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Inhibition of NK Cell Activity through TGF-β1 by Down-Regulation of NKG2D in a Murine Model of Head and Neck Cancer[1]

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In an orthotopic murine model of head and neck squamous cell carcinoma (SCC VII/SF) we studied NK cell-mediated immunity following vaccination with a recombinant vaccinia virus expressing IL-2 (rvv-IL-2). SCC VII/SF tumor cells were injected into the oral cavity of C3H/HeJ mice on day 0. Mice were vaccinated on days 7, 10, and 14 with rvv-IL-2 and control vaccines. Phenotypes, numbers, and biological activities of NK cells were determined following vaccination. Levels of expression of NK-activating receptor NKG2D and CD16 on NK cell surface were assayed in the vaccinated mice. Expression of NKG2D ligands, Rae1, and H60 on SCC VII/SF cells was also examined. Vaccination with rvv-IL-2 resulted in expansion of NK cells. NK cells isolated from rvv-IL-2-vaccinated mice had significantly higher biological activities compared with mice treated with control vaccines. NK cells from tumor-bearing mice expressed significantly lower levels of NKG2D and CD16 compared with rvv-IL-2 vaccinated mice. SCC VII/SF tumors expressed NKG2D ligand Rae 1, although H60 was not present. SCC VII/SF tumors expressed high levels of TGF-β1, which were down-modulated by vaccination with rvv-IL-2. Incubation of NK cells with tumor homogenate or cultured supernatant of SCC VII/SF cells reduced the expression of NKG2D and CD16. This inhibition appeared to be mediated by TGF-β1. SCC VII/SF tumors in the oral cavity of the mice secrete high quantities of TGF-β1, which reduce the expression of NK cell receptor NKG2D as well as CD16 and inhibits biological functions of NK cells. *The Journal of Immunology,* **2005, 175: 5541–5550.**

Head and neck squamous cell carcinoma (HNSCC)[3] is one of the most prevalent cancer types worldwide with over 500,000 annual incidences (1). Despite significant advancement in therapeutic regimens for treating HNSCC, 5-year survival rate remains poor over the last 20 years (2). Thus, there is an urgent need for an effective adjuvant therapy for the eradication of residual disease after initial conventional therapy. Immuno-gene therapy particularly with IL-2 is promising for these patients with minimal residual disease (3). Treatment with IL-2 has produced definite tumor regression in renal cell, melanoma, and colorectal carcinomas (4). Although systemic administration of rIL-2 in HNSCC patients caused little or no response, administration of natural or rIL-2 at the tumor site or around tumor draining lymph nodes showed initial response. Unfortunately, all patients had recurrent tumor after an interval of 3–5 mo and were refractory to further IL-2 treatment (5). Local administration of IL-2 probably was less effective for the therapy of HNSCC patients due to the short half-life of this cytokine (6).

Continuous production of IL-2 at the tumor site may be more effective for the treatment of HNSCC patients. One approach to achieve this goal would be the delivery of these cytokines by replication-competent viruses. We have demonstrated that intratumoral injection of recombinant vaccinia virus (rvv) expressing IL-2 (rvv-IL-2) led to the synthesis of IL-2 at the tumor site, which lasted for ~7 days (7). Moreover, vaccinia virus is a strong immune adjuvant and invokes humoral and cellular immunity against the transgene in animal and patients (8). We are exploring the feasibility of using rvv expressing cytokines for cancer therapy. We demonstrated the antitumor efficacy of rvv expressing the cytokines GM-CSF and IL-2 in a number of animal models (7, 9–14).

Because HNSCC patients are severely immunocompromised, we are exploring the potential of this vaccine in an orthotopic murine model of head and neck cancer (SCC VII/SF). The SCC VII/SF tumor model closely resembles human HNSCC by the histological characteristics, actual anatomical site, initial locoregional aggressiveness, and lymph node/pulmonary metastasis (15).

In an earlier study using this model we demonstrated that a combined s.c. and intratumoral vaccination with rvv-IL-2 resulted in enhanced survival and tumor regression compared with intratumoral rvv-IL-2 injection alone (11). However, complete eradication of tumors was not achieved by this treatment (11). A number of immunomodulatory substances, such as TGF-β1 or IL-10, were found in the tumor homogenate, which could cause immunosuppression in this model (10).

By immunohistochemical analysis we detected considerable loss of MHC class I molecules in tumor sections (10). Tumor cells become susceptible to lysis by NK cells, when they lose MHC class I expression (16). Peritumoral injection of IL-2-activated NK cells consistently induced regression of xenografts of HNSCC (17). NKG2D has recently been shown to be the primary cytotoxicity receptor for the NK cells (16). Ligands for the NKG2D receptor in

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[3] Abbreviations used in this paper: HNSCC, head and neck squamous cell carcinoma; rvv, recombinant vaccinia virus; ADCC, Ab-mediated cellular cytotoxicity.

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humans are MHC class I-related chain A and chain B and H60/Rae1 in mice. We studied the role of NK cells, NKG2D expression, and the effect of TGF-β1 on NK cells in this murine model of HNSCC. Immunization of the mice with rvv-IL-2 led to expansion of NK cells and enhanced their cytolytic activities. Tumors from untreated mice expressed high levels of TGF-β1, which appear to inactivate NK cell activities by down-regulation of NKG2D and CD16.

Materials and Methods

Animals and reagents

We purchased tissue culture media and reagents from Invitrogen Life Technologies and 6- to 8-wk-old female, syngeneic C3H/HeJ mice from The Jackson Laboratory. We obtained monoclonal anti-mouse pan-NK/CD49b (clone DX5), rat IgM, rat IgG, anti-CD3, and anti-CD16 Abs from BD Pharmingen. We also obtained the streptavidin-HRP kit and recombinant human IL-2 (rhIL-2) from BD Pharmingen. IFN-γ, TNF-α, IL-5, and IL-10 ELISA kits, purified TGF-β1, cell staining kit, anti-mouse NKG2D, H60, Rae1, and TGF-β1 Abs were purchased from R&D Systems. FITC-labeled goat anti-rat IgG was procured from Jackson ImmunoResearch Laboratories. We obtained anti-mouse CD4, CD8, CD11b, and DX5 magnetic beads from Miltenyi Biotec.

Cell culture

We cultured YAC-1 lymphoma, EL-4 thymoma, and RMA lymphoma cells in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml amphotericin B, and 2 mM L-glutamate. Murine SCC VII tumor cells were cultured in DMEM containing 10% FCS, 100 U/ml penicillin, 100 μg/ml amphotericin B, and 2 mM L-glutamate.

Immunotherapy with rvv

We used rvv-IL-2 and control rvv, rvv expressing *Escherichia coli* β-galactosidase (rvv-lacZ), for the preparation of the vaccines. The procedures for the preparation of rvv-IL-2 and rvv-lacZ have been previously described (11). For mock vaccination, we used 100 μl of PBS (10 mM phosphate buffer (pH 7.2), 0.15 M NaCl) injected in the flank or at the tumor site of the mice as appropriate.

For the development of oral tumor, we anesthetized syngeneic C3H/HeJ mice with 3% isoflurane (Abbott Laboratories) and then injected SCC VII/SF cells (1×10^5) slowly into the floor of mouth of the mouse with a 23-gauge needle at the depth of the mylohyoid muscle (11). All procedures were performed in accordance with the University of Cincinnati Institutional Guidelines for the care and use of laboratory animals.

Mice received s.c. injection of irradiated, rvv-infected SCC VII/SF cells and intratumoral vaccination with rvv at the same time. The control group received rvv-lacZ instead of rvv-IL-2. For s.c. vaccination, we irradiated monolayers of SCC VII/SF cells with a total of 10,000 rad of gamma radiation (Cesium-167). The cells were then infected with 1×10^5 PFU/ml rvv. After 24 h of infection, we injected 1×10^4 cells in 100 μl PBS s.c. into the left flank of the mice. For intratumoral vaccination, 1×10^9 PFU of either rvv-IL-2 or rvv-lacZ were injected directly into the tumor in 100 μl of PBS. We prepared and injected the tumor cells as described on day 0. First vaccination was given on day 7 when tumor weight reached ~40–50 mg. Subsequent vaccinations were given on days 10 and 14. Tumor-free normal mice also received s.c. and intratumoral vaccination with rvv-IL2 as described.

