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Signaling Through NK Cell-Associated CD137 Promotes Both Helper Function for CD8$^+$ Cytolytic T Cells and Responsiveness to IL-2 But Not Cytolytic Activity[1]

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NK cells possess both effector and regulatory activities that may be important during the antitumor immune response. In fact, the generation of antitumor immunity by the administration of an agonistic mAb against CD137 is NK cell-dependent. In this study, we report that NK cells could be induced by IL-2 and IL-15 to express CD137 and ligation of CD137-stimulated NK cell proliferation and IFN-γ secretion, but not their cytolytic activity. Importantly, CD137-stimulated NK cells promoted the expansion of activated T cells in vitro, demonstrating immunoregulatory or "helper" activity for CD8$^+$CTL. Furthermore, tumor-specific CTL activity against P815 tumor Ags was abrogated following anti-CD137 treatment in NK-depleted mice. We further demonstrate that CD137-stimulated helper NK cells expressed the high-affinity IL-2R and were hyperresponsive to IL-2. Taken together with previous findings that CD137 is a critical receptor for costimulation of T cells, our findings suggest that CD137 is a stimulatory receptor for NK cells involved in the crosstalk between innate and adaptive immunity. *The Journal of Immunology*, **2002, 169: 4230–4236.**

Natural killer cells, so named because of their ability to spontaneously kill tumor cells in vitro, are important effector cells in the innate immune response to virally infected or transformed cells (1–6). During an innate immune response, NK cells may not only kill target cells, but they may also secrete proinflammatory cytokines, including IFN-γ and TNF-α (5, 7, 8). Although the mechanisms are poorly understood, the NK cell's role as an immunoregulatory cell capable of modulating the adaptive immune response may be an equally important contribution to the immune response (9). For example, NK cell depletion studies in mice have demonstrated the importance of NK cells in the induction of both influenza virus-specific and B16 melanoma-specific CTLs (10, 11). Furthermore, studies performed in vitro suggest that NK cells may be required for the differentiation of fully competent effector CTLs in mixed lymphocyte cultures (12). These studies highlight the important role NK cells may play in modulating a CTL response.

NK cell reactivity is controlled by both inhibitory and stimulatory receptors (13, 14). Identification of inhibitory receptors capable of binding MHC class I supports the "missing self" hypothesis which, simply stated, suggests that NK cells survey potential targets for MHC class I expression. Upon encountering cells that fail to express MHC class I, the loss of any inhibition renders the NK cell capable of initiating its activation program. Recent studies have also highlighted the importance of stimulatory receptors, like NKG2D, in NK killing (15, 16). NKG2D is a type II dimer with lectin-like domains capable of binding the HLA class Ib molecules MICA and MICB in the human, and H-60 and Rae1 in the mouse (15, 17–19). The ability of these ligands (preferentially expressed on many tumors but notably absent on normal cells) to stimulate NK cells was suggested by the finding that NK-insensitive targets were rendered sensitive to NK cell-mediated lysis upon transfection with either H-60 or Rae1 (18, 19).

CD137, also called 4-1BB, is a member of the TNF superfamily expressed by activated T cells, monocytes, and dendritic cells (20, 21). Interestingly, CD137 was also found on the surface of activated mouse NK cells (22). Studies performed with either agonistic mAbs against CD137 or with CD137 ligand (CD137L)[3](4-1BBL) have shown that CD137 is a potent costimulatory molecule capable of stimulating T cell proliferation and cytokine production (23–26). The importance of CD137 in the generation of a fully competent T cell response was shown in both graft vs host disease and in viral models using CD137 or CD137L-deficient mice (27–29). Administration of agonistic CD137 mAb either alone or following peptide vaccination is capable of stimulating a potent tumor-specific CTL response, leading to regression of established tumors in various mouse models (30, 31). Interestingly, tumor eradication by CD137 mAb is NK cell-dependent, as demonstrated in studies using mice depleted of either NK1.1$^+$ or AsialoGM1$^+$ cells (22). However, the effect of CD137 on NK cell function remains elusive. In this report, we examine the functional consequence of CD137 triggering on NK cells.

Materials and Methods

Mice and cell lines

Female C57BL/6 (B6) and B6D2F1 mice were purchased from the National Cancer Institute (Frederick, MD). Female C57BL/6J-*Rag1*^{tm1Mom} mice (recombination-activating gene 1 knockout; RAG-1 KO) are deficient in T and B cells and were purchased from The Jackson Laboratory (Bar Harbor, ME). The OT-1 mice carrying TCR transgenic T cells specific for a H-2K^b-restricted CTL epitope were a generous gift from Dr. E. Celis

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[3] Abbreviations used in this paper: CD137L, CD137 ligand; RAG-1 KO, recombination-activating gene 1 knockout.

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(Mayo Clinic, Rochester, MN). Mock-transfected and mouse CD137L-transfected P815 (22), YAC-1, RMA-S, EL4, L1210, and C3 cells were maintained in a complete medium of RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate.

NK cell isolation and FACS analysis

NK cells were isolated from RAG-1 KO mice as previously described (32). Briefly, splenocytes were incubated at 37°C for 1–2 h. Nonadherent cells were collected and determined to be at least 85% NK1.1$^+$ by FACS analysis. IL-2-activated NK cells were also isolated from mice that had received 1×10^6 IU human IL-2 (Chiron, Emeryville, CA) each day for 3 days before NK cell isolation using the same method. In some experiments, mice were given 100 μg of rat IgG or CD137 mAb (clone 2A, rat IgG2a) i.p. 24 and 48 h before NK cell isolation. Freshly isolated NK cells were cultured overnight with 300 IU/ml human IL-2 or 20 ng/ml IL-15 (PeproTech, Rocky Hill, NJ). Cells were stained with FITC-conjugated isotype control rat Ig or CD137 mAb. Fluorescence was analyzed by a FACSCalibur flow cytometer with CellQuest software (both from BD Biosciences, Mountain View, CA). Alternatively, B6 mice were inoculated with 1×10^7 irradiated (10 Gy) RMA-S cells i.p., as previously described (33). Peritoneal exudate cells were isolated 3 days later and stained with both FITC-conjugated anti-NK1.1 and either anti-CD69, biotinylated rat IgG, or biotinylated anti-CD137 (2A). Cells were stained with streptavidin PE and analyzed by FACS.

