti-ICOS mAb (at the concentrations indicated) together with anti-NK1.1 mAb (0.3 μg/ml); ▨, the amount of IFN-γ produced by stimulation with only anti-NK1.1 mAb (0.3 μg/ml); ▨, the amount of IFN-γ produced by NK cells cultured only with rat control IgG1 (0.3 μg/ml).

In contrast, LY294002 completely blocked IFN-γ production from activated NK cells induced by anti-ICOS mAb stimulation. Therefore, PI3 kinase activity is essential for ICOS-dependent IFN-γ production from activated NK cells.

Furthermore, to investigate whether ICOS augments the IFN-γ production induced by anti-NK1.1 mAb cross-linking, we measured the amount of IFN-γ secreted by NK cells stimulated with

both anti-NK1.1 and anti-ICOS mAbs. As shown in Fig. 5, *C* and *D*, ICOS enhanced IFN-γ production from activated NK cells in a dose-dependent manner. Thus, ICOS functions as a costimulator of IFN-γ production by NK cells. Taken together, ICOS promotes IFN-γ production and cytotoxicity through the PI3 kinase pathway.

Expression of ICOS ligand slows tumor cell growth in vivo

To examine whether ICOS contributes to tumor immunity in vivo, mice were injected with either the B7RP-1$^+$ RMA tumor (RMA B7RP-1) or the RMA mock transfectants (RMA mock). As shown in Fig. 6*A*, the survival of mice injected with RMA B7RP-1 cells was significantly prolonged, by ~6 days, compared with that of mice inoculated with the RMA mock transfectants ($p < 0.001$). We sacrificed moribund mice to examine the cause of death and observed extensive growth of the tumors in the liver and spleen (data not shown). To examine whether B7RP-1 influences the growth of the tumor in liver and spleen, we sacrificed tumor-bearing mice on day 14 and measured liver and spleen weights (Fig. 6*B*). The average weight of the livers from mice injected with RMA mock tumors was about twice that of normal liver. In contrast, the average weight of the livers from mice inoculated with RMA B7RP-1 was about the same as that of normal liver. Similar results were obtained by examining the spleen (Fig. 6*B*). These findings indicate that ICOS may slow the growth and metastasis of tumors expressing B7RP-1. Furthermore, to examine whether NK1.1-positive cells are involved in slowing the growth of the B7RP-1$^+$ RMA tumors, we performed tumor experiments using mice depleted of NK cells by treatment with anti-NK1.1 mAb. As shown in Fig. 6*C*, when mice were depleted of NK1.1$^+$ cells, the growth of RMA B7RP-1 tumor cells in the spleen and liver was equivalent to the growth of mock-transfected RMA cells. These results indicated that NK1.1$^+$ cells (i.e., NK cells and/or NK-T