Purification of NK cells

We isolated splenocytes from vaccinated mice on days 9 and 12 as previously described (11). Each group contains four mice and each mouse was assayed independently. For the purification of NK cells, we washed the splenocytes twice with the culture medium followed by depletion of $CD4^+CD8^+$ T cells, $CD11b^+$ cells from the bulk splenocytes using anti-CD4, anti-CD8, and anti-CD11b magnetic beads. We separated NK cells from the $CD4^-CD8^-Cd11b^-$ splenocytes pool using anti-DX5 magnetic beads. The cells were passed through the column twice according to the manufacturer's instruction and washed three times with the culture medium. Cell purity was ≥90% as assessed by flow cytometry.

Immune flow cytometry

Splenocytes were isolated from individual mouse of each experimental group on day 9 or 12. NK cells were purified from the total splenocytes as described. For NKG2D staining, a mAb specific for NKG2D was used. FcR binding was prevented by preincubation of the cells with FcR-specific Ab. We harvested ~1×10^6 $CD4^-CD8^-CD11b^-DX5^+$ NK cells from each experimental group and suspended them in 100 μl of PBS containing 2% FCS. Purified anti-mouse anti-NKG2D Ab (20 ng/tube) was added to the cells and cells were incubated for 30 min at 4°C. After two washings, cells were incubated with 250 ng of FITC-labeled goat anti-rat IgG and incubated for 30 min at 4°C. For NKG2D ligand analysis, 1×10^6 SCC VII/SF, YAC-1, or RMA tumor cells were stained with purified anti-mouse H60 or Rae1 Ab (20 ng). Secondary Ab treatment was performed as previously described for NKG2D. For phenotyping NKT cells, we used a CD3-FITC/DX5-PE combination of Abs, whereas for staining FcR on the NK cells, a CD16-FITC/DX5-PE combination of Abs was used. Before analysis, cells were washed twice in cold buffer and fixed in 1% paraformaldehyde. Immunoflow was performed on a FACSCalibur microfluorometer (BD Biosciences).

Proliferation of NK cells

We incubated 2×10^5 purified NK cells in each well of a 96-well flat-bottom tissue culture plate in the presence of irradiated YAC-1 or EL-4 cells (1×10^3) in a total volume of 200 μl for 5 days. For irradiation, a monolayer of YAC-1 or EL-4 cells was treated with a total of 8000 rad of gamma radiation (Cesium-167). We pulsed the cells with 1 μCi of [^{3}H]thymidine for 18 h before harvesting. We performed each assay in triplicate and determined the stimulation indices by dividing the mean CPM of each sample by the CPM in presence of medium only as described earlier (18).

Determination of cytotoxicity of total splenocytes and purified NK cells

Total splenocytes or purified NK cells were prepared on day 9 after first vaccination as previously described from tumor-bearing PBS, rvv-lacZ-, and rvv-IL-2-vaccinated as well as tumor-free normal mice. Tumor-free normal mice vaccinated with rvv-IL-2 were also included in the experiment. Following two washes, we cultured bulk splenocytes and $DX5^+$ NK cells in RPMI 1640 medium in the presence of irradiated YAC-1 cells as stimulant, supplemented with rhIL-2 (1 ng/well) for 5 days. For the preparation of the target cells, we labeled 1×10^6 YAC-1 or control EL-4 cells with 200 μCi of Na_2 [$^{51}CrO_4$] and incubated the cells at 37°C for 1 h. We washed the cells four to five times with cold medium and adjusted the volume to have a target cell concentration of 4×10^4/ml. We placed 100 μl of labeled target cells in a well of a 96-well round-bottom plate. We added total splenocytes or purified $DX5^+$ cells (effector) from individual mouse to obtain E:T ratios of 12.5:1, 25:1, 50:1, and 100:1 in a final volume of 200 μl. The assays were performed in triplicate. Plates were incubated for 6 h in a tissue culture incubator and the culture supernatant was assayed for ^{51}Cr release using a gamma radiation counter. Percentage of specific lysis was determined by the formula: [(sample release − spontaneous release)/(maximum release − spontaneous release)] × 100.

To specifically block NKG2D receptor on the NK cells, in a parallel experiment, we used a mAb specific for NKG2D at a concentration of 2 μg/ml according to the manufacturer's instruction (R&D Systems). Purified NK cells prepared from the rvv-IL-2 group were used for this experiment. Cells were incubated with anti-NKG2D Ab for 1 h in a tissue culture incubator before cytotoxicity assay. Appropriate control Ab was also used in the blocking experiments. Cytotoxicity assay and determination of the percentage of specific lysis was as previously discussed.

Secretion of cytokines by stimulated NK cells

Amounts of IFN-γ, TNF-α, IL-5, and IL-10 in the tissue culture supernatant were measured by commercially available ELISA kits (R&D Systems). Purified NK cells were stimulated with irradiated YAC-1 or control EL-4 cells for 48 h for the determination of TNF-α, IL-5, and IL-10 and for 72 h for IFN-γ. All experiments were performed in triplicate. The lower limit for the cytokine detection was 5 pg/ml.

Immunohistochemistry

Fresh tumor tissues were paraffin embedded following standard protocols. Serial sections (5 μm) were deparaffinized with xylene and rehydrated in water. Sections were incubated in PBS for 2 min and stained with anti-TGF-β1 Ab as described earlier (10). Briefly, after three washes with PBS, slides were incubated in 0.3% H_2O_2 followed by blocking with rabbit serum for 15 min. We incubated the tumor sections with anti-TGF-β1 for 1 h and washed the slides extensively with PBS. Biotin-labeled anti-chicken IgY was used as the secondary Ab. After extensive PBS wash we incubated the slides with streptavidin-HRP followed by diaminobenzidine solution. The sections were counterstained in Harry's hematoxylin. Matched isotypic controls were used in each case. We repeated each experiment at least three times. For counting the positive cells and intensity measurements we

used Metamorph software (Universal Imaging) as described earlier (10). At least 10 fields were chosen at random for counting and data are expressed as mean ± SE. For intensity measurements, the lowest value (≤50 cells) was represented by a plus sign and each fold increase in intensity was represented by an additional plus sign (10). For comparison, all sections were processed in parallel. At least five mice from each group were analyzed.

Preparation of tumor homogenate

Fresh primary oral tumors (100 mg) were thoroughly washed and homogenized in a hand-held homogenizer in 5 ml serum-free DMEM as described (10). Cell-free supernatant was collected and stored frozen at −80°C.

Collection of SCC VII/SF culture supernatant

Primary tumor was minced into small pieces and gently passed through 40-μm nylon mesh to have a suspension. Cells were separated by centrifugation at 1000 rpm for 5 min, and cultured for 5 days in complete DMEM. Cell-free supernatant (50 μl) was collected on days 2, 3, 4, and 5, and TGF-β1 was assayed using ELISA kit (R&D Systems).