Peptide and Abs

The OVA (258–265) peptide (SIINFEKL) is a H-2K^b-restricted CTL epitope derived from chicken OVA. This peptide was synthesized by the Mayo Molecular Biology Core Facility and the purity of the peptide was >90% by reverse-phase HPLC purification. Preparation and growth of the hybridoma and purification of a rat IgG2a mAb specific for mouse CD137 has been described previously (30). Control rat IgG control Ab was purchased from Sigma-Aldrich (Gilbertsville, PA). The mAb specific for mouse CD137L was also described previously (21). Purified FITC-conjugated CD3, CD137, and isotype-matched control mAb were purchased from BD PharMingen (San Diego, CA). PE- and FITC-conjugated NK1.1 was purchased from BD PharMingen. Anti-CD132 was purchased from BD PharMingen and anti-CD25 (PC61) was a generous gift from Dr. S. Shu (Cleveland Clinic, Cleveland, OH). The depleting anti-NK1.1 mAb (PK136) and control mouse IgG mAb were previously described (22).

NK cell proliferation, IFN-γ secretion, and cytolytic activity

Flat-bottom 96-well plates were coated overnight with 10 μg/ml rat IgG, anti-NK1.1 mAb, or CD137 mAb. NK cells at 8×10^4/well were added to each well supplemented with 150 IU/ml human IL-2. Alternatively, NK cells were cocultured in triplicate with 3×10^4 irradiated (10 Gy) mock- or CD137L-transfected P815 cells in the presence of 5 μg/ml rat IgG or anti-CD137L mAb. Supernatants were collected after 48 h and IFN-γ concentration determined by sandwich ELISA following the manufacturer's instructions (BD PharMingen). NK cell proliferation was assessed by the addition of 1 μCi/well [^{3}H]TdR during the last 15 h of the 3-day culture. [^{3}H]TdR incorporation was measured in a MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland). For assay of cytolytic activity, NK cells were isolated from RAG-1 KO mice that had received IL-2 and rat IgG or CD137 mAb, as described above. NK cytotoxicity was measured in a standard 4-h ^{51}Cr-release assay (30). Briefly, NK cells were cocultured with 4×10^3 ^{51}Cr-labeled EL4, YAC-1, or C3 cells in a 96-well V-bottom plate at various E:T cell ratios.

Transwell experiments and CTL assay

CD8$^+$OT-1 cells were purified from the lymph nodes of OT-1 mice using magnetic anti-CD8 microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). OT-1 cells at 4×10^5/well were cocultured with 5×10^6 irradiated B6 splenocytes in triplicate wells of a 24-well plate. Cells were cultured in 1.2 ml complete RPMI alone or media supplemented with 1 ng/ml OVA peptide. A transwell insert (6.5-mm diameter, 0.4-mm pore size; Costar, Corning, NY) was added to each well. NK cells purified from RAG-1 KO mice that had received IL-2 and either rat IgG or CD137 mAb were added to the transwell insert at a density of 2×10^4 cells/well. [^{3}H]TdR at 10 μCi/well was added to each well during the last 15 h of the 3-day culture. NK cells were isolated from the transwell insert and transferred into a new 96-well plate. Likewise, 180 μl of the OT-1/splenocyte cocultures was transferred into a separate 96-well plate, and thymidine incorporation in both the OT-1 cells and NK cells was measured.

B6D2F1 mice were given 1×10^6 P815 cells s.c. Mice were given either a control mouse IgG or anti-NK1.1 (0.5 mg) 24 and 48 h following tumor inoculation (NK cell depletion was confirmed by FACS in all experiments). On days 4 and 7, mice were given 100 μg anti-CD137 (2A). Two weeks later, the mice (two in each group) were sacrificed and their spleens and tumor-draining lymph nodes harvested. Cell suspensions (5.5×10^6/well) were restimulated with 2.5×10^5 irradiated (10 Gy) P815 cells in a 24-well plate for 4 days. Effector cells were cocultured for 4 h with ^{51}Cr-labeled P815 or L1210 cells in 96-well, V-bottom plates at various E:T ratios.

Results

Expression of CD137 on NK cells in vitro and in vivo

Incubation of whole mouse spleen cells in the presence of IL-2 induces expression of CD137 on NK1.1$^+$ cells (22). However, this result could be interpreted as an indirect effect mediated by IL-2-activated T cells. Therefore, whether or not IL-2 may directly induce CD137 expression on NK cells was examined. We reported in this study that freshly isolated NK cells (>85% pure) from RAG-1 KO mice rapidly up-regulate CD137 expression on the cell surface within 24 h in the presence of IL-2 (Fig. 1*A*). A similar observation was also obtained after incubation with IL-15, a potent activator of NK cells (34). Our results suggest that CD137 is inducibly expressed on NK cells in direct response to IL-2 or IL-15. However, whether NK cells express CD137 in vivo remained unclear. To address that question, CD137 expression was examined on NK cells present within the peritoneal exudate of mice bearing an MHC class I-deficient tumor (RMA-S). These NK cells are cytolytic (33) and express the activation marker CD69 (Fig. 1*B*). CD137 expression was also observed on many of these NK cells (Fig. 1*C*). Therefore, activated NK cells inducibly express CD137 both in vitro and in vivo.

