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CD27, a member of the TNF receptor superfamily, has been implicated in T cell activation, T cell development, and T cell-dependent Ab production by B cells. In the present study we examined the expression and function of CD27 on murine NK cells. Murine NK cells constitutively expressed CD27 on their surface. Stimulation with immobilized anti-CD27 mAb or murine CD27 ligand (CD70) transfectants solely could induce proliferation and IFN-γ production of freshly isolated NK cells and enhanced the proliferation and IFN-γ production of anti-NK1.1-simulated NK cells. Although NK cell cytotoxicity was not triggered by anti-CD27 mAb or against CD70 transfectants, pretreatment via CD27 enhanced the cytotoxic activity of NK cells in an IFN-γ-dependent manner. These results suggest that CD27-mediated activation may be involved in the NK cell-mediated innate immunity against virus-infected or transformed cells expressing CD70. The Journal of Immunology, 2000, 164: 1741–1745.

Abs and reagents

Purified anti-CD16/32 mAb (2.4 G2), FITC-conjugated or no azide/low endotoxin anti-mouse NK1.1 mAb (PK 136), biotin-conjugated or no azide/low endotoxin anti-mouse CD27 mAb (LG.3A10), peridinin chlorophyll protein (PerCP)-conjugated anti-mouse CD3e mAb (145-2C11), biotin-conjugated anti-mouse CD30 mAb (mCD30.1), biotin-conjugated anti-mouse CD40 mAb (3/23), biotin-conjugated anti-mouse 4-1 BB mAb (1A2), no azide/low endotoxin anti-mouse CD70 mAb (FR-70), isotype control for hamster IgG (G235-2356), rat IgGl (R3-34), rat IgG2a (R35-95) and rat IgG2b (R95-1), and PE-conjugated streptavidin were purchased from Pharmingen (San Diego, CA). The hybridoma-producing anti-mouse OX-40 mAb (OX-86) was obtained from European Cell Culture Collection (Wilshire, U.K.), and the hybridomas producing mouse anti-IFN-γ-neutralizing mAb (R4-6A2), anti-mouse MHC class II mAb (M5/114), anti-mouse B220 mAb (RA3-3A1), anti-mouse CD8 mAb (3.155), and anti-mouse CD4 mAb (RL172.4) were obtained from American Type Culture Collection (Manassas, VA). The mAbs were prepared and biotinylated in our laboratory followings described standard method (13). Human RIL-2 was provided by Shionogi Pharmaceutical (Osaka, Japan). Recombinant murine IL-12 (4.9 × 10^6 U/mg) was provided by Genetic Institute (Andover, MA).

Cell preparation

Splenocyte mononuclear cells were prepared by pressing spleens through a stainless steel mesh followed by treatment with RBC lysis solution as previously described (14). NK cells were purified as previously described (8). In brief, B and T cells were depleted from splenocytes by passage through a nylon wool column (Wako, Osaka, Japan) and then by treatment with a mixture of hybridoma supernatants (anti-MHC class II, anti-B220, anti-CD4, and anti-CD8) and low toxic rabbit complement (Cedarlane, Hornby, Canada). After Percoll (Pharmacia Biotech) gradient centrifugation, the purity of NK (NK1.1^+ CD3^-)cells was >95% as determined by flow cytometry.

Cell lines

NK-susceptible YAC-1 target cells and NK-resistant P815 target cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 25 mM NaHCO3 in humidified 5% CO2 at 37°C. P815 cells stably transfected with mouse CD70 cDNA (P815-CD70) were prepared as previously described (15).

Flow cytometry

Expression of respective molecules on NK cells was analyzed by three-color flow cytometry as described previously (14, 16). To avoid the non-specific binding of Abs to FcγR on NK cells, the cells were preincubated with anti-mouse CD16/32 (2.4G2) mAb before staining, which diminished

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Materials and Methods

Mice

Male C57BL/6 (B6) mice, 6 wk of age, were purchased from Clear Japan (Tokyo, Japan). B6 RAG-2^-/^- mice were bred in Central Institute for Experimental Animals (Kawasaki, Japan) and used at 6–8 wk of age (12). All mice were maintained under specific pathogen-free conditions.

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3 Abbreviations used in this paper: PerCP, peridinin chlorophyll protein; TRAF, TNF receptor-associated factor.
the binding of isotype controls to NK cells. Then the cells were incubated with a saturating amount of biotinylated mAbs. After washing with PBS twice, the cells were incubated with FITC-conjugated anti-NK1.1 mAb, PerCP-conjugated anti-CD3 ε mAb, and PE-conjugated streptavidin. Expression of respective molecules on NK1.1 + CD3 ε cells was analyzed by flow cytometry. Solid lines indicate the staining with respective mAb, and broken lines indicate the staining with isotype-matched control IgG.

