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Evasion from NK Cell Immunity by MHC Class I Chain-Related Molecules Expressing Colon Adenocarcinoma

Ekaterina S. Doubrovina,* Mikhail M. Doubrovin,† Elena Vider,‡ Richard B. Sisson,* Richard J. O’Reilly,* Bo Dupont,§ and Yatin M. Vyas*§

Evasion of host immune responses is well documented for viruses and may also occur during tumor immunosurveillance. The mechanisms involve alterations in MHC class I expression, Ag processing and presentation, chemokine and cytokine production, and lymphocyte receptor expression. Epithelial tumors overexpress MHC class I chain-related (MIC) molecules, which are ligands for the activating receptor NKG2D on NK and T cells. We report that NK cells from patients with colorectal cancer lack expression of activating NKG2D and chemokine CXCR1 receptors, both of which are internalized. Serum levels of soluble MIC (sMIC) are elevated and are responsible for down-modulation of NKG2D and CXCR1. In contrast, high serum levels of CXC ligands, IL-8, and epithelial-neutrophil-activating peptide (ENA-78) do not down-modulate CXCR1. In vitro, internalization of NKG2D and CXCR1 occurs within 4 and 24 h, respectively, of incubating normal NK cells with sMIC-containing serum. Furthermore, natural cytotoxicity receptor Nkp44 and chemokine receptor CCR7 are also down-modulated in IL-2-activated NK cells cocultured in MIC-containing serum—an effect secondary to the down-modulation of NKG2D and not directly caused by physical association with sMIC. The patients’ NK cells up-regulate expression of NKG2D, Nkp44, CXCR1, and CCR7 when cultured in normal serum or anti-MIC Ab-treated autologous serum. NKG2D+ but not NKG2D− NK cells are tumoricidal in vitro, and in vivo they selectively traffic to the xenografted carcinoma, form immunological synapse with tumor cells, and significantly retard tumor growth in the SCID mice. These results suggest that circulating sMIC in the cancer patients deactivates NK immunity by down-modulating important activating and chemokine receptors. The Journal of Immunology, 2003, 171: 6891–6899.

Natural killer cells constitute a separate lineage of lymphocytes capable of mediating early innate immune responses to viral infection and recognition of transformed malignant cells (1, 2). A role for NK cells in tumor rejection and their cytolytic activity in vitro against tumor cell lines was recognized very early (3, 4). It is now becoming evident that several characteristics of tumor cells may induce NK cell-mediated antitumor reactivity. NK cells may recognize target cells, including tumor cells, that have down-regulated MHC class I Ag expression (i.e., “missing self-recognition”) (5). Through their activating receptors, NK cells may also recognize target cell structures that normally are not expressed or are present in very low density but become up-regulated in transformed cells. Among such molecules, in humans, are the MHC class I homologs MHC class I chain-related molecule A (MICA) and MICB, which are induced by cellular stress (6).

Another family includes the UL16 binding proteins, which originally were identified by their binding to human cytomegalovirus protein UL16 (7). MIC homologs have not been identified in mice. The retinoic acid early inducible proteins and H60, however, serve a similar function in this species (8–10). It is now recognized that MIC, UL16 binding protein, retinoic acid early inducible protein, and H60 are all ligands for the activating NKG2D receptor, which in humans is expressed on most NK cells, NKT cells, and CD8 T cells (11–13).

Ectopic expression of NKG2D ligands induces rejection of tumors by NK cells and CTLs in syngeneic mice (14, 15). MIC Ags are broadly expressed on epithelial tumors, and their expression is associated with an increased infiltration of NKG2D+ lymphocytes (6). Engagement of NKG2D by MIC enhances cytolytic and cytokine responses of CD8 T cells (16). Despite this, many MIC-expressing tumors rapidly progress, implying that NKG2D-MIC interactions are impaired. It has recently been demonstrated that tumor-derived soluble MIC (sMIC) down-regulates expression of NKG2D on CD8 T cells and suppresses T cell activation (17). However, the effects of down-regulation of NKG2D-mediated activation signals on NK cell effector function in vivo remains to be explored.

Another group of activating NK cell receptors, the natural cytotoxicity receptors (NCRs) Nkp46, Nkp30, and Nkp44, recognize tumor cells in an MHC-independent manner. The ligands for the NCRs are not elucidated, although they are thought to recognize certain tumors of the neuro-epithelial origin (18). Putative viral ligands for the NCR have also been reported (19). The majority of peripheral blood NK cells constitutively express Nkp46 and Nkp30 but not Nkp44, which is up-regulated upon activation with IL-2 (20).