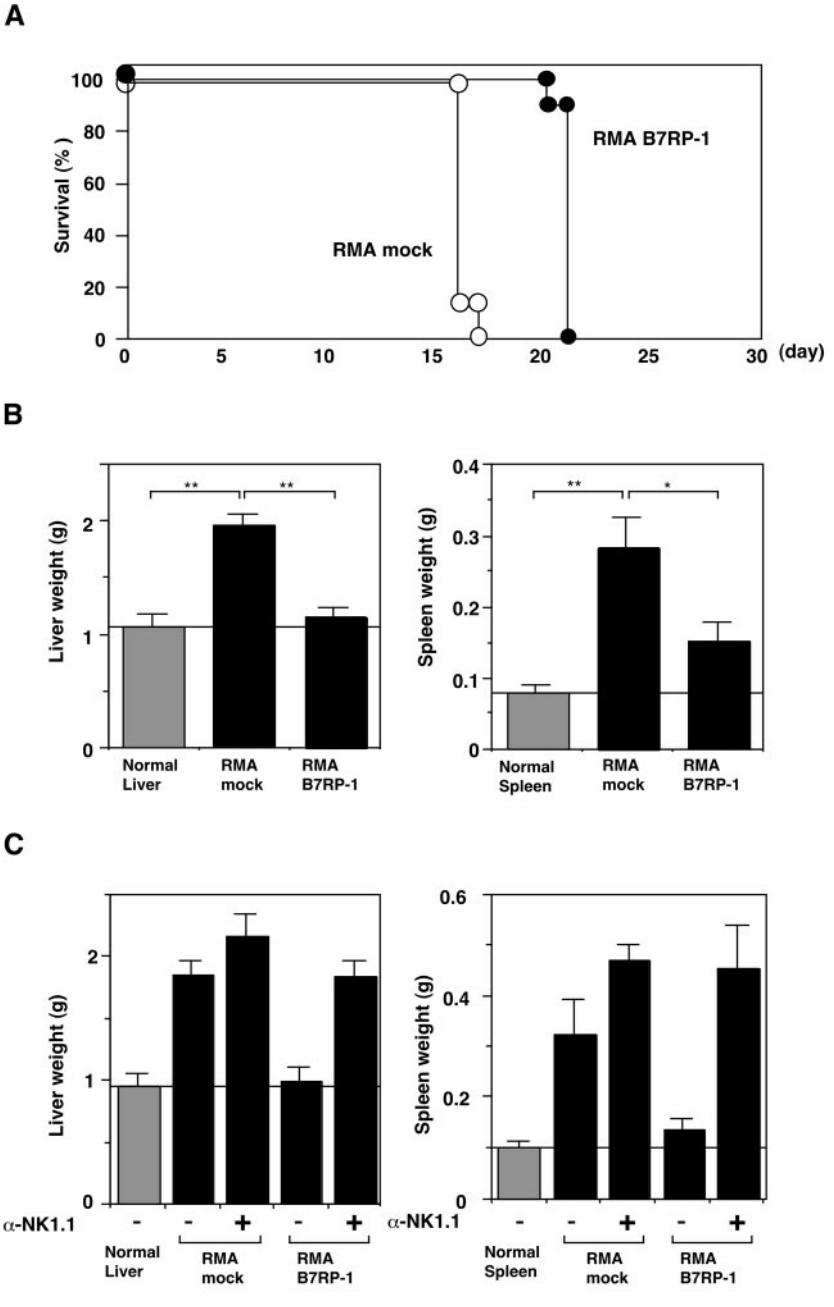

FIGURE 6. The expression of ICOS ligand slows tumor cell growth in vivo. *A*, Survival is prolonged in mice bearing B7RP-1-transduced tumors. C57BL/6 mice were injected in the tail vein with 1×10^4 RMA mock (○) or RMA B7RP-1 tumor cells (●). Survival (*y*-axis) indicates the date when animals were sacrificed due to excessive tumor burden. Statistical analysis was performed by using a log-rank test ($p < 0.001$). Survival data from a representative experiment with eight animals per group are shown. *B*, ICOS ligand expression inhibits tumor growth in liver and spleen. C57BL/6 mice were injected i.v. with 5×10^4 tumor cells. On day 14 tumor-bearing mice were sacrificed, and liver and spleen weights were determined. The mean weight ± SD ($n = 5$) for each group are presented. ▨, Weights of normal liver and spleen. Differences between mice injected with mock and B7RP-1 RMA tumors were highly significant: ∗, $p < 0.01$; ∗∗, $p < 0.001$. *C*, NK1.1$^+$ cells are involved in the inhibition of tumor growth by ICOS in the liver and spleen. C57BL/6 mice were injected i. p. with anti-NK1.1 Ab (200 μg) or PBS on days −2 and 1. On day 0, 1×10^4 RMA mock or RMA B7RP-1 tumor cells were injected i.v. in anti-NK1.1 Ab- or PBS-treated mice. On day 14 tumor-bearing mice were sacrificed, and liver and spleen weights were determined. The mean weight ± SD (n = 5) for each group are presented. Differences between the experimental groups were statistically significant: normal liver vs RMA mock, $p < 0.001$; normal liver vs RMA mock and anti-NK1.1, $p < 0.001$; normal liver vs RMA B7RP-1 and anti-NK1.1, $p < 0.001$; RMA mock vs RMA B7RP-1, $p < 0.001$; RMA B7RP-1 vs RMA B7RP-1 and anti-NK1.1, $p < 0.001$; normal spleen vs RMA mock, $p < 0.001$; normal spleen vs RMA mock and anti-NK1.1, $p < 0.001$; normal spleen vs RMA B7RP-1 and anti-NK1.1, $p < 0.001$; RMA mock vs RMA B7RP-1, $p < 0.001$; RMA B7RP-1 vs RMA B7RP-1 and anti-NK1.1, $p < 0.001$.

cells) are involved in slowing the growth of B7RP-1$^+$ RMA tumors in vivo.

Discussion

In this study we demonstrated that ICOS augmented the functions of activated mouse NK cells, but not resting NK cells, probably because resting NK cells express only low levels of ICOS. Similarly, ICOS is not present on naive T cells (11), but is up-regulated by TCR stimulation and retained on memory T cells (11). ICOS was induced on NK cells by cytokines such as IL-2, IL-12, and IL-15. Notably, IL-12 can also induce CD28 on mouse NK cells and permit CD28-dependent cytotoxicity (9). Thus, cytokines may be necessary to induce or up-regulate the expression of certain costimulatory molecules for the full activation of NK cells.