Effect of cell-free tumor homogenate and culture supernatant on NK cells

For the determination of the effect of tumor homogenate or culture supernatant on NK cells, NK cells (3×10^6) from the mice vaccinated with rvv-IL-2 were cultured with rhIL-2 (1 ng/well) in the presence of tumor homogenate/culture supernatant (100 μl/ml) or recombinant active TGF-β1 protein (10 ng/ml) for 72 h. Before addition of the tumor homogenate or culture supernatant, NK cells were treated with 1 N HCl for 10 min at room temperature to generate immunoreactive active TGF-β1. In a parallel experimental set up, anti-TGF-β1 mAb (1 μg/ml) was also added to the triplicate wells containing tumor homogenate/culture supernatant or TGF-β1 protein to neutralize the biological activities of TGF-β1. Following incubation of the cells in a tissue culture incubator for 72 h, we determined NKG2D expression on the treated NK cells at the mRNA and

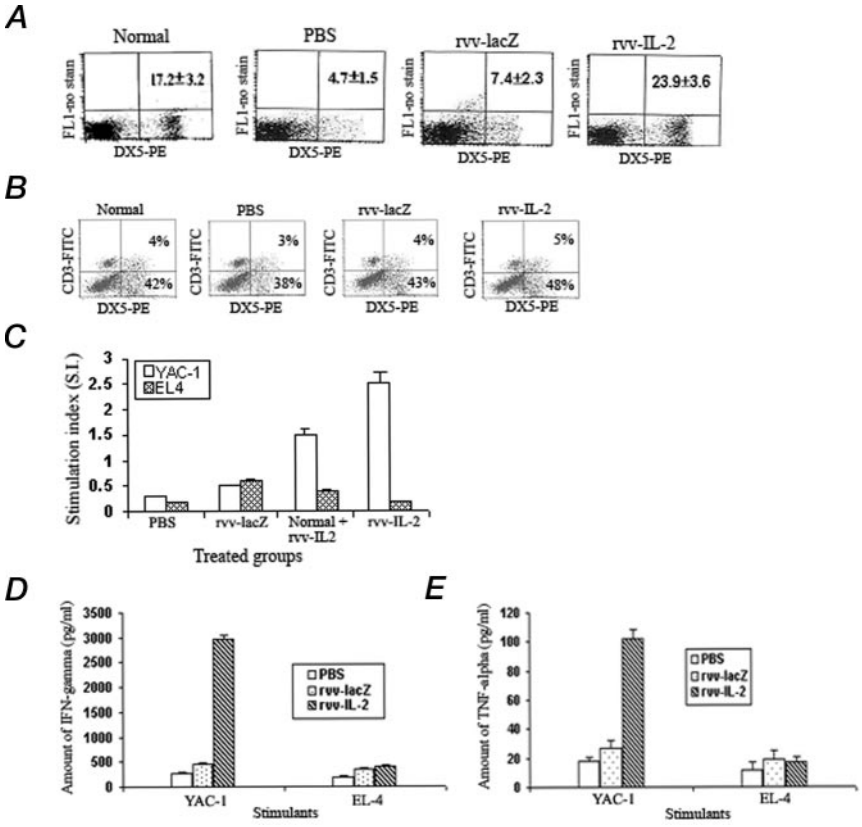

FIGURE 1. Analysis of phenotypes of NK cells and their biological activities. Total splenocytes were isolated from tumor-bearing individual mouse vaccinated with PBS, rvv-lacZ, or rvv-IL-2 on day 9, and their phenotypes were determined by flow cytometry. Total splenocytes isolated from tumor-free normal mice were also phenotyped. For the determination of biological activities (*C–E*), NK cells were purified immunomagnetically from total splenocytes. Each group contained four mice. *A*, Total splenocytes were stained with PE-conjugated anti-mouse NK cell (DX5) and isotype-matched Ab. Positive NK cell population is shown in the *lower right quadrant* (FL2) for each group and the value shown in the *upper right quadrant* represents the mean ± SE of four mice analyzed independently. No Ab was used for FL1 channel (FL1-no stain) analysis. Percentage of NK cells was significantly reduced in tumor-bearing mice and mice treated with rvv-lacZ. In the splenocytes of the rvv-IL-2-vaccinated group, the number of NK cells was increased to a normal level. *B*, Number of NKT cells in purified NK population was determined by double staining with DX5-PE/CD3-FITC Abs along with appropriate isotype controls. NK cells were purified from tumor-bearing mice vaccinated with PBS, rvv-lacZ, or rvv-IL-2 on day 9. Total splenocytes isolated from tumor-free mice were also phenotyped. The double positive cells are shown in the *upper right quadrant*. The number of NKT cells did not change either by tumor transplantation or vaccination. *C*, Proliferation (Stimulation Index) of NK cells in the presence of irradiated YAC-1 cells was determined using purified NK cells harvested on day 9. PBS-, rvv-lacZ-, and rvv-IL-2-vaccinated tumor-free normal mice served as controls. Irradiated EL-4 cells served as control stimulant. We incubated 2×10^5 cells in each well of a 96-well flat-bottom tissue culture plate in the presence of irradiated YAC-1 or EL-4 cells (1×10^3) in a total volume of 200 μl for 5 days. For irradiation, monolayer of YAC-1 or EL-4 cells was treated with a total of 8000 rad of gamma radiation (Cesium-167). Stimulation index of the purified NK cells from tumor-bearing rvv-IL-2-vaccinated mice in the presence of YAC-1 stimulant was significantly higher compared with control PBS-, rvv-lacZ-, or rvv-IL-2-vaccinated tumor-free normal mice ($p < 0.001$). Stimulation was negligible in the presence of control EL-4 cells. For cytokine ELISA, NK cells were harvested from PBS-, rvv-lacZ-, and rvv-IL-2-vaccinated mice and stimulated for 48 h in a tissue culture incubator for the determination of TNF-α, IL-5, and IL-10 and 72 h for IFN-γ. Purified NK cells from rvv-IL-2 group secreted significantly higher ($p < 0.002$) amounts of IFN-γ (*D*) and TNF-α (*E*) compared with the control PBS or rvv-lacZ vaccination groups when stimulated with irradiated YAC-1 cells. Similar results were obtained by repeating the experiments two more times.

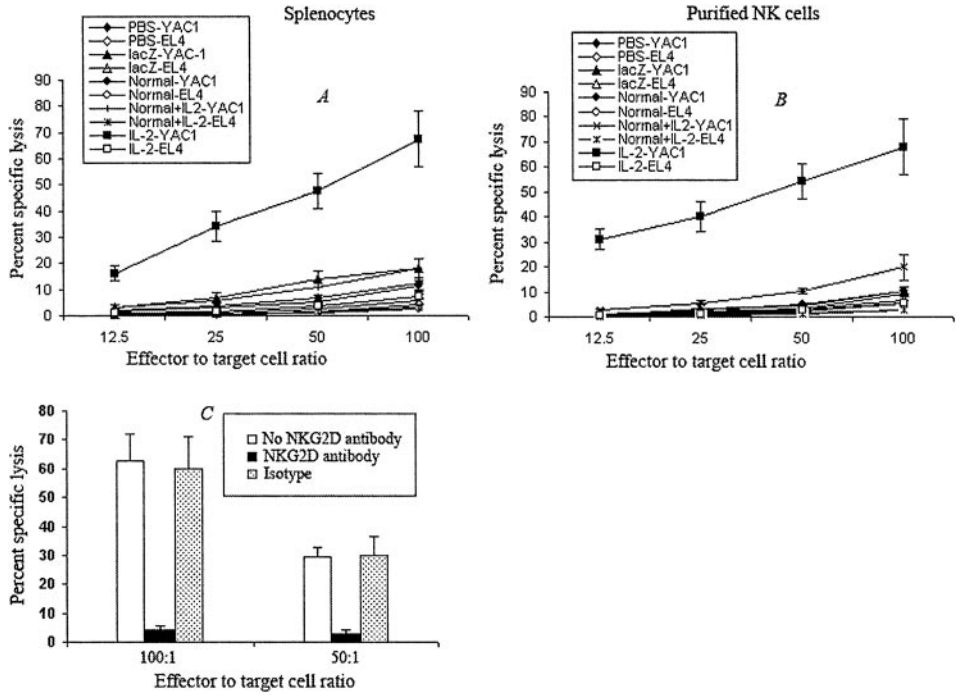

FIGURE 2. Cytolysis of YAC-1 cells by total splenocytes or purified NK cells. Total splenocytes or purified NK cells were prepared on day 9 after first vaccination from tumor-bearing PBS, rvv-lacZ (lacZ), and rvv-IL-2 (IL-2) as well as tumor-free normal mice (Normal). Tumor-free normal mice vaccinated with rvv-IL-2 (Normal+IL-2) were also included in the experiment. Cytotoxicity was determined by incubating total splenocytes or NK cells (effector) with ^{51}Cr-labeled YAC-1 or control EL-4 cells (target) for 6 h in a tissue culture incubator as described in *Materials and Methods*. Cytolysis of the YAC-1 cells by either splenocytes (*A*) or purified NK cells (*B*) was significantly higher in rvv-IL-2-vaccinated mice compared with all the control vaccination groups ($p < 0.0001$). *C*, In a select set of experiments, cytotoxicity of the purified NK cells isolated from rvv-IL-2-vaccinated mice on day 9 was examined in the presence of a mAb specific for NKG2D receptor. Isotype-matched irrelevant Ab was used as control in this experiment. Cytolysis of YAC-1 cells by purified NK cells was significantly inhibited ($p < 0.0001$) by anti-NKG2D mAb. Addition of isotype-matched Ab had no effect on NK cell-mediated cytolysis. The results shown are representative of two independent experiments.

protein levels by real-time RT-PCR and flow cytometry, respectively. Biological activities of the treated NK cells were determined by in vitro lysis of YAC–1 cells.