Migratory and homing characteristics of the NK cells will depend upon the expression of a variety of specific chemokine receptors (21–23). The CC chemokine receptor CCR7, which is important for T cell homing into secondary lymphoid organs, is not...
expressed on resting NK cells, but is up-regulated in activated NK cells. CCR7 is a shared receptor for CCL19 and CCL21 and participates in the recruitment and proliferation of activated NK cells (24). Another CC chemokine receptor, CCR5, is not expressed in either unactivated or activated NK cells (21–23). NK cells also express the CXC receptors, i.e., CXCR1, which binds to IL-8, and CXCR2, which binds to epithelial-neutrophil-activating peptide (ENA-78) (25). High levels of ENA-78 in fresh lung cancer specimens have correlated with tumor vascularity and spontaneous metastasis (26). IL-8 is also frequently produced by highly metastatic tumors (27, 28) and potentiates release of matrix metalloproteinases (MMPs) from the tumor cell (29), which in turn can enhance IL-8 activity by proteolytic cleavage (30).

MMPs are synthesized and secreted by cancer cells and by adjacent stromal cells and play important roles in degradation of extracellular matrix proteins (31–33). It was recently demonstrated that MMPs also release MICA/B from tumor cells (34). MMP-2 and MMP-9, the two secreted MMPs, are expressed at high levels in metastatic tumors (31–33). Taken together, these findings would suggest that tumor cell evasion from NK cell immunity during tumor development could be mediated by the combined effects of the release of MIC Ags and CXC ligands from tumor cells.

Here we test the hypothesis that some human epithelial tumors evade NK cell-mediated immune responses by down-modulating functionally important NK receptors by release of soluble decoy ligands. We demonstrate, for the first time in NK cells, that the soluble NKG2D ligands present in the sera of patients with colorectal cancer down-modulate both the activating and chemokine receptor groups, resulting in inability of NK cells to target the tumor and inactivation of NK cell effector functions in vitro and in vivo.

Materials and Methods

Cells

FACS-purified NK cells were isolated from PBMCs of three normal donors and three patients with colorectal cancer. For patient number 1 (localized primary tumor), samples were obtained at diagnosis and at 29, 32, and 38 mo thereafter. For patient number 2 (metastatic lung nodules), two samples were obtained 5 mo apart. For patient number 3 (metastatic disease in the peritoneum and liver), two samples were obtained 4 mo apart. Written informed consent was obtained from all patients and normal donors through the guidelines of the institutional review board. For colorectal tumor cells, fresh cell suspensions of surgically removed tumors from patients 1 and 2 were prepared by standard techniques. The EBV-transformed B-lymphoblastoid cell line (BLCL) was generated from patients 1 and 2 (i.e., autologous BLCL) by standard techniques.

Antibodies

**Primary.** Murine anti-human CD56 and CD3 (BD Biosciences, Mountain View, CA), goat polyclonal anti-human NKG2D and MIC (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-human CXCR1, CCR7, and CCR5 (R&D Systems, Minneapolis, MN) Abs were purchased. F(ab')2 fragments of anti-NKG2D Ab were generated after pepsin digestion of goat polyclonal NKG2D Ab per the standard procedure. The purity of the F(ab')2 fragment was determined after ascertaining the correct band size on gel electrophoresis. Anti-NKp46 and anti-NKp44 Abs were a kind gift from the University of California, Davis, CA.

**Secondary.** Affinity-purified second Abs and species-absorbed conjugates for dual-labeling were purchased from Chemicon International (Temecula, CA).

**In vitro culture conditions**

Condition A: PBMC NK cells were directly used for assays. Condition B: PBMC NK cells were cocultured with irradiated autologous PBMCs and EBV-BLCL in pooled human serum (from blood group AB individuals) and activated from day 7–21 with IL-2 (provided by the National Cancer Institute/Biological Response Modifiers Program, Frederick, MD) as previously described (35). Condition C: PBMC NK cells were expanded in culture condition B until day 14, when human AB serum was replaced with autologous patient serum. For the three normal donors, NK cells were propagated from 0–21 days in serum obtained from patient number 1. Condition D: PBMC NK cells were expanded in culture condition B until day 14, when human AB serum was replaced with autologous patient serum pretreated for 1 h with anti-MIC Ab at 1/10 dilution. Condition E: PBMC NK cells were expanded in culture condition B until day 14, when human AB serum was replaced with autologous patient serum pretreated with anti-IgG isotype control Ab at 1/10 dilution. Condition F: PBMC NK cells were cultured from day 0–21 in autologous patient serum and activated with IL-2 from day 7–21. NK cells cultured in each of these conditions were fed with fresh medium, IL-2, and the designated serum every 2–3 days. For conditions B–F, NK cells were analyzed on day 21.

