**Microenvironment and Immunology**

**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 (LIlig-B1/LILT2). Activating receptors include NKP46, NKP90, NKP44 (4), NKG2D (5), and DNAx accessory molecule (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 Th1 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...
establishment of immune tolerance within the tumor microenvironment. 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 cytometric analysis
Monoclonal antibodies (mAb) used in this study are listed in Supplementary Materials and Methods. For flow cytometric 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 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.).

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
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 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 ± SD of MRFIs. *P < 0.05; **P < 0.001 by Mann–Whitney test.

Next, we assessed the effect of melanoma cells on NK cell cytotoxicity induced via NKp30, NKp44, and NKG2D using the FcγR+ 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
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. 1MT (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. 1MT was sufficient to prevent the inhibitory effect of MeCoP cell line on NKp30, NKp44, and NKGD2 cell surface expression, whereas NS398 was able to inhibit, almost completely, the effect of MePA cell line on surface receptor expression. Finally, both 1MT 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 NKGD2 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 of 1MT. 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 1MT 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 1MT 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 1MT-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 ± 2% vs. 17.4 ± 2.6%, respectively, mean ± 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 NKGD2. NK cells cultured in the absence of tryptophan did not display any decrease expression of...
IDO1 expressed following treatment with IFN. We found that melanoma cells did not constitutively express IDO1, as tryptophan degradation is entirely dependent on IDO1 (28). We focused our analysis on IDO1, as tryptophan degradation is relevant to the presence of IL-2–activated NK cells (Fig. 6B). Next, we tested whether IDO expression 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 IFNγ. 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 IFNγ 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 IFNγRI. These experiments were conducted using MeCoP cell line, as its inhibitory function on NK cells was strictly IDO dependent. Anti-IFNγRI 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 IFNγ-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 a higher magnification. Panel I represents negative control.

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 conducted coculture experiments 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 ± SD obtained from 3 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001; 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 IFNg 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 context, 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
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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 IFNγ-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 IFNγ-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, IFNγ-treated melanoma cells, and melanoma cells cocultured in the presence of NK cells. Bars represent means ± 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, consequently, 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 IFNγ 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 IFNγ production by tumor-infiltrating NK cells. However, it should be stressed that IFNγ 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
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 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.

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