Quantitative real-time RT-PCR analysis of NKG2D receptor

Total RNA was prepared from individual mouse splenocytes or DX5$^+$ NK cells of rvv-vaccinated mice on day 12 as described earlier (7). For quantitative analysis, total RNA extracted was subjected to real-time RT-PCR using Cepheid SmartCycler system in triplicate as described earlier (10). The primers used were β-actin (432 bp) forward 5′-GGGACGACATG GAGAAGA-3′, reverse 5′-AAGTCTAGAGCAACATAGCA-3′; NKG2D (667 bp) forward 5′-CGACCTCAAGCCAGCAAAGTG-3′, reverse 5′-TGTTGCTGAGATGGGTAATG-3′. Before real-time RT-PCR analysis of the target gene, expression level of the internal control β-actin in all samples was normalized with respect to the threshold cycle number (Ct). Relative fold number of the target gene was then expressed with respect to the normalized internal control β-actin in each case. Lowest expression of NKG2D on non-IL-2-treated normal NK cells was considered as 1. PCR was conducted in 20 μl of reaction volume using SYBR Green QuantiTect RT-PCR kit (Qiagen). We set the reaction condition according to the manufacturer's instructions. Briefly, reverse transcription was conducted at 50°C for 20–30 min. Inactivation of reverse transcriptase, HotStarTaq DNA polymerase activation, and template cDNA denaturation were conducted at 95°C for 15 min. The final cDNA amplification step comprised of 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Data analysis was done using $2^{-\Delta\Delta Ct}$ method (19). Relative fold number of the target gene expression was determined by subtracting the threshold cycle value of the reference gene from the threshold cycle value of target gene in all cases.

Conventional RT-PCR

For conventional RT-PCR the reaction was conducted in a 50 μl of volume using Qiagen OneStep RT-PCR kit. The primers for β-actin and NKG2D genes and reaction conditions were same as discussed except for PCR cycle number, which was reduced to 25. For the determination of mRNA for H60/Rae1 ligands, we isolated total RNA from SCC VII/SF, YAC-1, or RMA tumor cells. Total RNA (10 ng) was used in a 50 μl of reaction volume as used earlier for 40 cycles using the following primers: H60 forward 5′-TTC TGA CAC CCA GGG TCT GAG-3′, reverse 5′-CAC CTG GAT AAA TCT GCA GG-3′ and Rae1 forward 5′-TTA TCC GCA AAG CCA GGG CC-3′, reverse 5′-TTC CCC ATC ATC AAA CCA TT-3′.

NK cell-mediated Ab-dependent cellular cytotoxicity (ADCC)

ADCC was determined as described earlier (18). NK cells purified from naive or PBS vaccinated mice on day 9 were activated with rhIL-2. For sensitizing the target cells (SCC VII/SF), sera from tumor-bearing mice vaccinated with PBS, rvv-lacZ, or rvv-IL-2 on day 9 were used. Target cells (1×10^6) in serum-free media were labeled with 200 μCi of $Na_2[^{51}CrO_4]$. IL-2-activated purified NK cells were used as effector cells. Labeled cells were added at a concentration of 1×10^4 cells/well in a 96-well plate followed by the addition of 100 μl of diluted mouse serum from vaccinated mice, and 1×10^6 of effector cells in 50 μl of serum-free DMEM. Plates were incubated for 6 h in a tissue culture incubator. Determination of percentage of specific lysis was as earlier described.

Statistical analysis

We used the Student *t* test for normally distributed variables. When the data did not fulfill the criteria of being normally distributed, we used nonparametric statistics (Mann-Whitney *U* rank sum test). We performed all statistical evaluations using SigmaStat software (Jandel), and values for $p < 0.05$ were considered to indicate statistical significance.

Results

Decrease of the numbers and biological activities of NK cells in tumor-bearing mice

As a first step to investigate the NK cell immunity in this model, we determined the numbers and phenotypes of NK cells in the

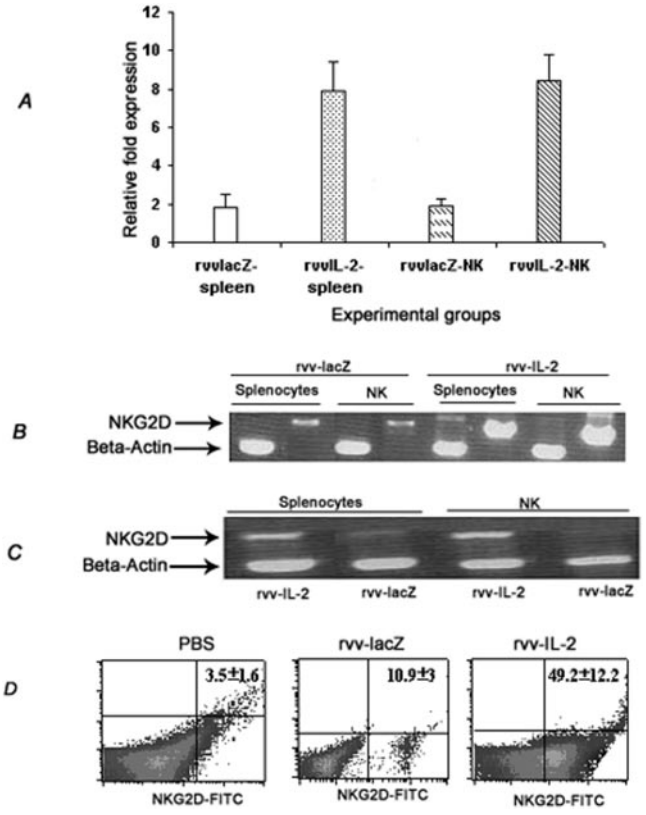

FIGURE 3. Expression of NKG2D in the splenocytes and purified NK cells of the vaccinated mice. Total RNA was isolated from splenocytes or purified NK cells of individual mice on day 9 and subjected to real-time as well as conventional multiplex RT-PCR analysis. The housekeeping gene β-actin was used as internal control in all RT-PCR analyses. In the real-time RT-PCR analysis, relative fold expression of the target NKG2D gene with respect to the normalized control β-actin was determined. Values are expressed as mean ± SE of relative mRNA content in splenocytes or purified NK cells from three individual mice analyzed. *A*, Expression of mRNA encoding NKG2D in both splenocytes and purified NK cells was significantly higher ($p < 0.001$) in the rvv-IL-2-vaccinated mice compared with control rvv-lacZ mice. *B*, Representative gel photographs of real-time analysis are shown. *C*, Using a 25-cycle conventional multiplex RT-PCR analysis, NKG2D expression was undetectable in the bulk splenocytes or NK cells of the control rvv-lacZ group, whereas the NKG2D band was clearly visible in both splenocytes and NK cells obtained from the rvv-IL-2 group. *D*, Purified NK cells harvested from the PBS-, rvv-lacZ-, and rvv-IL-2-vaccinated groups on day 9 were stained with a mAb specific for NKG2D receptor as described in *Materials and Methods*. The percentage of NKG2D expressing cells (*upper right quadrant*) was significantly higher in the rvv-IL-2-vaccinated group compared with the control rvv-lacZ or PBS groups ($p < 0.0001$). Representative data from two independent experiments are shown.

tumor-bearing mice. Data in Fig. 1*A* demonstrate that tumor-bearing, mock-vaccinated mice had approximately one-third the number of NK cells in splenocytes compared with naive mice on day 9 after tumor implantation and after one-time vaccination. Treatment with the control vaccine, rvv-lacZ, had negligible effect on NK cell number. However, vaccination with rvv-IL-2 restored the number of NK cells to the normal level. IL-2 is a growth and activation factor for NK cells. Rvv-IL-2 vaccination probably resulted in the expansion of NK cells. Like NK cells, NKT cells ($DX5^+/CD3^+$) may also serve as tumor killer cells. Analysis of phenotype (Fig. 1*B*) demonstrated that the number of NKT cells in this model was low and the numbers were similar in all groups of mice.