Recombinant human ENA-78 and IL-8 used for studying the temporal dynamics of CXCR1 internalization in NK cells were purchased from R&D Systems. For 2 and 24 h at 37°C, we exposed CXCRI+ NK cells to two concentrations of rIL-8, i.e., low dose (1300 pg/ml; highest concentration obtained in the patient serum) and high dose (1100 ng/ml; concentration that is known to internalize CXCR1 in neutrophils). Similarly, 1000 pg/ml and 1100 ng/ml concentrations of rENA-78 were used. NK cells were analyzed by flow cytometry.

**Flow cytometry**

Two-color FACS analysis was performed on NK cells labeled with anti-CD56 and either anti-NKG2D or anti-CXCR1 Abs, and the tumor cell suspensions were labeled with either anti-MIC or anti-CD56 Ab using a FACS sorter (BD Biosciences).

**Cytotoxicity assay**

Cell killing assays were performed using NK cells as effectors and 5 × 10⁶ labeled autologous EBV-BLCL, autologous colorectal cancer cells, and HLA class I-negative EBV-BLCL 721.221 as targets in the presence or absence of F(ab')2 fragment of NKG2D Ab. Assays were performed in triplicates for 4 h at an effector:target ratio of 10:1 and the percent specific lysis calculated as per the standard formula.

**ELISA**

**ELISA for sMICA/B.** Primary polyclonal goat anti-human MICA/B Ab (Santa Cruz Biotechnology) was added in 1/100 dilution to the plate precoated with the test serum or PBS and incubated for 1 h at room temperature. Secondary HRP-conjugated rabbit anti-goat IgG (Zymed Laboratories, San Francisco, CA) was added at 1/10000 dilution and incubated at room temperature. After 1 h, substrate reagent (R&D Systems) was added. The reactions were read at adsorption wavelengths of 450 nm and 570 nm using a microplate reader (SAFIRE, Tecan, Austria). Assays were performed in quadruplets and from at least two separate samples.

**ELISA for sMMPs, ENA-78, and IL-8.** Commercially available ELISA kits (R&D Systems) were used to quantify serum levels of MMP-2, MMP-9, ENA-78, and IL-8 per the manufacturer’s protocols. ELISA samples were read at a wavelength of 450 nm with a wavelength correction of either 570 or 630 nm, and OD values were used to construct a standard curve. Assays were performed in duplicate and from at least two to four separate samples.

**SCID mouse experiment**

Equal numbers of EBV-BLCL and colon adenocarcinoma cells (i.e., 5 × 10⁶) derived from patient number 1 were inoculated s.c. in the left and right thighs of the SCID mice (Taconic Farms, Germantown, NY), based on the previously described model (36). Animals were divided into three groups. Group 1 (n = 7) was treated with autologous NKG2D+ NK cells expanded in culture condition B. Group 2 (n = 9) was treated with autologous NKG2D+ NK cells expanded in culture condition F. Group 3 (n = 12) received no NK cells. Treatment of the animals was begun only when the tumor size reached ~0.25 cm³ in each of the three groups. NK cells were injected i.v. on days 6, 21, and 43 after tumor inoculation in a total dose of 8 × 10⁷/mouse/course. All animals were treated with 2000 IU of IL-2 mouse i.p. three times a week from days 6–43. Therapeutic effect of NK cells was assessed by measuring tumor volume using formula \( V = \pi D_{\text{avg}}^2 / 6 \), where \( D_{\text{avg}} \) is the average diameter (cm), \( D_{\text{avg}} \) is the average volume (cm³). The p values were calculated using the Wilcoxon nonparametric statistical analysis. In separate parallel experiments, three animals in each of the above groups were sacrificed on day 5 after the NK cell infusion to determine the homing characteristics of the injected NK cells. Fresh cell suspensions from adenocarcinoma and BLCL as well as liver, spleen, peripheral blood, and bone marrow were evaluated for the presence of NK cells.
Table I. Receptor profile of NK cells

<table>
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<th>Receptor</th>
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<th>Pt no. 3</th>
<th>Dn no. 1</th>
<th>Dn no. 2</th>
<th>Dn no. 3</th>
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* Expression of activating and chemokine receptors on NK cells obtained from PBMCs of three patients (Dn and three normal donors (Pt)) expanded in different culture conditions (B-E) is shown (see Materials and Methods for more details).

**Results**

**Tumor-derived sMIC reversibly down-modulates NK2G and renders NK cells selectively noncytotoxic against MIC+ tumor**

NK cells obtained from PBMCs of three patients with colorectal cancer were tested by flow cytometry for expression of NK2G. Only 3–7% of the NK cells were NK2G+, whereas 60–67% of NK cells from normal donors expressed NK2G (Table Ia). In the patients, decreased numbers of NK2G+ NK cells were observed in multiple samples obtained through the course of the disease.

NK cells from patients 1 and 2 were isolated and tested in vitro for cytotoxicity against fresh autologous tumor cells, which expressed MIC molecules (patient number 1, 48%, and patient number 2, 72% MIC positivity). In both cases, the patients’ NK cells did not display measurable cytotoxicity against the tumor cells but were cytolytic against the HLA class I-deficient EBV-B-lymphoblastoid cell line 721.221 (Fig. 1A; data not shown for patient number 1).

