

Melanoma Cells Inhibit Natural Killer Cell Function by Modulating the Expression of Activating Receptors and Cytolytic Activity

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Abstract

Natural killer (NK) cells play a key role in tumor immune surveillance. However, adoptive immunotherapy protocols using NK cells have shown limited clinical efficacy to date, possibly due to tumor escape mechanisms that inhibit NK cell function. In this study, we analyzed the effect of coculturing melanoma cells and NK cells on their phenotype and function. We found that melanoma cells inhibited the expression of major NK receptors that trigger their immune function, including Nkp30, Nkp44, and NKG2D, with consequent impairment of NK cell-mediated cytolytic activity against various melanoma cell lines. This inhibitory effect was primarily mediated by indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2). Together, our findings suggest that immunosuppressive barriers erected by tumors greatly hamper the antitumor activity of human NK cells, thereby favoring tumor outgrowth and progression. *Cancer Res*; 72(6); 1407–15. ©2012 AACR.

Introduction

Natural killers (NK) are cells of the innate immunity that play a fundamental role in antiviral and antitumor responses (1, 2). Their activation depends on the balance between activating and inhibitory signals mediated by cell surface receptors (3, 4). Inhibitory receptors are represented mainly by human leukocyte antigen (HLA) class I-binding receptors, including killer immunoglobulin-like receptors (KIR), CD94/NKG2A, and leukocyte Ig-like receptor B1 (LILR-B1/ILT2). Activating receptors include Nkp46, Nkp30, Nkp44 (4), NKG2D (5), and DNAX accessory molecule-1 (DNAM-1; ref. 6). Certain ligands for activating receptors are stress-inducible molecules, including MHC class I-related chain A/B (MICA/B) and UL16-binding proteins (ULBP) recognized by NKG2D (7, 8). Other ligands include the poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) that bind to DNAM-1 (9), the HLA-B-associated transcript 3 (BAT-3), and the recently discovered B7-H6 molecule both recognized by Nkp30 (10, 11). In most instances,

these ligands are absent on normal cells, whereas they become highly expressed in tumor cells. A series of costimulatory receptors and adhesion molecules such as 2B4, NTBA, Nkp80, and LFA-1 are also involved in the regulation of NK cell activation (12).

NK cells can recognize tumors that are resistant to T-cell killing due to loss or downregulation of MHC class I antigens, thus playing a complementary role in antitumor activity. NK cells are considered promising effector cells in the adoptive immunotherapy of cancer (13). A high degree of natural cytolytic activity of peripheral blood (PB) lymphocytes has been associated with a reduced cancer risk in an 11-year follow-up study (14). In addition, NK cell infiltration in solid tumors has been found to be associated with a better prognosis (15–17). Notably, recent evidence would suggest that NK cells, besides their direct cytolytic effect against tumor cells, may also shape the subsequent adaptive immune response toward a T_H1 profile, thought to favor antitumor responses (18, 19).

Despite the existence of different mechanisms by which NK cells may eliminate cancer cells, NK cell-based immunotherapy has resulted in limited clinical benefit (20). This may reflect the poor capacity of adoptively transferred NK cells to home to tumor sites. In addition, tumor cells may have developed various escape mechanisms to avoid NK-mediated killing. In this context, a number of cytokines, growth factors, and enzymes synthesized by tumor and/or stromal cells have been reported to exert suppressive effects on cells involved in immune response (21, 22). For example, transforming TGF- β , interleukin (IL)-10, and prostaglandin E2 (PGE2) as well as the activity of the tryptophan metabolizing enzyme indoleamine 2,3-dioxygenase (IDO) may contribute to the

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-11-2544

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establishment of immune tolerance within the tumor micro-environment. To date, limited information is available on the effect of melanoma cells on NK cell function. Our study shows that melanoma cells cocultured with PB NK cells sharply downregulate the expression of major activating NK receptors including NKp30, NKp44, and NKG2D, thus markedly affecting the NK-mediated cytotoxicity against melanoma cells.

Materials and Methods

Monoclonal antibodies and flow cytofluorimetric analysis

Monoclonal antibodies (mAb) used in this study are listed in Supplementary Materials and Methods. For cytofluorimetric analysis cells were stained with the appropriate mAb or soluble receptor (NKp30-Fc and NKp44-Fc), washed and stained with PE-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated) or PE-conjugated F(ab')₂ goat anti-human IgG (Jackson ImmunoResearch), respectively. For intracytoplasmic staining cells were fixed (4% formaldehyde), permeabilized (0.1% saponin), and stained with the appropriate mAb. All samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). To compare the surface densities of NK receptors and the intracytoplasmic content of cytotoxic granules among NK cells cultured in different conditions, we calculated the mean ratio fluorescence intensity (MRFI), that is, the ratio between the mean fluorescence intensity (MFI) of cells stained with the selected mAb and that of cells stained with second reagent or isotypic control alone. Data analysis was done using FlowJo software (TreeStar Inc.).

Production of soluble receptors

Plasmids utilized for expression of NKp30-Fc and NKp44-Fc recombinant molecules were prepared as described in Supplementary Materials and Methods.

Melanoma cell lines

Primary melanoma cell lines MeBO, MeCoP, MeMI, MeMO, MeOV, MePA, MeTA, and MeTU were originated from metastatic lesions obtained in accordance with consent procedures (n. OMA09.001) approved by the Internal Ethics Board of the National Cancer Institute (IST, Genova, Italy). Tissue specimens were processed for establishment of the cell lines as previously described (23). FO-1 cell line was kindly provided by S. Ferrone (New York Medical College), MEWO was from ECACC cell bank (2009), authenticated by PCR/STR (short tandem repeat) analysis (ECACC) and was used within 6 months of resuscitation of original cultures.