Biological activities of the NK cells were first determined by proliferation of these cells in response to appropriate stimulants. NK cells were purified from bulk splenocytes of vaccinated mice and cocultured with irradiated YAC-1 or control EL-4 cells. As shown in Fig. 1*C*, the stimulation index of the NK cells on day 9 was significantly higher in the rvv-IL-2-vaccinated mice compared with rvv-lacZ- or mock-vaccinated groups when incubated in the presence of irradiated YAC-1 cells as stimulant ($p < 0.001$). Proliferation of NK cells harvested from tumor-free normal mice treated with rvv-IL-2 was significantly higher compared with PBS- and rvv-lacZ-treated mice ($p < 0.001$) but significantly lower compared with the tumor-bearing mice vaccinated with rvv-IL-2 ($p < 0.003$) using YAC-1 stimulant. Proliferation of NK cells in the presence of control EL-4 cells was negligible in all vaccinated groups.

Activation of NK cells results in the production of specific Th type cytokines (20): IFN-γ and TNF-α (Th1 type) or IL-3, IL-5, IL-10, IL-13 (Th2 type). Functionally mature NK cells lose the ability to produce Th2 cytokines and acquire the capacity to produce Th1 cytokines IFN-γ and TNF-α (20). To determine the type of Th cytokine production in this study, we determined the levels of IFN-γ, TNF-α, IL-5, and IL-10 in the culture supernatant of purified NK cells on day 9 as described in *Materials and Methods* when all control groups of mice were alive. As shown in Fig. 1, *D* and *E*, secretion of both IFN-γ and TNF-α was significantly higher in the rvv-IL-2-vaccinated mice compared with the control rvv-lacZ or PBS vaccinated mice ($p < 0.002$). The secretion of IL-5 and IL-10 was, however, negligible in all the treatment groups (data not shown). Cytokine secretion in presence of control EL-4 cells was negligible.

Cytotoxicity of NK cells

Stimulated and activated NK cells also acquire cytotoxic ability. Therefore, we determined cytotoxicity of the bulk splenocytes as well as purified NK cells on day 9 following 2 days after first vaccination on day 7. Lysis of the target YAC-1 cells by either splenocytes (Fig. 2*A*) or purified NK cells (Fig. 2*B*) was significantly higher on day 9 in the rvv-IL-2-vaccinated mice compared with the control rvv-lacZ, mock-vaccinated PBS group, or tumor-free normal mice treated or untreated with rvv-IL-2 vaccine ($p < 0.0001$). The percentage of lysis ranged from 67.4 to 68.1% in the rvv-IL-2 group compared with only 6.4 to 20% in the all the control groups. Lysis of the control target EL-4 cells was low and ranged from 5 to 7.7% in all the vaccinated groups.

To validate whether NKG2D receptor was involved in NK cell-mediated cytotoxicity in this model, we used a mAb specific for NKG2D as a blockade in the cytotoxicity assay. We used NK cells isolated from days 9 and 12 after the first (day 7) and second (day 10) rvv-IL-2 vaccinations. Results in Fig. 2*C* demonstrate that the YAC-1 lysis by NK cells was completely blocked by this mAb at all E:T cell ratios with NK cells isolated from mice on day 9 after tumor implantation. Similar results were obtained with NK cells isolated on day 12 (data not shown). The inhibition ranged from 96 to 98%. Addition of control isotype had negligible effect on the NK cell activity. These data support the evidence that this lysis is mediated by NKG2D receptor.

Analysis of NKG2D expression by the immune cells of rvv-vaccinated mice by real-time RT-PCR

Murine NKG2D plays a key role in developing antitumor immunity because it acts as a primary receptor for cytotoxicity of the activated NK cells. Results presented in Fig. 2*C* demonstrated the complete inhibition of NK-mediated lysis of the YAC-1 cells by the anti-NKG2D Ab. We determined the expression of NKG2D

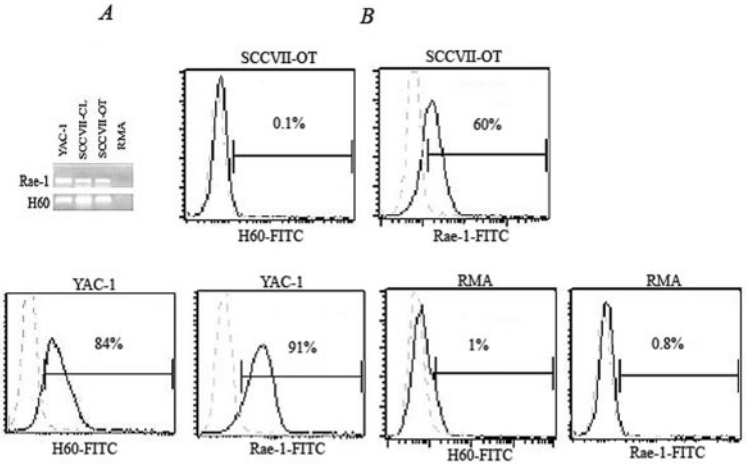

FIGURE 4. Expression of NKG2D receptor specific ligands H60 and Rae 1 by the tumor cells. *A*, Total RNA was isolated from cultured SCC VII/SF cells, (SCCVII-CL), single cell suspension of oral tumor (SCCVII-OT), YAC-1, and RMA cells, subjected to conventional RT-PCR, and analyzed by agarose gel electrophoresis as described in *Materials and Methods*. Cultured or in vivo oral tumor-derived SCC VII/SF cells expressed H60 and Rae1 ligands at the mRNA level. *B*, The cells were also stained with purified mAb specific for H60 or Rae1 ligands and subsequently treated with FITC-labeled goat anti-rat IgG as secondary Ab for flow cytometric analysis. Isotype-matched FITC-conjugated irrelevant Ab (dotted histogram) was used in each case as a negative control. SCC VII/SF cells from oral tumors do not express H60 at the protein level but 60% of the cells expressed Rae1. Cultured YAC-1 and RMA cells were used as positive and negative control in these experiments.

both at the mRNA and protein levels in the splenocytes and NK cells isolated from rvv-vaccinated mice. To determine the expression at the mRNA level we performed real-time RT-PCR analysis. Before real-time analysis of the target gene, expression of the internal control β-actin gene was normalized in all samples in terms of the threshold cycle number (Ct). As shown in Fig. 3*A*, relative expression fold level of NKG2D was significantly higher in the splenocytes of mice vaccinated with rvv-IL-2 compared with the control rvv-lacZ group (7.9- vs 1.8-fold; $p < 0.001$). Relative expression fold level of NKG2D in the purified NK cells was also significantly higher in the rvv-IL-2-vaccinated mice compared with control rvv-lacZ-vaccinated mice (8.4- vs 1.9-fold; $p < .0001$). Representative gel photograph of the real-time analysis are shown in Fig. 3*B*.