The cellular distribution of NK2G and MIC in NK cells from the three patients and normal donors was analyzed by digital immunofluorescence microscopy. The majority of NK cells from normal donors expressed NK2G uniformly on the cell surface with no surface or cytoplasmic MIC detected (Fig. 2, a and b). In contrast, NK2G2D was either absent (10–15%) or present only in the cytoplasm in the majority (60–65%) of NK cells obtained from the patients; few NK cells (20–25%) had both cytoplasmic and cell surface expression of this receptor (Fig. 2a). In addition, the majority of NK cells (~70%) displayed intracellular MIC; few NK cells (5–10%) displayed surface expression of MIC (Fig. 2b). The presence of MIC on the surface or within the cytoplasm of the NK cell suggests the presence of tumor-derived circulating sMIC in the patients with colorectal cancer.

To quantitate the amount of sMIC in circulation, sera from the patients were tested by a sandwich ELISA and found to contain levels of sMIC two- to sixfold higher than in normal serum (Fig. 3a). Furthermore, analysis of multiple samples from normal donors and patients revealed an inverse correlation between the number of NK2G+ NK cells and the amount of sMIC in the serum (Fig. 3, a and c). We infer from these data that the absence of significant cell surface expression of NK2G on PBMC NK cells in the three patients reflects the binding of sMIC to NK2G and subsequent internalization of NK2G2D-MIC receptor-ligand pair as demonstrated in Fig. 2, a and b.
The in vitro effects of tumor-derived sMIC on NK cell phenotype and function were analyzed. When NK cells from the patients were cultured with IL-2 in the presence of normal AB serum and analyzed on day 21, the proportion of NKG2D⁺ NK cells dramatically increased (Table 1B). However, only a minority of NK cells cultured under identical conditions and then transferred to medium containing IL-2 and autologous MIC-containing serum on day 14 expressed NKG2D by day 21. Similarly, NK cells from the patients cultured with IL-2 in the presence of autologous serum from day 0–21 continued to exhibit very low numbers (2–5%) of NKG2D⁺ NK cells (data not shown). Furthermore, NK cells from healthy controls cultured from day 0–21 in serum from patient number 1 (ninefold higher sMIC compared with healthy donors) also lost NKG2D expression (Table 1C). When human AB serum was replaced at day 14 with patient serum containing anti-MICA/B Ab, increasing numbers of NK cells from the patients continued to gain NKG2D expression (Table 1D). However, patient NK cells cultured in autologous serum with IgG control Ab continued to exhibit very low numbers of NKG2D⁺ NK cells (Table 1E).

The effect of modulation of NKG2D on NK cell-mediated cytotoxicity in vitro was analyzed by testing the tumoricidal activity of NK cells from patients 1 and 2 (data not shown for patient number 1). NK cytotoxicity against MICA⁺ autologous tumor cells was not observed with PBMC NK cells and with NK cells cultured in sMIC-containing autologous serum (Fig. 1, A and C), both of which were predominantly NKG2D⁻. However, NK cells cultured in either normal AB serum (Fig. 1B) or patient autologous serum pretreated with anti-MIC Ab (Fig. 1D) became NKG2D⁺ and were now cytotoxic to the tumor cells. Furthermore, F(ab)ᵡ fragment of anti-NKG2D Ab blocked antitumor cytotoxicity by NKG2D⁺ NK cells, whereas 721.221 cells remained susceptible (Fig. 1B, asterisk). NK cells cultured in autologous serum pretreated with IgG isotype control Ab failed to up-regulate NKG2D and failed to lyse carcinoma cells (Fig. 1D, star). Because 721.221 cells were satisfactorily lysed by IL-2-activated patient NK cells and because the activating receptor NKP46 is thought to mediate this killing (20), we determined the expression profile of NKP46 and NKP44 in circulating and IL-2-activated patient NK cells (Table 1). Although NKP46 was absent from PBMC NK cells of the patients, this receptor became up-regulated after propagation in IL-2 independently of the serum source, i.e., from healthy donors or patients (Table 1, B and C). As expected, NKP44 was not expressed in the circulating PBMC NK cells from healthy donors or patients but became expressed on a large fraction of normal NK cells and NK cells from the patients when cultured in human AB serum for 21 days (Table 1B). However, when the normal or patient NK cells were cultured in MIC-containing serum, NKP44 became down-regulated (Table 1C). In contrast, large numbers of NK cells continued to express NKP44 when cultured in MIC-containing patient serum coincubated with anti-MIC Abs but not with isotype control Ab (Table 1, D and E).