Cell culture
Spleen cells from B6 RAG-2−/− mice were incubated in a tissue culture grade polystyrene dish at 37°C for 1 h to deplete adherent cells. Harvested cells were routinely >95% NK1.1 + CD3 ε 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 NaHCO 3 in humidified 5% CO 2 at 37°C as previously reported (17). NK cell (3 × 10 5 ) were stimulated with immobilized anti-NK1.1 mAb (10 μg/ml) and/or anti-CD27 mAb (10 μg/ml) in the presence or the absence of IL-2 (25 U/ml) and/or IL-12 (5 U/ml) on a 96-well flat-bottom culture plate (Costar, Cambridge, MA). Abs in PBS were immobilized by overnight incubation at 4°C. In the coculture experiments, purified NK cells (3 × 10 7 /well) were cocultured with irradiated (15,000 rad) P815 or P815-CD70 cells (3 × 10 5 /well) in 96-well round-bottomed culture plate in the presence of anti-NK1.1 mAb (10 μg/ml) and/or anti-CD70 mAb (5 μg/ml). The cultures were incubated for 2 days and pulsed with 0.5 μCi/well of [ 3 H]thymidine (Amersham, Aylesbury, U.K.) for the last 16 h. Incorporated radioactivity was measured in a Microbeta counter (Microbeta Plus, Wallac, Turku, Finland). Cell-free culture supernatants were harvested after incubation for 3 days and subjected to cytokine assay.

ELISA
IFN-γ levels in the culture supernatants were evaluated by using a mouse IFN-γ-specific ELISA kit (OptEIA) purchased from PharMingen according to the manufacturer’s instruction.

Cytotoxicity assay
Cytotoxic activity was assessed by a standard 51 Cr release assay (14). Briefly, 51Cr-labeled target cells (5 × 10 5 ) were added to serial dilutions of effector cells. For assessing redirected lysis, anti-NK1.1 mAb or anti-CD27 mAb were added at 10 μg/ml. After 4-h incubation, supernatants were harvested and counted with a gamma counter. Specific cytotoxicity was calculated as previously described (14).

Results
Constitutive expression of CD27 on murine NK cells
To examine the expression of TNF receptor family molecules on NK cells, freshly isolated splenic NK cells from B6 mice and IL-2-activated NK cells were prepared from C57BL/6 splenocytes by depletion of B and T cells, and then stimulated with 500 U/ml IL-2 for 7 days. After Fc receptor blocking, fresh NK and IL-2-stimulated NK cells were stained with FITC-conjugated anti-NK1.1 mAb, PerCP-conjugated anti-CD3 ε mAb, and biotin-conjugated mAb against CD27, CD30, CD40, OX40(CD134), or 4-1BB(CD137) followed by PE-conjugated streptavidin. Expression of the respective TNF receptor family molecule was analyzed on gated NK1.1 + CD3 ε cells. While CD30, CD40, OX40, and CD134 were not detectable on either fresh or IL-2-activated NK cells, CD27 was constitutively expressed on both fresh and IL-2-activated NK cells (Fig. 1). As recently reported (18), CD137 was expressed on IL-2-activated NK cells, but not on fresh NK cells. These results indicated that CD27 is unique among the TNF receptor family molecules tested in that it is constitutively expressed on freshly isolated NK cells.

Proliferation of NK cells upon cross-linking of CD27
Since CD27 has been reported to provide a costimulatory signal for T cell proliferation (15, 19–24), the effect of CD27 cross-linking by immobilized anti-CD27 mAb on the proliferation of NK cells was first examined. To exclude the possible contribution of...
contaminated T cells, NK cells were purified from B6 RAG-2−/− splenocytes, which lack T cells and NKT cell (12). RAG-2−/− NK cells expressed CD27 at the same level as the B6 NK cells shown in Fig. 1 (data not shown). The cross-linking of CD27 induced a low, but significant, level of proliferation, which was comparable to that induced by NKR-P1 cross-linking by immobilized anti-NK1.1 mAb (Fig. 2A). Co-cross-linking of CD-27 and NKR-P1 showed an additive effect. In the presence of a suboptimal concentration of IL-2, anti-CD27 mAb also induced a substantial level of proliferation, albeit to a lesser extent than anti-NK1.1 mAb, and enhanced the proliferation induced by anti-NK1.1 mAb (Fig. 2B). These results suggested that CD27 expressed on freshly isolated NK cells is functional in inducing NK cell proliferation.