CD137 signaling stimulates NK cell proliferation and IFN-γ secretion

We next determined whether or not CD137 signaling directly stimulates NK cell functions. Cross-linking of freshly isolated NK cells from RAG-1 KO mice by CD137 mAb in the presence of IL-2 induced proliferation of NK cells to an extent similar to that observed upon cross-linking NK1.1 mAb (Fig. 2*A*), a known NK cell

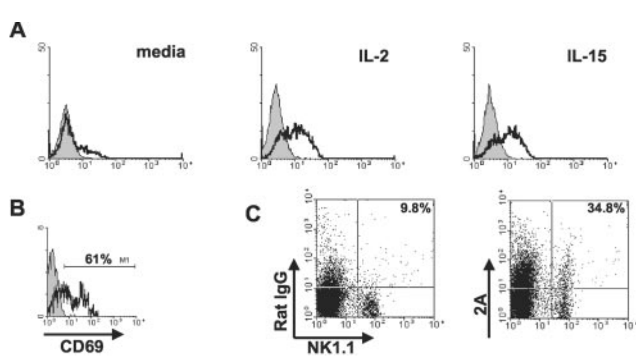

FIGURE 1. CD137 is expressed on NK cells. *A*, Freshly isolated splenic NK cells form RAG-1 KO mice were incubated in the presence or absence of IL-2 (300 U/ml) or IL-15 (20 ng/ml) at 1×10^6 cells/well in a 24-well plate. Twenty-four hours later, both the resting and cytokine-activated NK cells were stained with an isotype control (filled histogram) or anti-CD137 mAb (open histogram). *B*, B6 mice were inoculated i.p. with 1×10^7 irradiated RMA-S cells. Peritoneal exudate cells were isolated 3 days later and NK1.1$^+$ cells were stained with an isotype control (filled histogram) or anti-CD69 mAb (open histogram). *C*, Freshly isolated peritoneal exudate cells as described in *B* were stained with anti-NK1.1 mAb and either a biotinylated rat IgG control or anti-CD137 mAb (clone 2A). After washing, cells were stained with streptavidin PE.

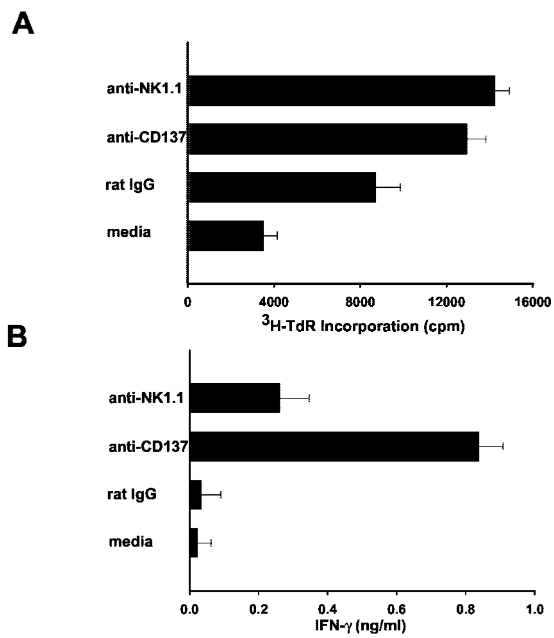

FIGURE 2. Stimulation of NK cell proliferation and IFN-γ secretion upon CD137 cross-linking. Freshly isolated NK cells from RAG-1 KO mice were stimulated for 3 days with plate-bound rat IgG, anti-CD137 mAb, or anti-NK1.1 mAb (10 μg/ml) in the presence of IL-2 (150 U/ml). Both the proliferative response (*A*) and IFN-γ secretion (*B*) were determined after 3 days of culture. Data are expressed as the mean ± SD of triplicate cultures. Differences in proliferation observed between rat IgG control and anti-CD137 or anti-NK1.1 groups was statistically significant ($p < 0.05$) by Student's *t* test. Data shown are representative of three independent experiments.

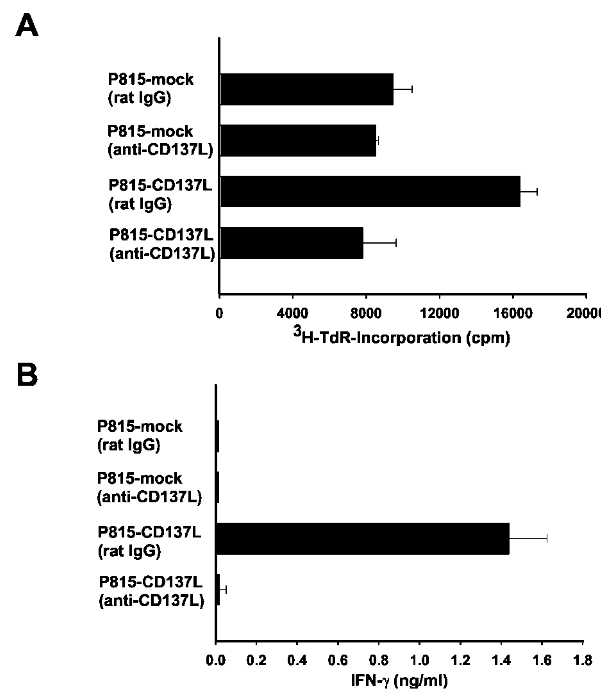

FIGURE 3. CD137L stimulates NK cell proliferation and IFN-γ secretion. Freshly isolated NK cells from RAG-1 KO mice were cocultured with irradiated mock-transfected or CD137L-transfected P815 cells in the presence of a control rat IgG or anti-CD137L (14B3) mAb (3 μg/ml) with 150 U/ml IL-2. After 3 days of culture, NK cell proliferation (*A*) and IFN-γ secretion (*B*) were determined. Data are expressed as the mean ± SD of triplicate cultures. Data shown are representative of three independent experiments.

activating receptor (35). Significant NK cell proliferation was also observed in the rat IgG control group, which may be attributed to the cross-linking of FcRs on NK cells (36, 37). However, CD137 cross-linking led to a high-level IFN-γ secretion from NK cells, whereas control rat IgG did not. CD137 stimulation led to a 4-fold increase in IFN-γ production when compared with those NK cells that had been stimulated with NK1.1 mAb (Fig. 2*B*).