We found that activated NK cells expressing ICOS efficiently killed RMA cells transduced with B7RP-1. Prior studies have established that RMA cells are protected against attack by NK cells because they express MHC class I, which engage inhibitory NK cell receptors (3). Our results suggest that the ICOS-dependent cytotoxicity may permit more efficient killing of class I-bearing tumors. Recent studies have shown that the stimulation of NK cells through NKG2D also allows NK cells to kill class I-positive tumors (35, 36); however, the effect of ICOS-dependent NK cell stimulation was less than that of NKG2D-dependent activation. In particular, resting freshly isolated NK cells killed RAE-1γ-transfected MHC class I-bearing RMA tumors (Fig. 3*B*), whereas ICOS-dependent cytotoxicity was evident only using cytokine-activated NK cells. This may be explained by the observation that NKG2D is constitutively expressed on all NK cells, whereas ICOS is expressed at low levels on resting NK cells. Therefore, these results suggest that the level of these receptors on NK cells may be important for their function.

The augmentation of NK cell-mediated cytotoxicity against ICOS ligand-bearing tumors might be due to either enhanced adhesion to target cells or signal transduction through ICOS. We demonstrated that adhesion alone is unlikely to be responsible for the enhanced killing, because the ICOS-dependent cytotoxicity required activating signals through PI3 kinase, which were blocked by a specific inhibitor. Two major activating signaling pathways in NK cells have been reported (31). One pathway uses PI3 kinase and the other uses the Syk family kinases, Syk and ZAP-70. ICOS and CD28 have a YxxM motif in their cytoplasmic regions, which upon tyrosine phosphorylation bind p85 PI3 kinase (12). NKG2D is associated with DAP10, which also has a YxxM motif (30). Thus, ICOS, CD28, and NKG2D may use a common signaling pathway. Interestingly, ICOS enhanced NKG2D-dependent cytotoxicity, although these receptors may use a common pathway. One possibility is that the stimulation of either receptor alone may result in suboptimal PI3 kinase activation. The relatively stronger signaling by NKG2D compared with ICOS might be due to their different signaling properties. Activation of the DAP10 adapter protein associated with NKG2D recruits both p85 PI3 kinase and Grb2 (37), similar to the ability of CD28 to bind these signaling molecules. By contrast, activated ICOS apparently binds to p85 PI3 kinase, but not Grb2 (12).

We found that the survival of mice injected i.v. with RMA B7RP-1 cells was significantly prolonged compared with that of mice inoculated with RMA mock transfectants. When tumors (B16, RMA, EL-4, and others) are injected i.v., the growth of the tumors in the spleen and liver may be inhibited by NK cells and NK-T cells (38–41). This process may be more efficient if the tumor expresses an ICOS ligand. In support of this concept, we have shown that activated NK cells and NK-T cells (data not shown) express ICOS, and that ICOS augmented the functions of activated NK cells in vitro (Figs. 2, 3, and 5). Therefore, our findings strongly suggest that NK cells and NK-T cells are the effector cells in the suppression of growth of ICOS ligand-bearing tumor in vivo. The suppressed growth of B7RP-1-transfected RMA tumor cells in the spleen and liver was abrogated by the depletion of NK1.1$^+$ cells in the tumor-bearing mice. Recent studies have also reported that ICOS costimulation can induce CD8$^+$ T cell memory (42, 43) and can enhance the anti-tumor responses mediated by CD8$^+$ T cells. Prior studies have shown that RMA tumors transfected with CD80 (B7.1) regress and induce CD8$^+$ T memory T cells (44).

What induces ICOS on NK cells in vivo? We have demonstrated ICOS-dependent cytotoxicity using NK cells from mice that were injected with Poly I:C or LPS (K. Ogasawara, unpublished data). These are potent inducers of cytokines; however, presently we do not know which host factors are involved in the ICOS induction of NK cells in vivo. Furthermore, how a tumor might up-regulate ICOS expression on NK cells in a physiological setting has not been addressed.

Our findings and other recent studies suggest the possibility that ICOS and NKG2D may both participate in the elimination of tumor cells by NK cells and may induce tumor-specific CD8$^+$ memory T cells. Since many tumors express NKG2D ligands (28, 29, 45), and certain tumors, especially leukemias, express the ICOS ligand (46), these costimulatory receptors may play important roles in immune surveillance against cancer.

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