Taken together, these studies demonstrate that sMIC in the serum of the patients with colorectal cancer binds to NKG2D, leading to its internalization. The resultant reduced expression of
NKG2D leads to impaired tumoricidal activity against MIC-expressing tumor cells in vitro, which require NKG2D activation for their cytotoxicity.

Expression of NKG2D determines the capacity of NK cells to prevent growth of MIC+ human colorectal cancer in SCID mice

The in vivo effects of NKG2D− and NKG2D+ NK cells on tumor growth were evaluated in SCID mice. Animals xenografted with the tumor from patient number 1 were treated with 1) autologous NKG2D− NK cells, 2) NKG2D− NK cells, or 3) IL-2 alone (Fig. 4a, left panel). NKG2D+ NK cell-treated animals demonstrated significantly smaller tumor size at each measurement done weekly over the period of observation, compared with either NKG2D− NK cell-treated or IL-2-treated animals. However, there was no difference in the growth of MIC− EBV-BLCL tumor derived from the same patient, which was concurrently implanted in the opposite flank of the animals (Fig. 4a, right panel).

In another set of experiments, similarly treated animals were sacrificed 5 days after NK cell infusion to evaluate the trafficking and homing characteristics of the infused NKG2D+ and NKG2D− NK cells. Flow cytometric analysis of fresh tumor suspensions demonstrated presence of NK cells (17–37%, n = 3) only in the NKG2D+ NK cell-treated tumor, but not in tumors of the mice treated with NKG2D− NK cells or with IL-2 alone (Fig. 4b). Moreover, infused NK cells were not detected in EBV-BLCL tumor suspensions and in other organs such as liver, spleen, bone marrow, or peripheral blood (data not shown).

Tissue sections of the xenografted carcinoma and EBV-BLCL tumors were also examined by immunohistochemistry and in situ digital immunofluorescence microscopy. Adenocarcinomas treated with NKG2D+ NK cells demonstrated infiltration of infused NK cells within the tumor substance, resulting in disruption of the tumor architecture and areas of necrosis (Fig. 4c, top left and middle panels), whereas this was not seen in mice treated with NKG2D− NK cells or IL-2 alone (data not shown). In addition, NK cell infiltration was not observed in the EBV-BLCL tumors treated with either NKG2D+ or NKG2D− NK cells (Fig. 4c, top right panel).

Similarly, immunofluorescence analysis of carcinoma xenografts demonstrated NK cell infiltration only in animals treated with NKG2D+ NK cells (Fig. 4c, bottom left panel). Furthermore, multiple NK cell–carcinoma cell conjugates demonstrated polarization of NKG2D2 to the cell-cell contact area, indicating formation of an activating NK cell immune synapse in situ (Fig. 4c, bottom right panel).

These in vivo experiments demonstrate significant growth retardation of implanted colorectal tumors when treated with autologous NKG2D+ NK cells, whereas mice treated with NKG2D− NK cells or IL-2 alone had tumor progression. Strikingly, whereas NKG2D+ NK cells infiltrated carcinoma xenografts, NKG2D− NK cells failed to do so.

NK cells from patients with MIC-expressing colorectal cancer lack expression of the chemokine receptors CXCR1 and CCR7

Because the infused NKG2D+ NK cells failed to localize to the MIC+ tumors, we analyzed cell surface expression of chemokine receptors important for cell migration and homing to inflammatory sites such as CXCR1, CCR5, and CCR7 (21–24). As expected, PBMC NK cells from the three patients and the normal donors had no significant expression of CCR7 (Table Ia) or CCR5. Although most normal NK cells expressed CXCR1, the proportion of circulating NK cells from the patients expressing this receptor was very low (Table Ia). Cell membrane expression of CXCR1 was below detectable levels by flow cytometry in the majority of NK cells. Immunofluorescence analysis of NK cells from the patients demonstrated increased CXCR1 in the cytoplasm (Fig. 2c). In contrast, NK cells from normal individuals expressed CXCR1 on the cell membrane. When the patient NK cells were cultured for 21 days with IL-2 and normal human AB serum, the number of CXCR1+ NK cells dramatically increased (Table Ib). Whereas culture of NK cells in the identical condition for 14 days induced membrane expression of CXCR1, subsequent transfer to autologous patient serum led to dramatic reduction in the number of CXCR1+ NK cells (Table Ic). CXCR1 was also down-modulated on normal NK cells cultured in serum from patient number 1 (containing high sMIC and ENA-78 but low IL-8 levels), contrasting with sustained high expression observed upon cultivating in normal serum (Table
IC). These findings parallel our studies of the modulation of NKG2D expression. In addition, however, the down-modulation of CXCR1 did not occur when NK cells from the patients were cultured in autologous serum coincubated with anti-MIC Ab (Table I, D and E). The CC chemokine receptor CCR7, which is expressed on activated NK cells, became expressed on a large fraction of normal NK cells and NK cells from the patients after culturing with IL-2 in human AB serum for 21 days (Table I B). In contrast, when the normal or patient NK cells were cultured in patient serum, this activation marker was down-regulated (Table I C). In contrast, the high level of expression of CCR7 was sustained when these NK cells were cultured in patient serum coincubated with anti-MIC Abs (Table I, D and E). Immunofluorescence analysis of IL-2-activated CCR7+ patient NK cells that were cultured in patient serum revealed strictly a cytoplasmic localization of CCR7 with no signals detected in the membrane, which is in contrast with normal IL-2-activated NK cells in which CCR7 is predominantly membrane localized with only a small fraction present in the cytoplasm close to the microtubule organizing center (data not shown).