To exclude the effect of FcR cross-linking, we next sought to determine the role of the physiologic ligand for CD137 (CD137L). To do so, mock-transfected and CD137L-transfected P815 cells were irradiated and used as stimulators for freshly isolated NK cells from RAG-1 KO mice. Compared with the proliferation observed using the mock-P815, a significant increase in proliferation of NK cells was observed after stimulation with CD137L-P815. This increase in NK cell proliferation was completely abrogated by inclusion of a neutralizing anti-CD137L mAb (Fig. 3*A*). Furthermore, while mock-transfected P815 cells were unable to stimulate the production of IFN-γ by NK cells, CD137L-transfected P815 stimulated the production of IFN-γ, and cytokine secretion was completely abrogated by the inclusion of anti-CD137L mAb (Fig. 3*B*). Similar NK cell responses, including proliferation and IFN-γ production, were also observed after stimulation by either mock-P815 or CD137L-P815 in the total absence of control Abs (data not shown). We concluded that CD137 signaling by either agonistic mAb or the physiologic ligand enhances NK cell proliferation and IFN-γ secretion.

NK cells are required for the generation of P815 tumor-specific CD8$^+$CTL following anti-CD137 administration

Our previous studies showed that depletion of NK1.1$^+$ cells completely eliminated the antitumor effect of CD137 mAb in a mouse P815 tumor model (22). Because P815 is resistant to NK-mediated cytolysis, it is possible that NK cells in this system provided a "helper" function. We tested this hypothesis by examining whether NK cells are required for the generation of CD8$^+$CTL specific for tumor Ags. F_1 mice (B6 and DBA/2) bearing a P815 tumor were all treated with CD137 mAb and the mice were divided into two groups, one group of the mice were injected with anti-NK1.1 mAb and another group of the mice were treated with control Ig. Although CTL activity was detected in control Ig-treated mice, P815-specific CTL activity was not detected in NK cell-depleted mice

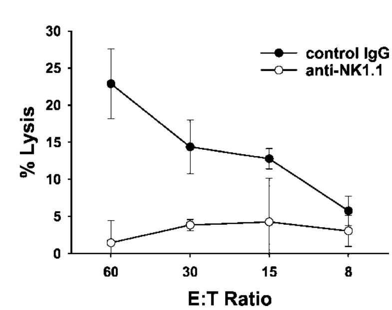

FIGURE 4. NK cells are required for the generation of tumor-specific CD8$^+$CTL following anti-CD137 administration. F_1 (B6 × DBA/2) mice were inoculated in the flank with 1×10^6 P815 cells s.c. Mice were given 0.5 mg of either a control Ab or a depleting anti-NK1.1 (PK136) mAb 24 and 48 h after tumor inoculation. On days 4 and 7, the mice were injected i.p. with 100 μg anti-CD137 mAb. Two weeks later, the spleens and tumor draining lymph nodes were harvested and restimulated with irradiated P815 cells in vitro. A standard 4-h ^{51}Cr-release cytotoxicity assay was performed 4 days later against P815 targets at the E:T ratios indicated. Nonspecific lysis of L1210 targets was <5% in all experiments performed.

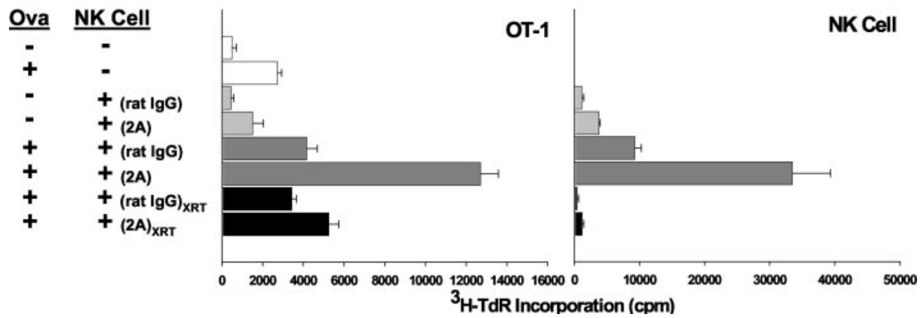

FIGURE 5. CD137-stimulated NK cells are hyperresponsive to soluble factor(s) secreted by activated T cells. A total of 4×10^5 purified OT-1 cells were cocultured with 5×10^6 irradiated splenocytes with or without OVA peptide (1 ng/ml) in 1.2 ml media in a 24-well plate. Both the resting and activated OT-1 cells were cocultured with 2×10^4 NK cells, which had been previously stimulated with rat IgG or anti-CD137 as described in the *Materials and Methods* that had been placed in a transwell insert. After 3 days, proliferation of both the OT-1 cells in the lower well and the NK cells in the upper transwell were measured and were expressed as the mean ± SD of triplicate cultures. Data shown are representative of at least three independent experiments.

(Fig. 4). CTL activity in mice treated with control Ig without anti-CD137 mAb was low (<10%, data not shown). Our results support that NK cells may serve as helper cells in the development of a P815 tumor-specific CD8$^+$CTL response.