Generation of polyclonal NK cell populations and cytolytic assays

NK cells were isolated from peripheral blood mononuclear cells using the NK Cell Enrichment Cocktail-RosetteSep (Stem-Cell Technologies Inc.). NK cells were tested for cytolytic activity in a 4-h ⁵¹Cr release assay against the FcγR⁺ P815 murine cell line and different melanoma cell lines. When indicated mAbs were added at the onset of redirected killing assays (1 μg/mL) and masking experiments (10 μg/mL).

NK/melanoma cocultures

NK cells were cultured for 6 days with irradiated melanoma cell lines in IL-2 (100 U/mL; at a Mel/NK ratio of 1:10). When indicated, the following specific inhibitors were added at the onset of cocultures: 1 mmol/L 1-methyl-DL-tryptophan (1MT; Sigma-Aldrich), 5 μmol/L NS398 (Cayman Chemicals), 400 ng/mL chicken anti-TGF-β-neutralizing antibody, 10 μg/mL anti-macrophage migration inhibitory factor (MIF), and 5 μg/mL anti-IFN-γ-RI-blocking mAbs (R&D System). Transwell studies were carried out by plating IL-2-activated NK cells and melanoma cells in the upper chamber (at a Mel/NK ratio of 1:10) plus melanoma cells in the lower chamber of a 0.4-μm transwell plate. Melanoma cells in the lower chamber were then analyzed. The presence of PGE2 in supernatants was measured using a commercially ELISA Kit (Cayman Chemicals).

When indicated, NK cells were cultured for 6 days with IL-2 in a tryptophan-free RPMI-1640 medium (PBI International) or with the indicated doses of PGE2 (Sigma-Aldrich).

Statistical analysis

Statistical significance was evaluated by Student *t* test or, where indicated, Mann-Whitney test. A *P* value of less than 0.05 (*), less than 0.01 (**), or less than 0.001 (***) was considered statistically significant.

Results

Melanoma cells inhibit the IL-2-induced expression of activating NK receptors

To investigate whether melanoma cells could affect the expression of NK cell receptors relevant for tumor cell killing, NK cells isolated from healthy donors were cocultured with 3 primary melanoma cell lines (i.e., MeCoP, MeMO, and MePA) derived from skin metastatic lesions. The surface expression of the main activating receptors was analyzed by flow cytometry both in freshly isolated NK cell populations and in NK cells that had been cultured for 6 days with IL-2 either in the absence or in the presence of melanoma cell lines. In particular, we analyzed the expression of NKp46, NKp30, NKp44, NKG2D, DNAM-1, CD69, and 2B4. In agreement with previous data, after culture in IL-2, NK cells displayed an increased expression of NKp30 and NKG2D and, partially, of NKp46, whereas NKp44 and CD69 were expressed *de novo*. Phenotypic analysis revealed that the expression of NKp30, NKp44, and NKG2D was lower in NK cells cocultured with melanoma cells as compared with those cultured in IL-2 alone. On the other hand, the surface density of NKp46, DNAM-1, CD69, and 2B4 was unaltered or only minimally modulated (Fig. 1A). Also the expression of KIRs (i.e., KIR2DL1/S1, KIR2DL2/L3/S2, and KIR3DL1/S1) and CD94/NKG2A was not substantially modified in NK cells cocultured with melanoma cells (data not shown). To assess the statistical significance of the phenotypic data, we conducted different experiments using NK cells derived from 3 healthy individuals and cultured in IL-2, either alone or in combination with 10 different melanoma cell lines. As shown in Fig. 1B, NKp30, NKp44, and NKG2D were significantly downregulated in NK cells cultured in the presence of melanoma cells as compared with those cultured in their

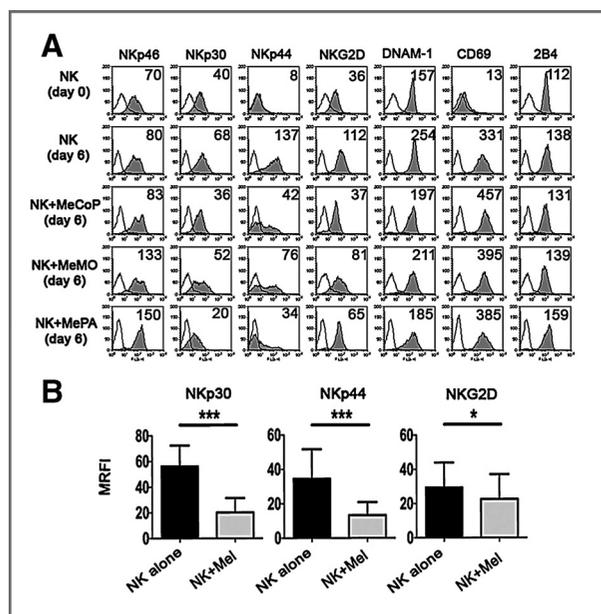


Figure 1. Effect of melanoma cells on the expression of activating NK receptors. A, the expression of activating receptors (filled profiles) was analyzed by fluorescence-activated cell sorting on NK cells freshly isolated (day 0) or cultured for 6 days in IL-2 either in the absence or in the presence of melanoma cell lines. White profiles represent negative controls. Numbers indicate MFI. A representative experiment out of 4 conducted is shown. B, surface expression of NKp30, NKp44, and NKG2D on NK cells derived from 3 healthy donors cultured with IL-2 for 6 days either alone (NK alone) or in the presence of different melanoma cell lines (NK+Mel). Each NK cell population has been cocultured with 10 different melanoma cell lines. Data are representative of those obtained from the 3 independent experiments. Y-axis denotes the MRFI. Results are represented as mean \pm SD of MRFIs. *, $P < 0.05$; ***, $P < 0.001$ by Mann-Whitney test.

absence. Taken together, these results showed that melanoma cells exert an inhibitory effect on the IL-2-induced upregulation of NKp30, NKp44, and NKG2D.