To further study the mechanism(s) of CXCR1 modulation in NK cells, we determined whether increased serum levels of the CXC chemokines were involved. Initial studies using sMIC-containing serum demonstrated that the internalization of CXCR1 from the membrane was not observed even after 24 h of coincubation (Figure 5, 6 h). To better understand the time course of CXCR1 internalization, cells were incubated with sMIC-containing serum for up to 48 h and their surface and intracellular pool of CXCR1 were monitored by triple-labeling with CD56 (in red), NKG2D (in blue), and CXCR1 (in green). Data in the figure represent 30 NK cells analyzed for each Ab combination from two independent experiments.
ligands were responsible for receptor-mediated down-modulation. Therefore, serum levels of IL-8 and ENA-78 were quantitatively analyzed by ELISA. The levels of IL-8 were significantly elevated in patients 2 and 3, whereas ENA-78 was increased in each of the three patients (Fig. 3b). Because MMP-9 is known to increase IL-8 activity in the tumor microenvironment (30) and both MMP-2 and MMP-9 are secreted by colon adenocarcinomas (31, 32), we tested the serum levels of these enzymes and found both markedly elevated in each of the patients studied (Fig. 3b, top panels).

Because incubating NK cells in serum (patient number 1) not containing high IL-8 resulted in internalization of CXCR1 and pretreatment of this serum with anti-MIC resulted in up-regulation of CXCR1, we designed experiments to address whether it is the CXC ligands or NKGD ligands that are involved in internalization of CXCR1. Therefore, CXC ligand-mediated modulation of CXCR1 on NK cells was evaluated in in vitro experiments. Because IL-8-induced receptor internalization has been demonstrated for CXCR1 in the neutrophils, we exposed CXCR1+ NK cells to low and high concentrations of rIL-8 and rENA-78 for 24 and 24 h as described in Materials and Methods. FACS and immunofluorescence analysis of NK cells incubated with either ligand at both doses and time points failed to demonstrate a decrease in the number of CXCR1+ NK cells or decreased surface expression and/or internalization of CXCR1 (data not shown).

Having demonstrated no role for IL-8 and ENA-78 in receptor internalization and because CXCR1 expression was influenced by the absence or presence of sMIC in the culture medium, we then determined the in vitro effect of sMIC-containing serum on the temporal relationship between NKGD2 and CXCR1 expression. CXCR1 and NKGD2-expressing IL-2-activated NK cells obtained from a normal donor were incubated for 4, 24, 65, and 140 h in medium containing serum from patient number 3 that contained a sevenfold higher concentration of sMIC than in sera from normal controls. In these experiments, as opposed to the experiment detailed in Table 1, the medium containing the serum was not changed over the 6 days of culture. The NK cells were then analyzed by immunofluorescent microscopy (Fig. 5). At 4 h, the majority of NK cells demonstrated internalization of NKGD2 but not of CXCR1. At 24 h, half of the NK cells showed almost complete absence of NKGD2, whereas the other half showed some degree of internalization of NKGD2. Interestingly, CXCR1 was seen internalized only in those NK cells in which NKGD2 was completely absent, whereas normal surface expression of CXCR1 was observed in the NK cells that contained NKGD2 either on the surface or in the cytoplasm. At 65 h, 20% of the NK cells showed complete absence of NKGD2 and internalization of CXCR1, whereas the rest of the NK cells showed internalization of NKGD2 only. By 6 days, the majority of NK cells regained normal surface expression of NKGD2 and CXCR1 similar to that of untreated normal NK cells, thereby reflecting depletion of sMIC from the medium over the course of culture (Fig. 5).

These results suggest that the MIC-NKGD2 interaction mediates internalization of CXCR1 in the NK cells. The resultant reduced surface expression of CXCR1 may contribute to defects in homing of NK cells to MIC-expressing tumor cells in vivo.

Discussion

Here we demonstrate in human NK cells that soluble NKGD ligands, MICA/B, which are elevated in the sera of patients with colorectal carcinoma, can interact with NKGD2 on NK cells from patients and normal individuals and induce internalization of NKGD2-MIC complex, resulting in marked reduction in cell surface expression of NKGD2. These changes in NKGD2 are associated with profound defects in NK cell-mediated antitumor cytotoxicity in vitro and both homing and tumor inhibiting activity in vivo.