CD137-activated NK cells provide help for CD8$^+$CTL through the release of soluble factors

To determine whether soluble factors may mediate the helper function of NK cells, we first activated NK cells in vivo by injecting RAG-1 KO mice with CD137 mAb together with IL-2 before NK cell isolation. Freshly isolated NK cells were plated into the upper chamber of a transwell while OT-1 TCR transgenic T cells were plated in the lower chamber. This system prevents the direct contact of NK cells and OT-1 cells, allowing soluble factors to pass through the transwell insert. Addition of OVA peptide in the presence of irradiated spleen cells as APCs led to OT-1 proliferation as expected. Low levels of T cell proliferation were observed in the cultures that did not contain OVA peptide. However, inclusion of freshly isolated NK cells in the upper well did not significantly increase proliferation of OT-1 T cells, indicating that the ability of IL-2-stimulated NK cells to enhance T cell proliferation is limited. A small increase in OT-1 proliferation was observed using NK cells from the mice that had been treated with the control rat IgG. However, a significant increase in T cell proliferation was observed by inclusion of NK cells from the mice that had been treated with CD137 mAb. The ability of CD137 mAb-stimulated NK cells to augment T cell proliferation was abrogated by irradiating (XRT) the NK cells before culture (Fig. 5, *left panel*). Our results thus indicate that CD137-triggered NK cells provide soluble factors to augment the proliferation of OT-1 T cells.

CD137 stimulation does not enhance cytolytic activity of NK cells

To examine whether CD137 stimulation also increased the cytolytic function of NK cells, we activated NK cells in vivo by injecting RAG-1 KO mice with CD137 mAb as described previously

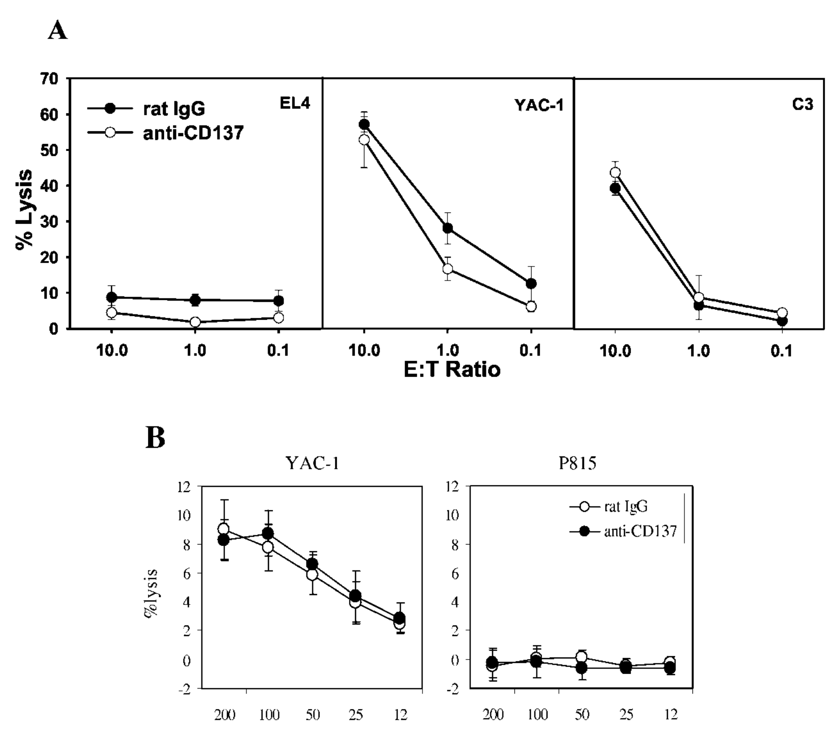

FIGURE 6. CD137 does not stimulate NK cell-mediated cytolysis. *A*, RAG-1-deficient mice were given IL-2 and a control rat IgG or anti-CD137 mAb as described in the *Materials and Methods*. Freshly isolated NK cells from these mice were cocultured for 4 h with 4×10^3 ^{51}Cr-labeled EL4, YAC-1, or C3 cells at the E:T ratios indicated. *B*, DBA/2 mice were inoculated in the flank with 1×10^6 P815 cells s.c. Mice were given 0.1 mg of either a control Ab (rat IgG) or anti-CD137 mAb 3 days after tumor inoculation. On day 7, the spleen cells were harvested and cytolytic activity against YAC-1 (*left panel*) and P815 (*right panel*) was accessed in a 4-h ^{51}Cr-release assay at the E:T ratios indicated. Two independent experiments were performed and the results were pooled.

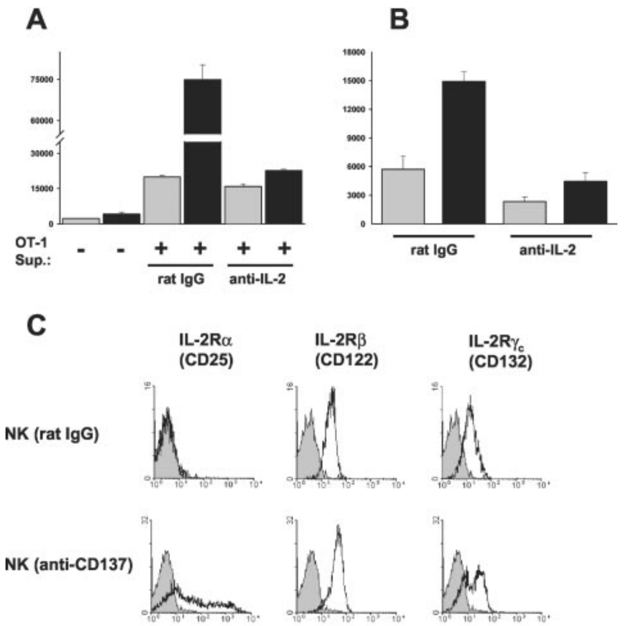

FIGURE 7. CD137-stimulated NK cells up-regulate CD25 and are hyperresponsive to IL-2. *A*, NK cells at 1×10^5, which had been stimulated by rat IgG (▨) or anti-CD137 mAb (■) in vivo, were cultured in a 96-well plate in 200 μl total volume with media alone, media supplemented with 100 μl of supernatant taken from a 48-h culture of activated OT-1 cells (*A*), or media supplemented with 2 ng/ml recombinant mouse IL-2 (*B*). Rat IgG or a neutralizing anti-IL-2 mAb (5 μg/ml) was added to each well, as indicated. After 3 days, NK cell proliferation was measured. *C*, A total of 1×10^6 rat IgG or anti-CD137-stimulated NK cells were cultured in 50 CU/ml IL-2 in a 24-well plate. NK cells were stained with anti-CD25, anti-CD122, and anti-CD132 (open histograms), or an isotype-matched mAb control (filled histogram) 48 h later.