NK cells cocultured with melanoma cells display a sharp impairment of cytolytic activity

Because NK cell cytotoxicity is known to depend on the engagement of different triggering receptors, we further analyzed whether the melanoma-induced downregulation of NKp30, NKp44, and NKG2D could result in an impairment of NK cell cytolytic activity. In addition, as the mechanism by which NK cells kill target cells is mostly based on the activity of perforin and granzymes A/B contained in NK cell granules, we also analyzed whether their expression level could be modified on melanoma-conditioned NK cells. As shown in Fig. 2A, IL-2-activated NK cells exhibited high intracytoplasmic levels of cytolytic granules containing both perforin and granzymes A/B. In contrast, when exposed to melanoma cells, IL-2-activated NK cells showed a low content of granzyme A. On the other hand, the expression of granzyme B and perforin was not affected (or only slightly modulated; Fig. 2A). Figure 2B shows the statistical analysis of the experiments done on 3 different NK cell populations cocultured with 10 different melanoma cell lines.

Next, we assessed the effect of melanoma cells on NK cell cytotoxicity induced via NKp30, NKp44, and NKG2D using the Fc γ R⁺ P815 cells as triggering target in a redirected killing assay upon addition of receptor-specific mAbs. NK cells that had been cocultured with MeCoP, MeMO, and MePA displayed an impaired triggering capability of NKp30, NKG2D, and NKp44 (Fig. 3A). We further analyzed whether the documented loss of function of NKp30, NKp44, and NKG2D could also affect the ability of NK cells to kill different melanoma cell lines. As shown in Fig. 3B, IL-2-activated NK cells could efficiently lyse all melanoma target cells. In contrast, NK cells cultured in the presence of MeCoP, MeMO, and MePA showed a reduced killing capability. Figure 3C shows the statistical analysis of the cytolytic activity mediated by NK cells cultured under different conditions. Although not shown, the different NK cytotoxicity between NK alone and melanoma-conditioned NK cells (Fig. 3 panels A and C) maintained statistical significance at different effector-to-target (E/T) ratios (ranging from 10:1 to 2.5:1). Thus, we measured the expression of NK cell ligands in

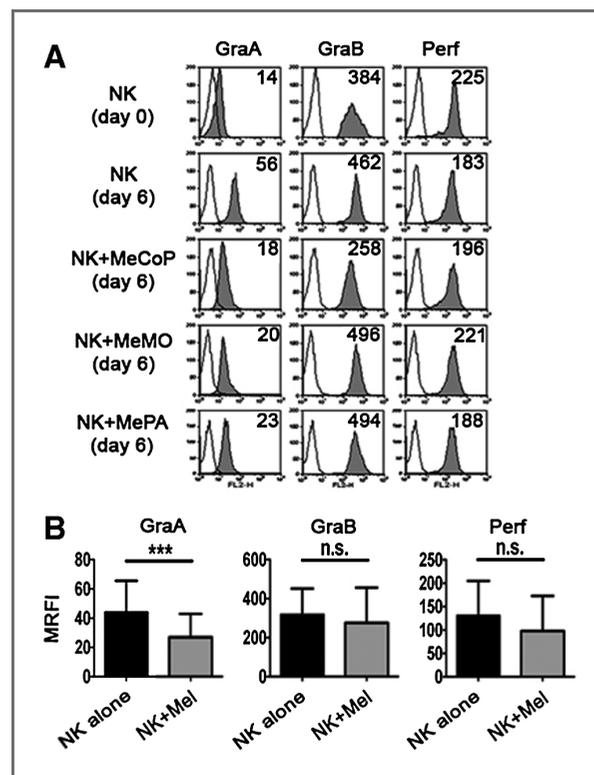


Figure 2. Intracellular perforin and granzymes A/B content in melanoma-conditioned NK cells. A, immunofluorescence analysis was done on freshly isolated NK cells (NK day 0) and on the same NK cells after 6-day culture in IL-2 (NK day 6) either in the absence or in the presence of the indicated melanoma cell lines. The expression of the intracellular markers was analyzed with mAbs to the indicated molecules (filled profiles). White profiles represent negative controls. Numbers indicate the MFI for each marker. Results of a representative experiment out of 4 conducted are shown. B, cytotoxic granules content in NK cells derived from 3 healthy donors cultured with IL-2 for 6 days either alone (NK alone) or in the presence of melanoma cell lines (NK+Mel; see legend to Fig. 1). Y-Axis denotes the MRFI. Results are represented as mean \pm SD of MRFIs. ***, $P < 0.001$; n.s., not significant by Mann-Whitney test.