**IFN-γ production by NK cells upon cross-linking of CD27**

We next examined the IFN-γ induction by NK cells upon CD27 cross-linking with anti-CD27 mAb. When NK cells were incubated with immobilized anti-CD27 mAb in the presence of 25 U/ml IL-2 for 3 days, a substantial level of IFN-γ was detected in the culture supernatant, which was comparable to that induced by anti-NK1.1 mAb (10 μg/ml) in the presence of 25 U/ml IL-2 without (A) or with (B) 5 U/ml IL-12. Cell-free culture supernatants were collected from the cultures after 3 days, and IFN-γ was measured by ELISA. Data are expressed as the mean ± SD of triplicate cultures. Data shown are representative of three independent experiments with similar results.

**CD27 ligand (CD70) stimulates NK cells to proliferate and to produce IFN-γ**

To exclude the possible contribution of Fc receptors on NK cells to the NK cell activation by immobilized mAb, we determined whether the natural ligand for CD27 (CD70) could induce the proliferation and IFN-γ production of NK cells. Purified NK cells from RAG-2−/− mice were cocultured with irradiated P815 or CD70-transfected P815 cells in the presence or the absence of anti-NK1.1 mAb (10 μg/ml) and/or anti-CD70 mAb (5 μg/ml) with 25 U/ml IL-2. After 2 days, the proliferative response was assessed by pulsing the culture with 0.5 μCi/well of [3H]thymidine ([3H]-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 ± SD of triplicate cultures. Data shown are representative of three independent experiments with similar results.

**CD27 cannot trigger NK cell cytotoxicity**

It has been reported that cross-linking of NK1.1 could trigger NK cell cytotoxicity (25–27). We then examined whether CD27 could trigger NK cell cytotoxicity by using anti-CD27 mAb-redirected cytotoxic assay against FcR-bearing P815 target cells or by using CD70-transfected P815 cells as the target cells. Freshly isolated NK cells lysed the classical NK target YAC-1 cells efficiently (data not shown), but not P815 cells. Although anti-NK1.1 mAb efficiently directed NK cell cytotoxicity against P815, anti-CD27 mAb did not (Fig. 5). Moreover, P815-CD70 cells did not show increased susceptibility to NK cells in the presence or the absence of anti-NK1.1 mAb (Fig. 5). These results indicated that CD27 cannot trigger cytotoxic activity to NK cells, unlike NK1.1.

**IFN-γ-mediated enhancement of NK cell cytotoxicity by prestimulation via CD27**

Since IFN-γ has been known to enhance the NK cell cytotoxicity (28, 29), it would be possible that NK cell cytotoxicity could be augmented by prestimulation via CD27, which induce IFN-γ production by NK cells as shown in Fig. 3. To address this possibility,
indicated that CD27-mediated stimulation can augment NK cell toxicity indirectly by inducing autocrine IFN-γ production. Unlike NKR-P1, it can augment NK cell cytotoxicity against both target cells to levels comparable to those induced by anti-NK1.1 pre-stimulation and further enhanced the anti-NK1.1-induced cytotoxicity. The enhancement by anti-CD27 pre-stimulation was completely abrogated by neutralizing anti-IFN-γ mAb. These results indicated that CD27-mediated stimulation can augment NK cell cytotoxicity indirectly by inducing IFN-γ production.

Discussion

In this study we demonstrated that murine NK cells express CD27 constitutively and that stimulation with immobilized anti-CD27 mAb or its ligand, CD70, induces proliferation and IFN-γ production of freshly isolated NK cells as potent as stimulation with CD27 mAb or CD70-transfected P815 cells in the presence of IL-2 (17, 24–26). Although CD27 stimulation does not directly trigger cytolytic function of NK cells, unlike NKR-P1, it can augment NK cell cytotoxicity indirectly by inducing autocrine IFN-γ production. These results suggest the involvement of CD27 in the regulation of NK cell expansion and functions.

Expression and function of CD27 on T cells have been well characterized in both human and murine systems (5, 6, 15, 19–24). CD27 is expressed on the majority of human peripheral blood T cells, and its engagement by anti-CD27 mAb or CD70 induced proliferation of naive T cells in the presence of a suboptimal dose of PMA, PHA, or anti-CD3 mAb (5, 6, 19–24). Also in the murine system, CD27 is expressed on the vast majority of mature T cells, and its engagement by anti-CD27 mAb or murine CD70 costimulated proliferation of Con A- or anti-CD3-stimulated T cells (15, 20). In contrast, the expression and function of CD27 on NK cells have not been well characterized. In the human system, it has been reported that CD27 was expressed at low levels by peripheral blood NK cells and was up-regulated on IL-2-activated NK cells (30). In the present study we showed that the great majority of murine NK cells express CD27 before and after IL-2 stimulation. Expression of CD27 on freshly isolated NK cells was unique among the TNF receptor family molecules tested, while CD137 was expressed on IL-2-activated NK cells as previously reported (18). Moreover, we demonstrated that CD27 on murine NK cells is functional in inducing proliferation and IFN-γ production, while such functions have not been explored in previous studies with human NK cells. Our present observation of the failure to trigger cytolytic function of murine NK cells by anti-CD27 mAb or against CD70 transfectants is consistent with previous observations, which reported that human NK cells did not show enhanced cytotoxicity against CD70 transfectants in a 4-h 51Cr release assay (31). Furthermore, we clarified that the enhanced cytotoxic activity of murine NK cells after prestimulation with immobilized anti-CD27 mAb or CD70-transfected P815 might be mediated by autocrine IFN-γ, suggesting that the increased NK cell activity after coculture with CD70 transfectants in the human system might be also mediated by IFN-γ (31). These results imply that CD27 plays a similar role on murine and human NK cells.