(Fig. 5). Freshly isolated NK cells were tested for their cytolytic activity against tumor cells in a standard 4-h chromium release assay. As shown in Fig. 6*A*, NK cell cytolytic activity against YAC-1 and C3, two NK-sensitive tumor lines, was unchanged. We have also examined the NK activity of spleen cells against P815 cells after exposure to anti-CD137 mAb. DBA/2 mice were inoculated with P815 tumor cells and subsequently treated with anti-CD137 mAb. Freshly isolated spleen cells were then examined for their NK activity against P815 cells. NK activity of spleen cells against P815 again was unchanged (Fig. 6*B*). Therefore, despite an increase in helper function, CD137 signaling does not modulate the cytolytic activity of NK cells.

CD137-stimulated NK cells up-regulate the IL-2Rα (CD25) chain and are hyperresponsive to IL-2

NK cell proliferation was also measured in the presence of activated OT-1 T cells (Fig. 5). Although low levels of NK cell proliferation were measured in the cultures that did not contain peptide, a significant (10-fold) increase in NK cell proliferation was observed in the cultures containing OVA peptide, suggesting that soluble factors from activated T cells induce NK cell proliferation. Interestingly, a further increase in proliferation was observed in the CD137-stimulated NK cells compared with the control NK cells (Fig. 5, *right panel*). Therefore, CD137-stimulated NK cells not only provide "help" to activated T cells, but also become hyperresponsive to factor(s) secreted by activated T cells.

To identify this factor(s), we first prepared cell-free supernatants of OT-1 cells activated for 48 h in the presence of an optimal concentration of OVA peptide in the presence of irradiated spleen cells as APC. NK cells that had been treated by CD137 mAb or control rat IgG in vivo were cultured in the presence of purified IL-2 or OT-1 supernatant. The proliferation of NK cells was measured. As shown in Fig. 7, while NK cells isolated from control Ig-treated mice responded to OT-1 supernatant, proliferation of the CD137 mAb-treated NK cells was at least 4-fold higher than that observed in the control Ig-treated NK cells. Inclusion of neutralizing mAb to mouse IL-2 completely neutralized the proliferation (Fig. 7*A*). Similar results were also observed in the cultures using recombinant mouse IL-2 (Fig. 7*B*). FACS analysis indicated that the IL-2Rα chain (CD25), but not IL-2Rβ (CD122) or IL-2Rγc (CD132), was up-regulated in the NK cells that were treated with CD137 mAb (Fig. 7*C*). Furthermore, neutralizing mAb against IL-2Rα and IL-2Rβ blocked the stimulatory effect of rIL-2 on CD137-stimulated NK cells (Fig. 8). We concluded that responsiveness to IL-2 is the mechanism by which CD137-stimulated NK cells proliferate in the presence of activated T cells.

Discussion

We reported previously that activated NK cells express CD137 (22). However, the functional consequence of this finding is unknown. By triggering NK-associated CD137 using either an agonistic mAb or CD137L-transfected cells, we show in this study that CD137 signaling stimulates proliferation and IFN-γ secretion in mouse NK cells, indicating that CD137 ligation delivers a signal for activation of NK cells. More importantly, by depleting NK1.1$^+$ cells in vivo, we demonstrate that NK cells are required for the generation of P815 tumor-specific CTL. Because P815 tumor cells are resistant to NK-mediated lysis, our results suggest a helper cell function for CD137-stimulated NK cells in the induction of CTL.

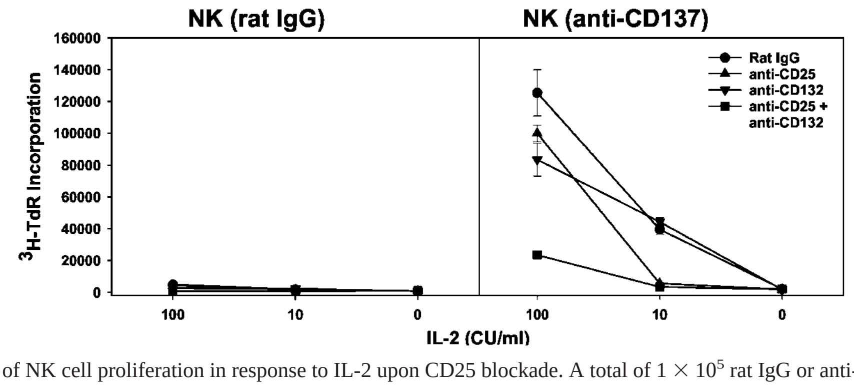

FIGURE 8. Abrogation of NK cell proliferation in response to IL-2 upon CD25 blockade. A total of 1×10^5 rat IgG or anti-CD137-stimulated NK cells were cultured with the concentrations of recombinant human IL-2 indicated. A total of 10 μg/ml of a control rat IgG or 5 μg/ml of anti-CD25 or anti-CD132 supplemented with 5 μg/ml rat IgG, and 5 μg/ml each of both anti-CD25 and anti-CD132 were included in the NK cell cultures, as indicated. Proliferation was measured 72 h later by thymidine incorporation.