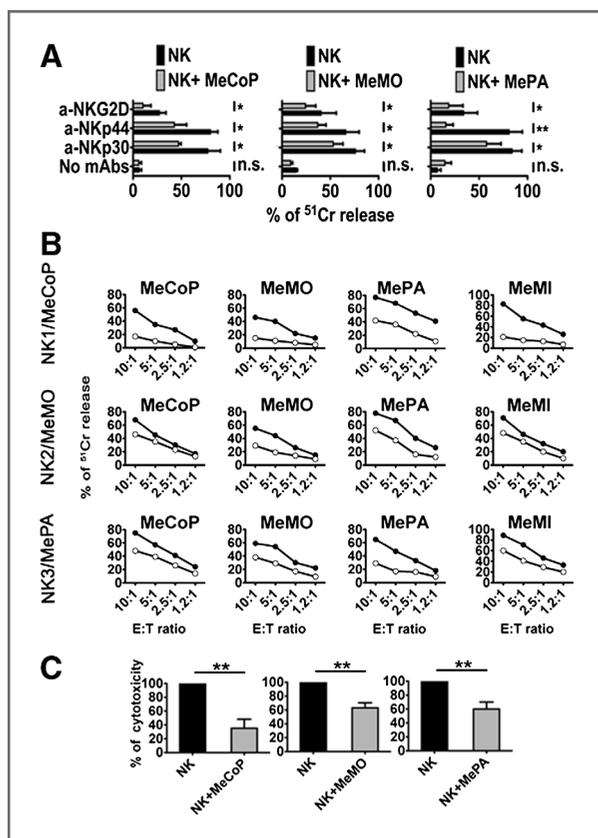


Figure 3. Impaired cytolytic function of melanoma-conditioned NK cells. **A**, NK cells cultured for 6 days with IL-2 either in the absence (black bars) or in the presence of the indicated melanoma cell lines (grey bars) were analyzed for cytolytic activity in a redirected killing assay against the Fc γ R⁺ P815 target cells in the presence of mAbs specific for the indicated triggering receptors. All the mAbs used in these experiments were of IgG1 isotype. Bars represent means \pm SD obtained from 3 independent experiments. The E/T ratio was 5:1. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant. **B**, antimelanoma cytotoxic activity of NK cells (derived from 3 healthy donors; NK1, NK2, and NK3) cultured with IL-2 for 6 days either alone (black circles) or in the presence of MeCoP, MeMO, or MePA, respectively (white circles). Results are representative of those obtained in 3 independent experiments. **C**, data represent the percentage of lysis mediated by NK cells cultured with the indicated melanoma with respect to NK cells cultured alone (100%). The E/T ratio was 10:1. Bars represent means \pm SD obtained from 3 independent experiments. **, $P < 0.01$.

melanoma cell lines used as targets in the cytolytic experiments. MeCoP, MeMI, MeMO, and MePA expressed, although at different levels, the ligands recognized by the activating NK receptors affected during NK/Mel coculture experiments (Fig. 4A). Furthermore, because B7-H6 has been described as a major NKp30 cellular ligand on tumor cells of various origins (11), we carried out reverse transcriptase PCR (RT-PCR) analysis of *B7-H6* mRNA on melanoma cell lines. MeCoP, MeMI, and MeMO were found to express *B7-H6* mRNA, whereas MePA was negative (Supplementary Fig. S1). This finding is not surprising, as in addition to B7-H6 other still unknown ligands of NKp30 must exist. mAb-mediated masking experiments showed that killing of melanoma cell lines involved a simultaneous engagement of NKp30, NKp44, and NKG2D (Fig. 4B; ref. 23).

Melanoma-derived soluble factors are involved in the modulation of activating NK cell receptors

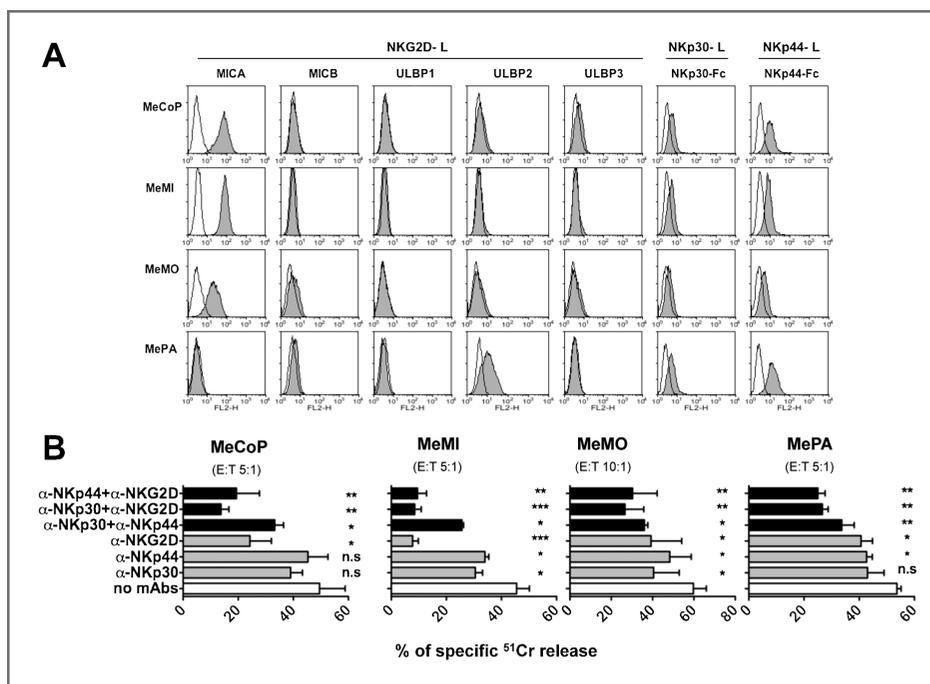
Previous reports showed that TGF- β , IDO, PGE₂, and MIF could inhibit NK cell function (24–27). To investigate whether the melanoma-mediated inhibition of NK cell receptor expression was mediated by soluble factors, we conducted NK/melanoma coculture experiments in the presence or in the absence of specific inhibitors of these mediators. IMT (an inhibitor of IDO enzymatic activity), NS398 (an inhibitor of PGE₂ synthesis), anti-TGF- β , or anti-MIF-neutralizing antibodies were added to NK/melanoma cocultures. NK cells were analyzed after 6 days for surface receptor expression. IMT was sufficient to prevent the inhibitory effect of MeCoP cell line on NKp30, NKp44, and NKG2D cell surface expression, whereas NS398 was able to inhibit, almost completely, the effect of MePA cell line on surface receptor expression. Finally, both IMT and NS398 were necessary to counteract the inhibitory effect mediated by MeMO cell line (Fig. 5A). In contrast, neither anti-TGF- β nor anti-MIF-neutralizing antibodies could restore expression of NKp30, NKp44, and NKG2D in melanoma-conditioned NK cells (Supplementary Fig. S2). These results suggested that both IDO and PGE₂ are involved in melanoma-mediated inhibition of activating receptor expression in NK cells.