We also performed conventional multiplex RT-PCR with normalized RNA samples from both splenocytes and purified NK cells from rvv-vaccinated groups, used in these experiment. By multiplex RT-PCR analysis with the same samples, we could not detect NKG2D expression by 25 cycles in the rvv-lacZ group, whereas a distinct band was visible in the rvv-IL-2-vaccinated group (Fig. 3*C*). These results further confirm relative lower expression of the NKG2D in the NK cells from the control rvv-lacZ-vaccinated group compared with those from mice vaccinated with rvv-IL-2. Expression of mRNA does not always correspond with protein expression due to instability of mRNA. Therefore, we stained purified NK cells from the vaccinated mice on day 9 and 12 with a mAb specific for NKG2D receptor. As shown in Fig. 3*D*, on day 9, a number of NKG2D expressing NK cells was significantly higher in the vaccinated rvv-IL-2 group compared with the control rvv-lacZ group (49.2 ± 12.2% vs 10.9 ± 3.1%; $p < 0.0001$) or PBS group (49.2 ± 12.2% vs 3.5 ± 1.6%; $p < 0.0001$). The NKG2D expressing NK cell number remained significantly higher on day 12 in the rvv-IL-2-vaccinated mice compared with the rvv-lacZ-treated mice (75.7 ± 19.7% vs 13.2 ± 4.3%; $p < 0.0001$) (data not shown). We could not compare further because all mice from the control groups were dead by day 12. Apart from NK cells, almost all the $CD4^+$ and $CD8^+$ T cells from the rvv-IL-2-vaccinated group also expressed NKG2D (data not shown).

Expression of H60 or Rae1 ligands by the SCC VII tumor cells

Tumor cells expressing ligands specific for NKG2D receptor are susceptible for lysis by NK cells. Two ligands, H60 and Rae1 are commonly expressed in NK susceptible mice tumors (21). To investigate whether the SCC VII/SF tumor cells express the H60 and/or Rae1 ligands, we set up experiments to determine the expression of these ligands both at the mRNA and protein levels. YAC-1 cells served as the positive and RMA cells as the negative controls for the presence of these ligands. At the mRNA level both the cultured and tumor-derived SCC VII/SF cells expressed both H60 and Rae1 (Fig. 4*A*). At the protein level, 60% of the tumor-derived SCC VII/SF cells expressed Rae1, whereas only 0.1% of these cells expressed H60 ligand (Fig. 4*B*).

TGF-β1 secretion by tumor cells and its effect on NKG2D expression and NK cell cytotoxicity

We reported previously that cultured SCC VII/SF cells do not express TGF-β1 protein, whereas tumors from oral cavity expressed TGF-β1 at the mRNA as well as at protein levels (7, 10). Immunohistochemical analysis of tumor samples for TGF-β1 showed high expression of active forms of TGF-β1 in tumors from mock- and rvv-lacZ-vaccinated mice (Fig. 5*A*). The expression of TGF-β1 was significantly reduced in tumors from mice vaccinated with rvv-IL-2. Single cell suspension of SCC VII/SF tumors from PBS mice secreted high amounts of TGF-β1 for at least 5 days (Fig. 5*B*). It has been recently reported that in cancer patients TGF-β1 impaired NK cytotoxicity by down-modulation of NKG2D (22). Therefore, we determined the effect of TGF-β1 on NKG2D expression of NK cells and the cytotoxicity of YAC-1 cells. We incubated NK cells purified from the rvv-IL-2-vaccinated group with either tumor homogenate or culture supernatant harvested from single cell suspension of SCC VII/SF tumors from PBS mice. As shown in Fig. 5, addition of either tumor homogenate or culture supernatant significantly down-regulated the expression of NKG2D at the mRNA level ($p < 0.001$) (Fig. 5*C*) as well as at the protein level ($p < 0.02$) (Fig. 5*D*). Moreover, tumor cell homogenate as well as cultured supernatant from the single cell suspension significantly reduced the NK cell-mediated lysis ($p < 0.001$) of YAC-1 cells (Fig. 5*E*). Purified TGF-β1 inhibited the expression of NKG2D and NK cell-mediated lysis of YAC-1 cells to similar extend. Both the inhibition of expression of NKG2D and the inhibition of lysis of YAC-1 cells could be partially restored by the monoclonal anti-TGF-β1 Ab, suggesting that the impairment of NK cells are mediated through TGF-β1 by down-modulation of NKG2D.

Expression of CD16 and ADCC by NK cells from vaccinated mice

ADCC is another mechanism for antitumor immunity mediated by the NK cells and macrophages. NK cells induce ADCC through CD16 molecule, which is present on its cell surface in conjunction