We further demonstrate by in vitro transwell experiments that CD137-stimulated NK cells support the growth of activated $CD8^+$OT-1 CTL through soluble factors. High-affinity IL-2 receptor is up-regulated on CD137-stimulated NK cells that become hyperresponsive to IL-2. Our results suggest an important role for CD137 signaling in the activation of NK cells and crosstalk between NK cells and CTL.

NK cells express a rich array of natural cytotoxic receptors that trigger cytolytic activity (13), a function believed to be involved in cancer surveillance and the control of viral infection. Our results indicate that CD137 receptor on NK cells, upon ligation, also delivers an activation signal, as demonstrated by increased proliferation and secretion of IFN-γ from NK cells (Fig. 2). However, CD137-mediated signaling is fundamentally different from natural cytotoxic receptor since cytolytic activity of NK cells following CD137 ligation did not increase (Fig. 6). Rather, CD137 signaling supports a helper function of NK cells for the generation of $CD8^+$CTL.

The generation of Ag-specific cytolytic T cells is a complicated process involving Th cells and many cytokines. It was reported that generation of CTL against alloantigens and influenza virus required regulatory NK cells for differentiation of effector CTL (11, 12). We have shown that eradication of established P815 tumors in syngeneic mice following anti-CD137 mAb administration is entirely NK cell-dependent (22). Similarly, eradication of the HPV-16-transformed C3 tumor by CD137 mAb was also largely dependent upon NK cells (30). However, P815 tumor cells express high levels of MHC class I and are resistant to lysis by freshly isolated NK cells from normal mice (22) or from the mice treated with CD137 mAb (Fig. 6*B*). By in vivo depletion and in vitro cytotoxic T cell assay, we demonstrated that $CD8^+$CTL are key effector cells for tumor eradication (22, 30, 31). These findings support the contention that CD137 signaling stimulates helper or regulatory activity in NK cells required for the generation of effector CTL following CD137 stimulation. In this study, we present direct evidence that depletion of NK cells in vivo prevents the generation of CTL against P815 cells. However, it should be noted that a role for NK T cells might not be excluded, as these cells could also express $NK1.1^+$. We showed previously that administration of anti-AsialoGM-1 Abs, which do not deplete NK T cells, also eliminated antitumor effect of CD137 mAb (22), suggesting that NK cells are likely the target cells in our experiments. Our results thus establish a helper function of CD137-stimulated NK cells in the induction of tumor-reactive CTL in vivo.

Using a transwell culture system to separate NK and T cells, we provide evidence that helper function of NK cells is largely mediated by soluble factors. Although the identities of these factors remains to be characterized, a significant increase in T cell proliferation was observed in those cultures containing CD137 mAb-stimulated NK cells. NK cells exposed to control rat IgG exhibit some helper activity, albeit to a lesser extant than that observed by CD137-stimulated NK cells. A nonspecific stimulatory role for control rat Ig was demonstrated in vitro (Fig. 2*A*) using various control Abs (data not shown) from various sources. It was reported that FcR ligation on NK cells may stimulate NK cell activity; our results suggest this possibility. Despite the possible effect of FcR ligation, our results support a unique role for CD137 signaling in the stimulation NK cells, as CD137 mAb stimulated NK cell proliferation, IFN-γ secretion (Fig. 2), and helper function (Fig. 5). More importantly, CD137L transfectants are a potent stimulator of NK cell proliferation and IFN-γ secretion. This effect could be completely neutralized upon inclusion of CD137L mAb.

Interestingly, CD137-stimulated NK cells, in contrast to the control cells, were able to up-regulate the expression of CD25, a component of the high-affinity IL-2R, in the presence of IL-2. Both groups of NK cells also expressed components of the intermediate affinity IL-2R, including CD122 and CD132. However, only those NK cells that had been previously stimulated with CD137 mAb were capable of expressing CD25. Expression of CD25 led to their hyperresponsiveness to IL-2, as confirmed in subsequent blocking experiments (Fig. 8). Proliferation of the CD137-stimulated NK cells was partially inhibited at 600 U/ml IL-2 by either the anti-CD25 or anti-CD132 mAbs. However, inclusion of both Abs in the culture almost completely inhibited NK cell proliferation at both the high and low doses of IL-2. In contrast, anti-CD25 completely blocked NK cell proliferation at a low concentration of IL-2 (60 U/ml), suggesting that expression of the high-affinity IL-2R is required for NK cell proliferation at this lower concentration of IL-2. Therefore, the ability of CD137-stimulated NK cells to proliferate in response to IL-2 may be attributed to the up-regulation of CD25 following CD137 stimulation.

In summary, in addition to providing costimulatory activity for T cells, the data presented in this study demonstrate that ligation of CD137 on NK cells also delivers an activation signal leading to growth, cytokine secretion, and enhanced regulatory function for $CD8^+$ T cells. Taken together with recent studies that ligation of CD137 also promotes the ability of dendritic cells to stimulate T cells and to secrete cytokines including IL-6 and IL-12 (21), our findings support the notion that CD137 receptor-ligand interactions regulate innate immune responses and may bridge the innate and adaptive immune responses.