Role of IDO and PGE₂ in the inhibition of NK cell activity

We further investigated whether IDO and PGE₂ could inhibit NK cell cytotoxicity. To this aim, NK cells were cultured for 6 days either alone or with MeCoP, MeMO, and MePA cell lines in the presence or in the absence of specific inhibitors of IDO and/or PGE₂. The same melanoma cell lines used in the coculture experiments were used as targets in the cytolytic assays. Melanoma-conditioned NK cells displayed a reduced capability of killing melanoma cells (Fig. 5B). In agreement with data above, the cytolytic activity of NK cells cultured in the presence of MeCoP cell line was fully restored when IDO was blocked by 1 mmol/L IMT. The dose of 1 mmol/L was selected on the basis of previous studies (25). Titration experiments confirmed that doses between 0.5 and 1 mmol/L of IMT induced a complete restoration of the cytolytic activity of NK cells cultured in the presence of MeCoP (Supplementary Fig. S3).

We also addressed whether the observed effect of IMT on NK cell activity was indeed related to IDO inhibition rather than to direct toxicity on melanoma cells. To this end, the percentages of early apoptotic (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺), and necrotic cells (Annexin V⁻/PI⁺) were evaluated in both IMT-treated and untreated MeCoP cell line. The proportion of apoptotic/necrotic melanoma cells accumulated in the 2 different conditions at 24 hours was comparable [$14.7 \pm 2\%$ vs. $17 \pm 2.6\%$, respectively, mean \pm SD, not significant (n.s.), data not shown, detailed procedures described in Supplementary Materials and Methods]. Because the inhibitory effect mediated by melanoma cell lines on NK cells might reflect the depletion of tryptophan by IDO, we investigated whether a tryptophan-free medium could result in impaired expression of NKp30, NKp44, and NKG2D. NK cells cultured in the absence of tryptophan did not display any decrease expression of

Figure 4. Melanoma cell lines express NKG2D-, NKp30-, and NKp44 ligands and are susceptible to NK cell-mediated lysis. **A**, melanoma cell lines were phenotyped for the ligands of the activating NK receptors, whose expression was affected during coculture experiments, using mAbs or soluble chimeric molecules specific for the marker of interest (filled profiles). White profiles represent negative controls. **B**, IL-2-activated NK cells were analyzed for cytolytic activity against the indicated melanoma cell lines either in the absence or in the presence of mAbs specific for different activating NK receptors. mAbs were used either alone (grey bars) or in combination (black bars). E/T ratios are indicated in the figure. Bars represent means \pm SD obtained from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.



NKp44, whereas only a marginal inhibitory effect in the expression of NKp30 and NKG2D was detected (data not shown). Thus, our data, together with the finding that L-kynurenine could affect NK cell function (27), suggest that the effect of 1MT on restoration of NK cell function is dependent on the inhibition of IDO catabolic activity on tryptophan. At variance with MeCoP, cytolytic activity against MePA was restored by the PGE2 inhibitor, whereas the one against MeMO required both IDO and PGE2 inhibitors (Fig. 5B). These functional data further confirmed that IDO and PGE2 may play a major role in the immunosuppressive activity mediated by melanoma cells.

Expression of IDO and production of PGE2 by melanoma cells

Given the involvement of IDO and PGE2 in melanoma-mediated inhibition of NK cell cytotoxicity, we further analyzed whether MeCoP, MeMO, and MePA cell lines were responsible for their expression/production. In particular, although both IDO1 and IDO2 may be expressed in human tumors, we focused our analysis on IDO1, as tryptophan degradation is entirely dependent on IDO1 (28). We found that melanoma cells did not constitutively expressed *IDO1* mRNA, but only following treatment with IFN γ . Remarkably, melanoma cells expressed *IDO1* mRNA even upon coculture with NK cells under transwell conditions (Fig. 6A). To determine whether melanoma cell lines expressed IDO enzyme upon coculture in the presence of IL-2-activated NK cells, Western blot analysis was carried out on protein lysates from untreated, IFN γ -treated melanoma cells and melanoma cells cocultured with NK cells. As expected, melanoma cells did not constitutively express IDO protein, whereas IFN γ -treated cells upregulated IDO expression. In line with the functional data (Fig. 5B),