The signaling mechanism by which CD27 activates NK cells remains to be determined in the future study. It has been shown that cross-linking of CD27 induced tyrosine phosphorylation of cytoplasmic proteins, including ZAP-70, in human T cells, might enhance the TCR/CD3-mediated signaling (22). It remains to be determined whether CD27 stimulation may also induce protein tyrosine phosphorylation of NK cells. In this respect, it may be

**FIGURE 5.** CD27 does not trigger NK cell cytotoxicity. NK cells were freshly isolated from RAG-2−/− spleen, and cytotoxicity was tested against 51Cr-labeled P815 or CD70-transfected P815 cells in the presence or the absence of anti-NK1.1 mAb (10 μg/ml) or anti-CD27 mAb (10 μg/ml) at the indicated E:T cell ratios. Data are expressed as the mean ± SD of triplicate wells and are representative of three independent experiments with similar results. *, p < 0.05 compared with P815 alone.

**FIGURE 6.** IFN-γ-mediated enhancement of NK cell cytotoxicity by prestimulation via CD27. Freshly isolated RAG-2−/− splenic NK cells were stimulated for 3 days with immobilized anti-CD27 mAb (10 μg/ml) and/or immobilized anti-NK1.1 mAb (10 μg/ml) in the presence of 25 U/ml IL-2. Neutralizing anti-IFN-γ mAb (10 μg/ml) was added to some wells. After 3 days, NK cells were harvested, and cytotoxicity against YAC-1 (A) and P815 (B) was tested at the indicated E:T cell ratios. Data are expressed as the mean ± SD of triplicate cultures. Data shown are representative of three independent experiments with similar results. *, p < 0.05 compared with IL-2 alone.
interesting to compare the signaling via CD27 with that via the NKR-P1 molecule, which not only induces IFN-γ production but also triggers NK cell lytic function. It has been shown that NKR-P1 is associated with the FcR γ-chain, which activates ZAP-70 and/or Syk and induces an increase in intracellular Ca²⁺ that is requisite for triggering NK cell lytic function (32–34). In contrast, it has been shown that CD27 ligation affected calcium mobilization in NK cells only in the induction mediated by CD27 (30), which might be responsible for the failure to trigger the cytolytic function of NK cells. In addition to the tyrosine phosphorylation events, we recently revealed that CD27 recruits TNF receptor-associated factor-2 (TRAF2) and TRAF5, which activate NF-κB and c-Jun N-terminal kinase and have been implicated in cellular proliferation and cytokine production (35). An important role for TRAF-mediated signaling in T cells has been substantiated by the impaired proliferation of TRAF5-deficient T cell in response to CD27-mediated costimulation (36). Further studies are underway to characterize the signaling pathway responsible for the CD27-mediated activation of NK cells.

It has been established that NK cells play important roles in the innate immunity against infection and tumor development (29, 37, 38). NK cells preferentially lyse virus-infected cells and transformed cells by their cytotoxic function. IFN-γ produced by NK cells not only exerts anti-viral and proinflammatory effects, but also induces Th1 and CTL-mediated acquired immunity. In the present study we showed that CD27-mediated stimulation by anti-CD27 mAb or CD27 enhanced IFN-γ production and the cytotoxic activity of NK cells. CD70 was originally characterized as a marker of Reed-Sternberg cells in Hodgkin’s disease and non-Hodgkin’s lymphomas (39). It has also been found that CD70 is highly expressed on EBV-transformed lymphoblastoid cells and human immunodeficiency virus-infected T cells (39, 40). Therefore, the CD27-mediated activation of NK cells may be involved in the innate immune surveillance against these virus-infected or transformed cells expressing CD70. Consistent with this idea, CD70-transfected murine tumor cells exhibited a reduced ability of lung or hepatic metastasis, which was predominantly mediated by CD70, transformed cells expressing CD70 and CD70-transfected murine tumor cells exhibited a reduced ability of lung or hepatic metastasis, which was predominantly mediated by CD70, transformed cells expressing CD70 and CD70. Consistent with this idea, CD70-transfected murine tumor cells exhibited a reduced ability of lung or hepatic metastasis, which was predominantly mediated by CD70, transformed cells expressing CD70 and CD70.

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