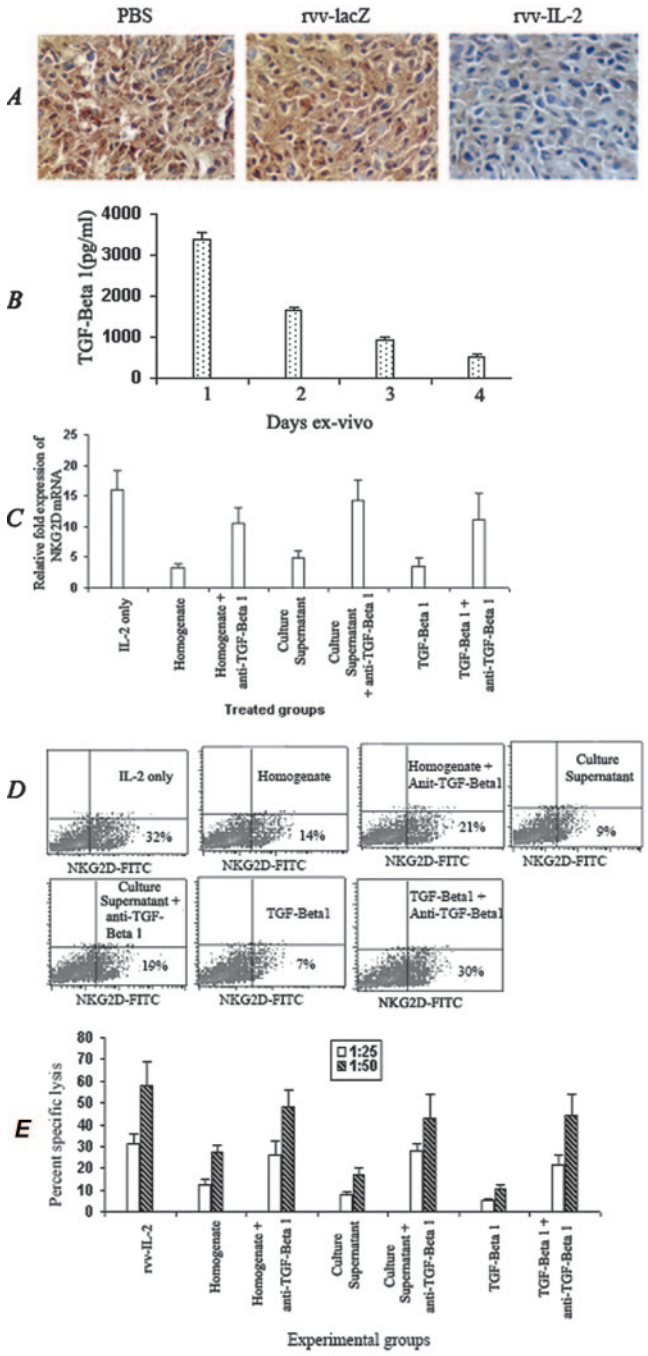

FIGURE 5. Effect of TGF-β1 on the expression of NKG2D receptor and NK-mediated cytotoxicity. *A*, Paraffin-embedded tumor sections (5 μm) from vaccinated mice were stained with anti-TGF-β1 mAb as described in *Materials and Methods*. Tumors from mock-vaccinated and rvv-lacZ-treated mice expressed significantly higher levels of TGF-β1 compared with the rvv-IL-2-vaccinated mice ($p < 0.0001$). Intensity measurements were represented by a plus sign and each fold increase in intensity was represented by an additional plus sign. *B*, Tumors from mock-vaccinated mice secrete high levels of TGF-β1 for several days. NK cells from individual mice in the rvv-IL-2-treated group were cultured with rhIL-2 (1 ng/well) in the presence or absence of activated cell-free tumor homogenate, culture supernatant of mock-vaccinated mice, or recombinant active TGF-β1. Expression of NKG2D mRNA (*C*) and protein (*D*) was determined by real-time RT-PCR and FACS analysis, respectively, after 72 h of incubation of the cells in a tissue culture incubator. Expression of NKG2D mRNA was significantly inhibited ($p < 0.001$) in the NK cells treated with the tumor homogenate, culture supernatant, or TGF-β1 protein compared with the rhIL-2-treated NK cells (*C*). The inhibition was considerably reversed by anti-TGF-β1 Ab. Values shown are mean $\pm$ SE of three mice analyzed. One pool of treated cells was stained with an anti-NKG2D mAb along with appropriate isotype control. For FcR block, anti-mouse CD16 Ab was used. As with mRNA, expression of NKG2D protein was also significantly inhibited ($p < 0.02$) in the presence of tumor homogenate, cultured supernatant, and recombinant TGF-β1 protein (*D*). The inhibition was considerably reversed by anti-TGF-β1 Ab. *E*, YAC-1 lysis by NK cells was also significantly inhibited ($p < 0.001$) by the treatment of the NK cells with tumor homogenate, cultured supernatant, or TGF-β1 protein at different E:T ratios (50:1, 25:1). Addition of anti-TGF-β1 Ab significantly restored cytolytic activities of the NK cells.

with Abs to the target cell. In the rvv-IL-2- and rvv-lacZ-vaccinated groups the proportion of $CD16^+$ NK cells was similar to that in the naive mice (Fig. 6*A*). However, the proportion of $CD16^+$ NK cells from tumor-bearing, mock-vaccinated mice was significantly reduced (9%). To determine whether the NK cells could lyse SCC VII/SF cells in presence of sera obtained from vaccinated mice, we performed ADCC assay with purified NK cells isolated from naive mice. Presumably tumor-specific Abs present in the serum would bind to the tumor cells and render them sensitive to killing by NK cells. As shown in Fig. 6*B*, ADCC by NK cells was significantly higher when target cells were preincubated with diluted sera (1/5 or 1/10) from the vaccinated rvv-IL-2 group compared with sera from rvv-lacZ ($p < 0.001$) or PBS group ($p < 0.001$). ADCC was negligible in the absence of serum. Incubation of NK cells with anti-CD16 Ab completely blocked the lysis of tumor cells by ADCC (data not shown).

When NK cells were isolated from tumor-bearing mock-vaccinated mice and ADCC was performed with sera from rvv-IL2-vaccinated mice, ADCC was significantly lower compared with NK cells from rvv-IL-2-vaccinated group (Fig. 6*C*). These data suggested that expression of CD16 on the NK cells of the tumor-bearing mice was down-modulated, which resulted in the reduction of ADCC mediated by these NK cells.

Tumor growth, number, and biological activities of NK cells following second and third vaccination of rvv-IL-2 mice

Therapy was started on day 7 when mean tumor weight reached 43 $\pm$ 4 mg. On day 12 after second vaccination, mean tumor weight was 123 $\pm$ 14 mg in the rvv-lacZ-vaccinated mice, whereas in the rvv-IL-2-vaccinated mice, tumor growth was significantly inhibited ($p < 0.001$) and the mean tumor weight was recorded as 53 $\pm$ 4 mg (Fig. 7). By day 16, definite tumor regression was noted in the rvv-IL-2-vaccinated mice when mean tumor weight was recorded as 25 $\pm$ 7 mg (Fig. 7). On day 12, the number of NK cells in the rvv-IL-2-vaccinated mice was significantly higher (41.8 $\pm$ 5.7 vs 8.4 $\pm$ 2.1, $p < 0.0001$) compared with the number found in rvv-lacZ-vaccinated mice (Fig. 7). Cytotoxicity of the NK cells was significantly higher in the rvv-IL-2-vaccinated group on day 12 compared with the control rvv-lacZ (45 $\pm$ 5.6% vs 9 $\pm$ 1.2%, $p < 0.0001$). The lysis of control target EL-4 cells ranged from 4.9 to 7.8% in both the rvv-vaccinated groups on day 12. We could not compare further because all mice died by day 12 in the rvv-lacZ-vaccinated group. On day 16, the number of NK cells was 57.2 $\pm$ 6.6 and cytotoxicity was recorded as high as 41 $\pm$ 7% in the rvv-IL-2-vaccinated group. Lysis of control EL-4 cells was negligible and at the background level.

Discussion

In an orthotopic murine model of HNSCC, we demonstrated that a combined s.c. plus intratumoral rvv-IL-2 injection induced significant tumor regression and enhanced survival (11). Because NK cell activity occurs predominantly during the early phases of the

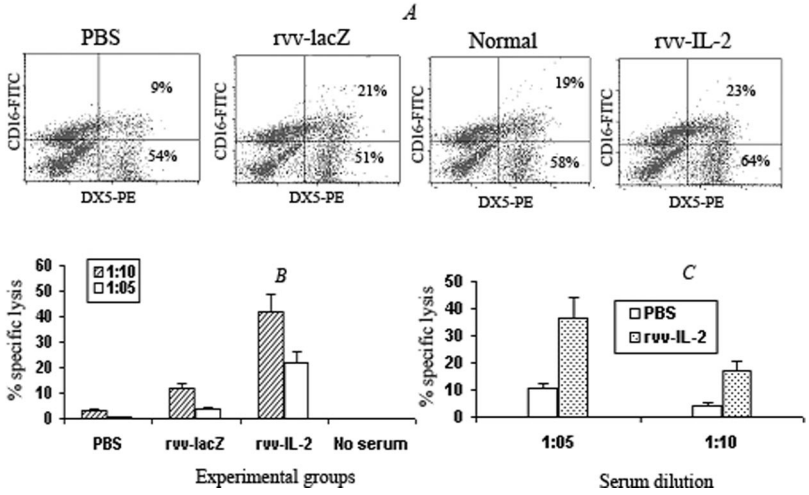

FIGURE 6. Expression of CD16 and ADCC of the purified NK cells from naive and vaccinated mice. *A*, Purified NK cells obtained from different groups of mice on day 9 were double stained with PE-DX5/FITC-CD16 Abs along with the appropriate isotype controls. Except for the PBS group (9%), 21–23% of NK cells from the rvv-vaccinated and naive mice expressed CD16. *B*, Purified NK cells from naive mice were used as effectors for ADCC of ^{51}Cr-labeled target SCC VII/SF cells. Before ADCC, SCC VII/SF cells were preincubated with diluted serum from PBS-, rvv-lacZ-, and rvv-IL-2-vaccinated groups as indicated. Values are mean ± SD in all vaccine groups each consisting of four mice. ADCC was significantly higher ($p < 0.0001$) for the target cells coated with serum from rvv-IL-2-vaccinated mice. *C*, ADCC was significantly lower ($p < 0.002$) when NK cells from tumor-bearing PBS-vaccinated mice together with sera from rvv-IL-2-vaccinated mice were used for ADCC, suggesting down-regulation of CD16 of the NK cells in a tumor microenvironment. The experiment was repeated two more times with similar results.