MeCoP and MeMO expressed IDO also upon coculture with IL-2-activated NK cells, whereas MePA cell line did not (Fig. 6B). Next, we tested whether IDO expressed by "NK-conditioned" melanomas was enzymatically active by detection of L-kynurenine in culture supernatants (C-SN). As shown in Fig. 6C, C-SNs from melanoma cells did not contain L-kynurenine, whereas L-kynurenine was detected in C-SNs from MeCoP, MeMO, and MePA cultured with IFN γ . Of note, also C-SNs from MeCoP and MeMO incubated in the presence of NK cells did contain L-kynurenine, whereas C-SN from MePA did not (Fig. 6C; detailed procedures described in Supplementary Materials and Methods). To assess whether IFN γ released by activated NK cells plays a role in the induction of IDO protein expression, we conducted coculture experiments in the presence of a blocking mAb specific for IFN γ RI. These experiments were conducted using MeCoP cell line, as its inhibitory function on NK cells was strictly IDO dependent. Anti-IFN γ RI mAb added to NK/melanoma cocultures completely abrogated both IDO expression by MeCoP (Supplementary Fig. S4A) and the inhibitory effect of MeCoP cell line on NK cells (Supplementary Fig. S4B). Thus, these data suggested that the inhibitory effect of MeCoP was dependent on IFN γ -mediated signaling.

To assess the expression of IDO by melanoma cells *in vivo*, immunofluorescent analysis was done on available biopsies of patients, from which the cell lines used in this study had been originated. Supplementary Fig. S5 shows a representative sample of metastatic skin melanoma stained with antibodies recognizing IDO (panel A) and HMB-45 (panel B). IDO is clearly expressed by HMB-45⁺ cells (panel C). Nuclei were stained with 4',6-diamidino-2-phenylindole (panel D). Panels E, F, G, and H represent the same staining of panels A, B, C, and D, respectively, at an higher magnification. Panel I represents negative control.

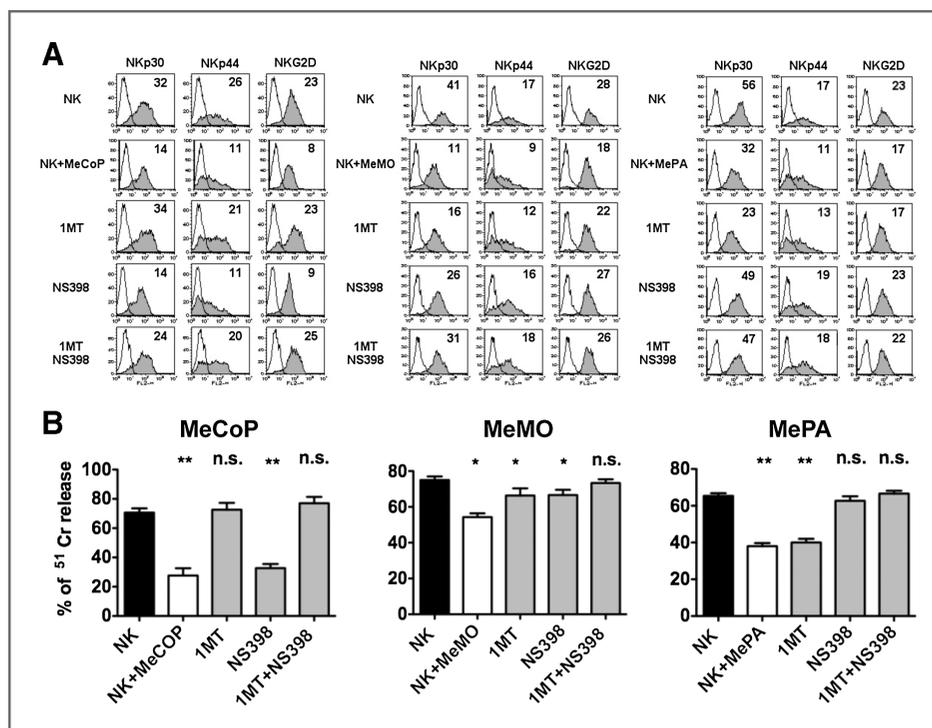


Figure 5. Melanoma-derived IDO and/or PGE2 inhibit the expression of activating receptors and the cytotoxic activity of NK cells. **A**, NK cells were cultured for 6 days with IL-2 either alone or in the presence of the indicated melanoma cell lines. NK/melanoma cell cocultures were set either in the absence or in the presence of the indicated inhibitors. The expression of the activating NK receptors was analyzed with mAbs to the indicated molecules (filled profiles). White profiles represent negative controls. Numbers indicate the MRFI for each receptor. A representative experiment out of 3 conducted is shown. **B**, cytotoxic activity of NK cells cultured with IL-2 for 6 days either alone (black bars) or in the presence of MeCoP, MeMO, and MePA (white bars). Cytotoxic activity of NK cells cocultured with MeCoP, MeMO, or MePA in the presence of the indicated inhibitors used either alone or in combination (grey bars). Bars represent means \pm SD obtained from 3 independent experiments. The E/T ratio was 10:1. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

Assessment of PGE2 in culture supernatants showed that it is released by MeMO and MePA but not by MeCoP cell line (Table 1). Interestingly, increments of PGE2 production by MePA and MeMO were induced both by addition of IFN γ and coculture with NK cells (Table 1). The PGE2 inhibitor NS398 abolished PGE2 production by MeMO and MePA upon coculture with NK cells. As shown in Supplementary Fig. S6, NK cells cultured with different concentrations of PGE2 displayed a reduced surface expression of NKp30, NKp44, and NKG2D as compared with NK cells cultured alone. Taken together, our data indicated that both IDO expression and PGE2 production differ among the cell lines analyzed when cultured with NK cells.