Functional deficiency of T cells, i.e., cytotoxicity and IFN-γ production in vitro, has been shown to correlate with expression of NKGD2 (17). Our results are the first to demonstrate functional impairment of the adoptively transferred NKGD2− NK cells in vivo.

The ability of NK cells to selectively traffic to tumor sites will depend on the chemokine receptor expression profile and chemokine responsiveness, both of which are different from those of T cells (22). Our studies have demonstrated that NKGD−CXCR1− and CCR7−infused NK cells were not detected in the carcinoma xenografts of the treated mice, whereas NKGD+CXCR1+ and CCR7+ NK cells were found infiltrating carcinoma xenografts. Whether this phenomenon is due to failure to traffic (chemokine receptor/chemokine defect) to the tumor sites or failure to remain adhered to the tumor cells (adhesion defect) after successfully trafficking cannot be ascertained from our experiments, although we did observe elevated levels of soluble ICAM-1 in the sera of patients with colorectal cancer (our unpublished observations), as have others (37), suggesting that multiple mechanisms must play a role in failure to home to tumor sites.

Release of tumor-derived soluble ligands for important receptors on the immune cells is an effective mechanism of evading tumor immunity, as is depleting the tumor cell surface of the molecular targets for NK cells or CTLs. MMP-mediated proteolytic shedding of MIC molecules is at least one of the mechanisms involved in the release of sMIC in the circulation and consequent reduced expression of MIC on the surface of the tumor cell (34). Although we did not attempt to correlate surface density of MIC molecules with serum levels of sMIC, one recent study failed to demonstrate such a relationship (38). We hypothesize that production of MMPs in the tumor microenvironment leads to proteolytic cleavage and release of NKGD2Ls, particularly MIC molecules. Alternatively, MIC molecules can be secreted into the tumor microenvironment as alternatively spliced variants (39). In either case, soluble ligand(s) would bind to NKGD2, inducing receptor internalization and abolishing NK cell effector functions. Secretion of tumor-derived sMIC is not confined to solid epithelial tumors but is also seen with leukemia cells in vivo (38).

Internalization of NK receptor expression in vivo and in vitro is not limited to NKGD2 but is also observed for the chemokine receptors CXCR1 and CCR7. The immunofluorescence analysis of circulating PBMC NK cells clearly demonstrated internalization of CXCR1. Although serum levels of the CXC glutamic acid-leucine-arginine−/− chemokines IL-8, a strong agonist for CXCR1, and ENA-78, a weak agonist for CXCR1 (P.M. Murphy, unpublished observations), were elevated in the patients, our studies indicate that down-modulation of CXCR1 is probably not mediated by these soluble CXC ligands, at least at the time points studied. These findings are in contrast with neutrophils, in which dose-dependent IL-8-mediated receptor internalization is observed (40). However, we cannot exclude the possibility that incubation with these and other (i.e., granulocyte chemotactic protein 2) (41) chemokine ligands at additional time points, earlier or later, may result in desensitization and internalization of CXCR1.

The mechanism(s) responsible for up-regulation of CXCR1 on NK cells incubated in patient sera pretreated with anti-MIC is less clear. Studies on the temporal kinetics of sMIC-induced phosphorylation-dephosphorylation of the chemokine receptors are likely to throw additional light on the mechanism for receptor internalization (40–42). Our in vitro experiments do demonstrate, however, a temporal relationship between expression of NKGD2 and CXCR1, and they also show modulation of receptor expression of
both NKG2D and CXCR1 by sMIC. Upon incubating NK cells with sMIC-containing serum, we observed the first indications of internalization of NKG2D at ~4 h and maximum internalization by 24 h, with over half of the analyzed NK cells showing total absence of NKG2D. These results are in agreement with the observations made in CD8+ T cells incubated with purified recombinant sMICA (17). In contrast, internalization of CXCR1 was not observed before 24 h of incubation with sMIC-containing serum. It is of note that CXCR1 was internalized only in those NK cells with complete internalization and no surface expression of NKG2D. Partial internalization with residual surface expression of NKG2D was not enough to modulate expression of CXCR1. These results and results from the temporal analysis of NKG2D and CXCR1 internalization would suggest that sMIC-mediated down-modulation of the triggering receptor NKG2D is rapid and precedes down-modulation of chemokine receptors. Our results also imply that reduction in surface density of NKG2D must reach a critical threshold before secondary effects on other receptors can be observed.