immune response in which IL-2 could act as an important growth and activation factor, we rationalized that NK cells may be involved in generating antitumor immunity. Tumor bearing, mock-vaccinated mice had reduced number of NK cells. Following vaccination, NK cell population increased rapidly in the rvv-IL-2-treated mice (Fig. 1*A*). Increase in the numbers of NK cells was not likely due to the action of vaccinia virus because rvv-lacZ vaccination did not result in a higher number of NK cells. This result suggested that the IL-2 secreted from the rvv-IL-2 vaccine following infection of tumor cells led to expansion of NK cells at the early phases of immune response. NK cells secrete a number of Th1 and Th2 effector cytokine upon activation. Functionally mature NK cells lose the ability to produce Th2 cytokine and acquire the capacity to produce IFN-γ and TNF-α (20). IFN-γ has tumoricidal activity and induces other cells of the innate immune system, including macrophages and dendritic cells, whereas TNF-α directly promotes NK cell-mediated killing (20, 23). There is now increasing evidence that IFN-γ also plays an essential role in effector functions against tumor metastasis and methylcholanthrene-induced sarcomas (24). IFN-γ produced by NK cells also acts as a

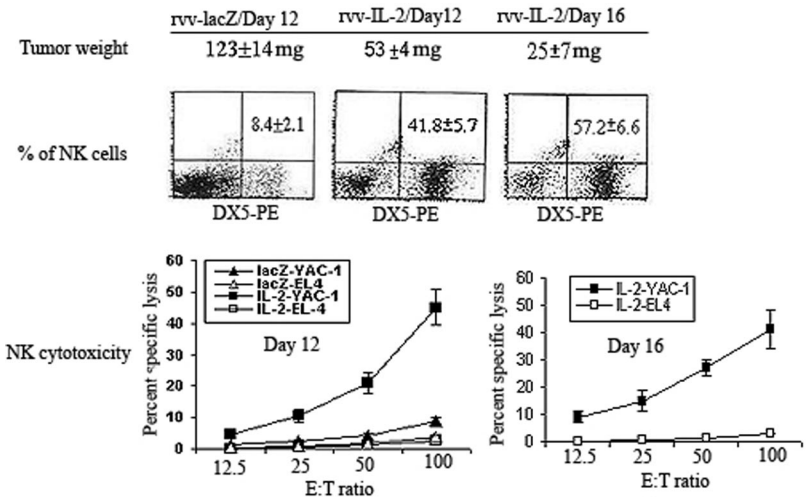

FIGURE 7. Tumor regression and increase in number of NK cells. Five mice from the rvv-lacZ- or rvv-IL-2-vaccinated group were sacrificed on the indicated days and mean tumor weights were recorded. Significant tumor growth inhibition and regression ($p < 0.001$) was noted on days 12 and 16 in mice vaccinated with rvv-IL-2. Total splenocytes were isolated from rvv-vaccinated mice on days 12 and 16 and stained with PE-labeled anti-mouse DX5 Ab. Isotype-matched control Ab was also used. Values shown are mean ± SE of the three mice individually analyzed. Positive cells are represented in the *lower right quadrant* and the mean ± SE value is given in the *upper right quadrant*. The number of NK cells was significantly higher ($p < 0.0001$) in the rvv-IL-2-vaccinated mice compared with control rvv-lacZ on day 12 and were nearly 20% higher on day 16. Lysis of the ^{51}Cr-labeled target YAC-1 cells was determined using purified NK effector cells harvested from rvv-lacZ- or rvv-IL-2-vaccinated mice on the indicated days at different E:T ratios. Cytotoxicity of the NK cells obtained from rvv-IL-2-vaccinated mice was significantly higher ($p < 0.0001$) on day 12 compared with rvv-lacZ-vaccinated mice. Almost similar cytotoxicity was observed in the rvv-IL-2-vaccinated mice on day 16.

self-activating molecule because impairment of NK cell activation correlates with reduced secretion of IFN-γ and limited NK cell-mediated killing (25). In the present study, NK cells from the rvv-IL-2-vaccinated group responded to YAC-1 stimulation and secreted significantly higher amount of IFN-γ and TNF-α compared with the control vaccination groups (Fig. 1, *D* and *E*). These results suggested that rvv-IL-2-stimulated NK cells were functionally mature and Th1-biased and potentially antitumoricidal.

Cytotoxicity is the major effector pathway of NK cell function. Results presented in Fig. 2, *A* and *B*, show that cytotoxicity of splenocytes or NK cells were significantly higher when the cells were isolated from the rvv-IL-2-vaccinated mice compared with the control groups.

Several studies have now clearly indicated that the NKG2D is an important non-MHC-specific primary cytotoxicity receptor for murine NK cells (26, 27). NKG2D functions as a triggering receptor involved in natural cytotoxicity mediated by normal NK cells against a variety of tumors (28). To validate whether NKG2D receptor was involved in NK cell-mediated cytotoxicity in this model, we used a mAb specific for NKG2D as a blockade in the cytotoxicity assay. Results in Fig. 2*C* demonstrate that the YAC-1 lysis by the NK cells was completely blocked by this mAb, providing evidence that this lysis is mediated by the NKG2D receptor. We also examined NKG2D expression in both mRNA and protein level in the vaccinated mice. Results in Fig. 3 demonstrate that NKG2D expression was mostly restricted to the splenocytes or purified NK cells of the rvv-IL-2-treated mice. Although the splenocytes contain cells other than the NK cells, such as $CD4^+CD8^+$ $\gamma\delta^+$ T cells and macrophages, which also express NKG2D when activated (26, 29), results presented in Fig. 3 demonstrated that the NKG2D up-regulation observed is most likely in the NK cells. Purified NK cells from the rvv-lacZ-vaccinated group showed significantly lower expression of NKG2D at the mRNA and protein levels compared with the rvv-IL-2-vaccinated group (Fig. 3). These results suggested that the stimulation by IL-2 from the rvv-IL-2 vaccine was necessary for the activation of the NKG2D on the NK cells.

For NKG2D-mediated lysis of the tumor cells it is necessary that these cells express one or more of the NKG2D ligands. Results presented in Fig. 4 demonstrated that at the mRNA level, cultured as well as single cell suspension of oral SCC VII/SF tumors expressed Rae1 and H60 ligands. At the protein level, however, only Rae1 was expressed significantly.

To improve upon the cancer immunotherapy in this model and eventual translation into the patients, it is necessary to study the mechanism of NKG2D down-modulation. Lee et al. (22) recently reported that sera of cancer patients contain high levels of TGF-β1. They presented data demonstrating the down-modulation of NKG2D by TGF-β1. We have previously reported that although cultured SCC VII/SF cells do not express high levels of TGF-β1, fresh SCC VII/SF tumors obtained from the oral cavity express high levels of TGF-β1 at the mRNA and protein levels (7, 10). Results presented in Fig. 5 demonstrate that tumors of mock-vaccinated mice contain high levels of active TGF-β1. The levels of TGF-β1, however, were down-modulated after vaccination with rvv-IL-2. Moreover, single cell suspension of fresh SCC VII/SF tumor secretes TGF-β1 at least for 5 days in culture (Fig. 5*B*). Expression of NKG2D decreased on incubation with tumor homogenate as well as supernatant from cultured cells (Fig. 5, *C* and *D*). Recombinant TGF-β1 also inhibits NKG2D expression. The expression of NKG2D can be restored by treatment with anti-TGF-β1 Ab. These results suggested that TGF-β1 might be responsible for down-modulation of NKG2D. Significant ADCC by the NK cells from the rvv-IL-2-vaccinated mice (Fig. 6*B*) suggested the presence of SCC VII/SF tumor-specific Abs in the serum. ADCC is mediated through CD16 receptor on NK cells. Phenotype analysis of NK cells demonstrated that the percentage of $CD16^+$ NK cells was low in the tumor-bearing mock-vaccinated mice. When the NK cells from the tumor-bearing mice were used for ADCC using sera from the rvv-IL-2-treated mice, ADCC was significantly reduced (Fig. 6*C*). These results suggest vaccination with rvv-IL-2 restored or prevented the loss of CD16 on NK cells and of CD16 biological activity.

We could not perform in vivo NK cell depletion to further confirm the antitumor functions of NK cells because Abs suitable for depletion of NK cells are not available for C3H mice. However, when tumor size was reduced to 50% on day 16 compared with day 12, the number of NK cells had increased by ~20% (Fig. 7). These data suggest the possible role of NK cells in tumor regression for this model.

Disclosures

The authors have no financial conflict of interest.

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