Discussion

In this study we show that melanoma cells may interfere with NK cell function by downregulating the surface expression of activating receptors, including NKp30, NKp44, and NKG2D. Receptor modulation results in an impaired ability of NK cells to kill melanoma cells. We also show that the inhibitory effect is primarily mediated by IDO and PGE2.

It is generally accepted that immune surveillance plays a role in the control of tumor development. However, effector cells can be inhibited by different mechanisms, including immune evasion at the level of tumor microenvironment. In this con-

text, it has been shown that the effect of antitumor responses can be counteracted by a variety of immunosuppressive mechanisms, predominantly occurring at the effector phase of antitumor immune responses (29). Thus, although various potentially defensive effector cells are recruited at the tumor site, their antitumor activity may be downregulated mostly by factors produced by tumor cells or even by tumor-associated macrophages or stromal cells (21, 22, 30). These factors include soluble mediators (such as TGF- β and PGE2) and enzymes (such as IDO) which inhibit both T and NK cell function.

Although melanoma-associated antigens may elicit T-cell responses, various attempts to enhance such responses including (i) vaccination to increase the number of tumor-reactive T cells and (ii) direct adoptive transfer of effector cells had limited success (31). Therefore, new immunotherapeutic strategies are required. The low surface levels of HLA class I molecules on melanoma cells, combined with the fact that they often express ligands recognized by major activating NK receptors (32), suggest that NK cells could represent potentially useful effector cells in the therapy of melanomas. In this context, although T cells represent the majority of infiltrating lymphocytes in biopsies of metastatic melanomas, CD56⁺ NK cells have also been detected (33). However, tumor-infiltrating NK cells were found to be impaired in their functional capability (34, 35). Notably, even PB NK cell function has been

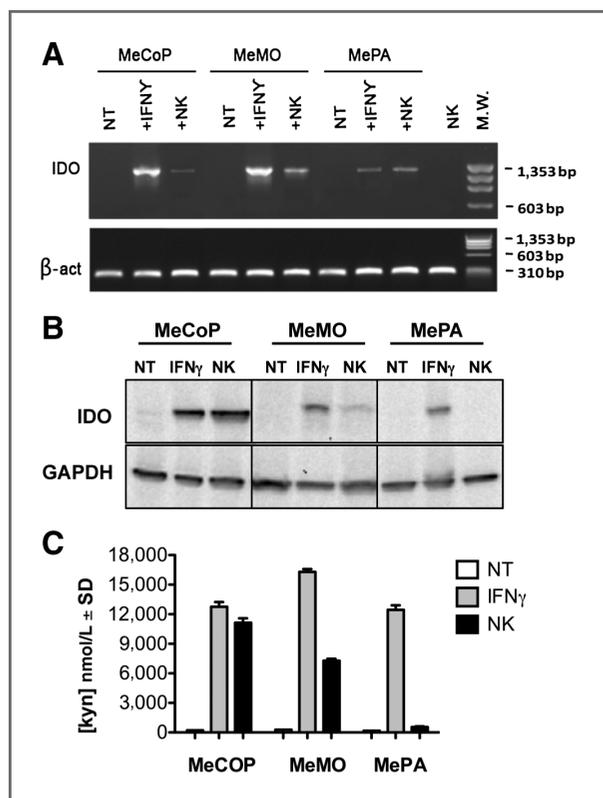


Figure 6. Expression of IDO and production of L-kynurenine by melanoma cell lines. A, IDO mRNA expression was assessed by RT-PCR on: untreated and IFN γ -treated melanoma cells; melanoma cells cocultured for 96 hours in the presence of NK cells (under transwell conditions) and NK cells alone. RT-PCR with specific β -actin primers was used as an internal control. B, the expression of IDO enzyme was assessed by Western blot analysis on: untreated and IFN γ -treated melanoma cells; melanoma cells cocultured in the presence of NK cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. C, L-kynurenine concentration in C-SNs from untreated, IFN γ -treated melanoma cells, and melanoma cells cocultured in the presence of NK cells. Bars represent means \pm SD obtained from 3 independent experiments.

reported to be highly compromised in different malignancies, including acute myeloid leukemias, lung and colorectal cancers, and melanomas (36–38). In these cases, the impaired NK cell function was associated with downregulation of triggering NK receptors. The molecular mechanisms underlying this modulation are only partially understood. In this context, mechanisms such as ligand-induced receptor downmodulation may play a relevant role. This may be consequent either to the release of soluble ligands from the tumor cells or to their intercellular transfer (39, 40). Also a sustained surface expression of NKG2D ligands has been proposed as a possible mechanism of suppression of NK cell function (41). Different soluble factors could also inhibit the NK cell function. For example, the ovarian tumor-derived MUC16 glycoprotein has been shown to downregulate CD16 surface expression and to inhibit the NK cytotoxicity (42). In addition, cytokines or soluble mediators such as TGF- β and PGE2 downregulate the surface expression of NKp30, NKp44, and NKG2D and, conse-

quently, NK cell cytotoxicity and cytokine production (24, 25). Furthermore, NK cell function may be suppressed by IDO-generated L-kynurenine (27). Finally, also MIF has been shown to inhibit the NKG2D surface expression in PB NK cells derived from ovarian cancer patients (26). Because these immunosuppressive factors seem to be produced by tumors of different histotypes, including melanomas, we analyzed whether one or more of these factors were responsible for the modulation of NKp30, NKp44, and NKG2D in NK cells that had been cocultured with melanoma cells. Indeed, by the use of specific inhibitors, we show that the modulation of activating NK receptors is dependent on melanoma-derived IDO and PGE2. On the other hand, neither anti-MIF nor anti-TGF- β -neutralizing antibodies had any effect. Notably, coculture of NK cells with melanoma cells did not result in downregulation of other activating NK cell receptors or coreceptors, including NKp46, DNAM-1, and 2B4. These data are reminiscent of previous results on mesenchymal stem cell–NK cell interaction (25).