Furthermore, we observed down-modulation in the IL-2 activated patient NK cells of other activation markers, such as the CC chemokine receptor CCR7 and the NCR NKP44, which are normally up-regulated in IL-activated NK cells. A causal relationship between soluble ligand-mediated down-modulation of NKG2D and down-modulation of other receptors has not been established, but may reflect NKG2D-mediated general inactivation of the NK cells. Similar findings of activating receptor down-modulation in NK cells from patients with acute leukemia have been described (43).

Alternatively, NKG2D-DNAX-activating protein 10 (DAP10)-mediated activation pathways in humans may be involved in modulation of CXCR1 and CCR7, and extinguishing the NKG2D-DAP10 signals will not only prevent NKG2D-mediated cytotoxicity but will also perturb chemokine receptor expression. The observation that the triggering receptor expressed on myeloid cell-2/DAP12-mediated activation pathway regulates the expression of CC chemokine receptor CCR7 in murine dendritic cells is another example of cross-talk between activation and chemokine receptors (44).

It is important to emphasize that the observed down-modulation of NKP44 and the chemokine receptors are events that are perhaps secondary to the down-modulation of NKG2D and are likely not directly caused by physical association with sMIC present in the sera of the patients.

Our in vitro studies demonstrate re-expression of NKG2D and recovery of NK cytotoxicity in vitro after culture of NK cells in normal serum. Our observation that selective inhibition of NK cytotoxicity against MIC+ tumor cells, but not 721.221 cells, occurs upon blocking NKG2D receptor with anti-NKG2D-F(ab')2, clearly indicates that functional NKG2D is necessary for tumor cell killing in vitro in these autologous combinations. These findings are in agreement with studies in mice bearing tumors transduced to express NKG2D ligands (NKG2DL) (14, 15). In these models, NK cells were able to reject tumors despite their expression of self-MHC class I.

It is of particular interest that NKG2D-expressing NK cells, in our studies, were able to mediate effector responses against tumors that constitutively express NKG2DL.

Our study is the first in demonstrating in vivo polarization of NKG2D to cell-cell contact area and formation of productive immune synapse in the in situ NK cell-tumor cell conjugates.

Recently, reversible down-modulation of NKG2D upon exposure to NKG2DL was seen in NK cells from nonobese diabetic mice, resulting in impairment of NK cytotoxicity and cytokine production (45). These findings support the notion that the NKG2D-NKG2DL receptor-ligand pair is a powerful regulator of NK cell effector functions. The prolonged changes in NKG2D expression and slow reappearance are in agreement with observations made in a recent study of TGFβ1-induced modulation of NKG2D expression in NK cells. Here the decreased surface expression of NKG2D correlated with the decrease in NKG2D transcript expression in some but not in all NK clones or in polyclonal NK cells (46). This study also demonstrated regulation of triggering receptor expression on NK cells by cytokine TGF-β, which is secreted by a variety of malignant tumors and imparts an aggressive phenotype (47–49).

In the experiments presented in Table I, the medium containing patient serum was replaced every 2–3 days, thus maintaining high concentrations of sMIC all through 21 days of culture. That NKG2D can be re-expressed by NK cells is illustrated by the effect of a single pulse of medium containing patient serum on normal NK cells described in Fig. 5. In this experiment, the NK cells gradually regained surface expression of NKG2D between 65 and 140 h of culture. Whether re-expression of NKG2D is a result of receptor recycling or de novo protein synthesis in the NK cells or their progeny cannot be ascertained from our experiments. However, a recent study in CD8 T cells demonstrated that MIC-mediated internalized NKG2D was not recycled but was degraded (17).

The described tumor escape mechanisms involving production and release of soluble decoy ligands for lymphocyte receptors are very similar to processes used by viruses in their evasion of immune responses (50–52). It should also be emphasized that the adaptive immune response to MIC-expressing tumors such as breast, colon, prostate, and lung cancers as well as leukemias (38) can be evaded by mechanisms similar to escape from NK immunity because CD8+ T cells also use NKG2D as an activating receptor (35, 53, 54). Our ongoing studies have shown that in other epithelial tumors such as ovarian cancer, the circulating PBMC-derived NK cells were also significantly deficient of NKG2D (data not shown).

Although it is intriguing how tumors have developed a mechanism(s) to evade the immune system by receptor perturbation mediated by soluble decoy ligands, the timing and the mechanism of shedding of the surface MIC by tumor cells during tumor genesis will be interesting to explore. It would be especially interesting to determine whether this requires a large tumor cell mass or reflects specific changes in the tumor cells. It is possible that early tumor cells do not shed this ligand, making them an easy target for NK and T cell cytolytic function, whereas secondary mutations in the tumor cells are responsible for this effect. Our studies stress the need to carefully select lymphocytes, based on their phenotypic characteristics, for successful implementation of adoptive cell-based therapies as a means of treating cancers. Alternatively, manipulation of enzymes responsible for generation of soluble decoy ligands may offer attractive future approaches.

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References