References

1. Kiessling, R., E. Klein, and H. Wigzell. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells: specificity and distribution according to genotype. *Eur. J. Immunol. 5:112.*
2. Kiessling, R., E. Klein, H. Pross, and H. Wigzell. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells: characteristics of the killer cell. *Eur. J. Immunol. 5:117.*
3. Herberman, R. B., M. E. Nunn, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer 16:216.*
4. Herberman, R. B., M. E. Nunn, H. T. Holden, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer 16:230.*
5. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol. 47:187.*
6. Biron, C. A. 1997. Activation and function of natural killer cell responses during viral infections. *Curr. Opin. Immunol. 9:24.*
7. Smyth, M. J., J. M. Kelly, A. G. Baxter, H. Korner, and J. D. Sedgwick. 1998. An essential role for tumor necrosis factor in natural killer cell- mediated tumor rejection in the peritoneum. *J. Exp. Med. 188:1611.*
8. Trinchieri, G. 1995. Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis. *Semin. Immunol. 7:83.*
9. Kos, F. J. 1998. Regulation of adaptive immunity by natural killer cells. *Immunol. Res. 17:303.*
10. Kurosawa, S., M. Harada, G. Matsuzaki, Y. Shinomiya, H. Terao, N. Kobayashi, and K. Nomoto. 1995. Early appearing tumour-infiltrating natural killer cells play a crucial role in the generation of anti-tumour T lymphocytes. *Immunology 85: 338.*
11. Kos, F. J., and E. G. Engleman. 1996. Role of natural killer cells in the generation of influenza virus-specific cytotoxic T cells. *Cell Immunol. 173:1.*
12. Kos, F. J., and E. G. Engleman. 1995. Requirement for natural killer cells in the induction of cytotoxic T cells. *J. Immunol. 155:578.*
13. Soloski, M. J. 2001. Recognition of tumor cells by the innate immune system. *Curr. Opin. Immunol. 13:154.*
14. Ravetch, J. V., and L. L. Lanier. 2000. Immune inhibitory receptors. *Science 290:84.*
15. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science 285:727.*
16. Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science 285:730.*
17. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature 413: 165.*
18. Diefenbach, A., A. M. Jamieson, S. D. Liu, N. Shastri, and D. H. Raulet. 2000. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol. 1:119.*

19. Cerwenka, A., A. B. Bakker, T. McClanahan, J. Wagner, J. Wu, J. H. Phillips, and L. L. Lanier. 2000. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity 12:721.*
20. Sica, G., and L. Chen. 2000. Modulation of the immune response through 4-1BB. *Adv. Exp. Med. Biol. 465:355.*
21. Wilcox, R. A., A. I. Chapoval, K. S. Gorski, M. Otsuji, T. Shin, D. B. Flies, K. Tamada, R. S. Mittler, H. Tsuchiya, D. M. Pardoll, and L. Chen. 2002. Expression of functional CD137 by murine dendritic cells. *J. Immunol. 168:4262.*
22. Melero, I., J. V. Johnston, W. W. Shufford, R. S. Mittler, and L. Chen. 1998. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell. Immunol. 190:167.*
23. Cannons, J. L., P. Lau, B. Ghumman, M. A. DeBenedette, H. Yagita, K. Okumura, and T. H. Watts. 2001. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. *J. Immunol. 167:1313.*
24. Takahashi, C., R. S. Mittler, and A. T. Vella. 2001. Differential clonal expansion of CD4 and CD8 T cells in response to 4-1BB ligation: contribution of 4-1BB during inflammatory responses. *Immunol. Lett. 76:183.*
25. Saoulli, K., S. Y. Lee, J. L. Cannons, W. C. Yeh, A. Santana, M. D. Goldstein, N. Bangia, M. A. DeBenedette, T. W. Mak, Y. Choi, and T. H. Watts. 1998. CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. *J. Exp. Med. 187:1849.*
26. DeBenedette, M. A., A. Shahinian, T. W. Mak, and T. H. Watts. 1997. Costimulation of CD28-T lymphocytes by 4-1BB ligand. *J. Immunol. 158:551.*
27. Tan, J. T., J. K. Whitmire, K. Murali-Krishna, R. Ahmed, J. D. Altman, R. S. Mittler, A. Sette, T. C. Pearson, and C. P. Larsen. 2000. 4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination. *J. Immunol. 164:2320.*
28. DeBenedette, M. A., T. Wen, M. F. Bachmann, P. S. Ohashi, B. H. Barber, K. L. Stocking, J. J. Peschon, and T. H. Watts. 1999. Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J. Immunol. 163:4833.*
29. Blazar, B. R., B. S. Kwon, A. Panoskaltsis-Mortari, K. B. Kwak, J. J. Peschon, and P. A. Taylor. 2001. Ligation of 4-1BB (CDw137) regulates graft-versus-host disease, graft-versus-leukemia, and graft rejection in allogeneic bone marrow transplant recipients. *J. Immunol. 166:3174.*
30. Wilcox, R. A., D. B. Flies, G. Zhu, A. J. Johnson, K. Tamada, A. I. Chapoval, S. E. Strome, L. R. Pease, and L. Chen. 2002. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. *J. Clin. Invest. 109:651.*
31. Melero, I., W. W. Shuford, S. A. Newby, A. Aruffo, J. A. Ledbetter, K. E. Hellstrom, R. S. Mittler, and L. Chen. 1997. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med. 3:682.*
32. Takeda, K., H. Oshima, Y. Hayakawa, H. Akiba, M. Atsuta, T. Kobata, K. Kobayashi, M. Ito, H. Yagita, and K. Okumura. 2000. CD27-mediated activation of murine NK cells. *J. Immunol. 164:1741.*
33. Glas, R., L. Franksson, C. Une, M. L. Eloranta, C. Ohlen, A. Orn, and K. Karre. 2000. Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype: an adaptive component of NK cell-mediated responses. *J. Exp. Med. 191:129.*
34. Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med. 180:1395.*
35. Ryan, J. C., J. Turck, E. C. Niemi, W. M. Yokoyama, and W. E. Seaman. 1992. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. *J. Immunol. 149:1631.*
36. Warren, H. S., and B. F. Kinnear. 1999. Quantitative analysis of the effect of CD16 ligation on human NK cell proliferation. *J. Immunol. 162:735.*
37. Sulica, A., D. Metes, M. Gherman, T. L. Whiteside, and R. B. Herberman. 1996. Divergent effects of FcγRIIIA ligands on the functional activities of human natural killer cells in vitro. *Eur. J. Immunol. 26:1199.*