Because ligands of both NKp30 and NKG2D can be expressed by melanomas (11, 23, 43), it is not surprising that the impaired expression of these activating receptors may affect the NK-mediated killing of melanoma cells. Notably, the melanoma cell lines used in coculture experiments (i.e., MeCoP, MeMO, and MePA) induced a different degree of NKp30, NKp44, and NKG2D downmodulation on NK cells (Fig. 1A). On the other hand, melanoma-induced modulation of NKp30, NKp44, and NKG2D did not completely prevent killing of the melanoma cell lines analyzed. In this context, both NKp46 and DNAM-1 have been shown to play a relevant role in melanoma cell lysis (33).

Recently, Balsamo and colleagues reported that melanoma-associated fibroblasts could inhibit NK cell function by modulating the surface expression of NKp30, NKp44, and DNAM-1 (22). Interestingly, DNAM-1 modulation was dependent on cell-to-cell interactions and did not involve soluble mediators. Thus, one could speculate that tumor cells and the associated stromal cells may have evolved complementary strategies to favor tumor escape.

Notably, our present findings suggest that the melanoma-mediated downregulation of NKp30 on NK cells could also impair their functional interaction with dendritic cells (DC). Accordingly, downregulation of NKp30 expression may result in altered DC editing, which, in turn, could favor T-cell tolerance toward tumor antigens.

The fact that IDO expression (Fig. 6A and Supplementary Fig. S7) can be induced in melanoma cells by IFN γ suggests that T or NK cell responses at the tumor site may result in amplification of the suppressive effect. In this context, one may argue that downregulation of activating receptors could impair IFN γ production by tumor-infiltrating NK cells. However, it should be stressed that IFN γ production may be induced also upon NK cell activation by cytokines such as IL-15, which may be produced at the tumor site by macrophages or even by melanoma cells themselves (44).

Immunofluorescence revealed that IDO is expressed *in vivo* in metastatic melanoma lesions. Notably, high expression of IDO in metastatic melanomas has been reported to be

Table 1. Production of PGE2 by melanoma cell lines cultured in the absence and in the presence of IFN γ or cocultured with NK cells (in the presence or in the absence of the PGE2 inhibitor NS-398)

Melanoma cell line	Alone	Plus IFN γ ^a	Plus NK cells ^b	Plus NK cells plus NS-398 ^c
MeCoP	ND ^d	ND	48 \pm 18	67.5 \pm 52.5
MeMO	2,914 \pm 1,253	10,876 \pm 3,104	12,650 \pm 919.2	27.5 \pm 17.7
MePA	511 \pm 140	592 \pm 192	4,127 \pm 282	ND

Data refer to the levels of PGE2 expressed at pg/mL. Results are the means \pm SD of 3 independent experiments.

^aThe levels of PGE2 were evaluated after 6 days of culture in supernatants derived from melanoma cells stimulated with IFN γ .

^bThe levels of PGE2 were evaluated after 6 days of culture in supernatants derived from melanoma cells cocultured with NK cells.

^cThe levels of PGE2 were evaluated after 6 days of culture in supernatants derived from melanoma cells cocultured with NK cells in the presence of the PGE2 inhibitor NS-398.

^dND, not detectable.

associated with a poor patient survival (45). Moreover, a direct correlation with poor prognosis could be established in patients with decreased serum concentration of tryptophan (i.e., the IDO substrate; ref. 46).

Finally, although PGE2 production greatly varied among the cell lines analyzed, its release was enhanced in the presence of NK cells (Table 1 and Supplementary Table S1). As recently reported, higher expression of COX-2 has been detected in various subtypes of melanomas as compared with benign melanocytic nevi (47). This data supports the notion that PGE2 production by melanoma cells *in vivo* may further promote local immunosuppression.

In conclusion, our results help to better understand the molecular mechanisms responsible for the inhibition of NK cell function upon interaction with melanoma cells. In view of this immunosuppressive effect, new strategies might be developed to prevent inhibition of potentially efficient antitumor effector cells (e.g., by blocking IDO or PGE2). Notably, these strategies may be developed also to the design of novel protocols of NK

cell-based adoptive immunotherapy to treat melanoma and possibly other tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by grants awarded by AIRC: IG project n. 4725 (L. Moretta), MFAG project n. 6384 (G. Pietra), IG project n. 5282 (M. Vitale) and Special Project 5 \times 1000 n. 9962 (L. Moretta); Ministero dell'Istruzione, dell'Università e della Ricerca: MIUR-FIRB 2003 project RBLA039LSF-001 (L. Moretta), MIUR-PRIN 2007 projects 2007473C72_002 (G. Pietra) and 20077NFBH8_005 (M.C. Mingari), MIUR-PRIN 2008 project prot. 2008PTB3HC_005 (L. Moretta); Ministero della Salute: RF2006-RO strategici 3/07 (L. Moretta) and RO strategici 8/07 (M.C. Mingari, G. Pietra, and M. Vitale). C. Manzini is a recipient of a fellowship awarded by FIRCC.

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Received July 31, 2011; revised December 16, 2011; accepted January 4, 2012; published OnlineFirst January 18, 2012.

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Cancer Res 2012;72:1407-1415. Published OnlineFirst January 18, 2012.

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