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NK-cell Editing Mediates Epithelial-to-Mesenchymal Transition via Phenotypic and Proteomic Changes in Melanoma Cell Lines

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Abstract

Tumor cell plasticity is a major obstacle for the cure of malignancies as it makes tumor cells highly adaptable to microenvironmental changes, enables their phenotype switching among different forms, and favors the generation of prometastatic tumor cell subsets. Phenotype switching toward more aggressive forms involves different functional, phenotypic, and morphologic changes, which are often related to the process known as epithelial–mesenchymal transition (EMT). In this study, we report natural killer (NK) cells may increase the malignancy of melanoma cells by inducing changes relevant to EMT and, more broadly, to phenotype switching from proliferative to invasive forms. In coculture, NK cells induced effects on tumor cells similar to those induced by EMT-promoting cytokines, including upregulation of stemness and EMT markers, morphologic transition, inhibition of proliferation, and increased capacity for Matrigel invasion. Most changes were dependent on the engagement of NKp30 or NKG2D and the release of cytokines including IFNγ and TNFα. Moreover, EMT induction also favored escape from NK-cell attack. Melanoma cells undergoing EMT either increased NK-protective HLA-I expression on their surface or downregulated several tumor-recognizing activating receptors on NK cells. Mass spectrometry–based proteomic analysis revealed in two different melanoma cell lines a partial overlap between proteomic profiles induced by NK cells or by EMT cytokines, indicating that various processes or pathways related to tumor progression are induced by exposure to NK cells.

Significance: NK cells can induce prometastatic properties on melanoma cells that escape from killing, providing important clues to improve the efficacy of NK cells in innovative antitumor therapies. *Cancer Res; 78(14); 3913–25. ©2018 AACR.*

Introduction

Attempts to exploit the natural killer (NK) cell antitumor potential for the cure of malignancies recently have proven highly promising for the therapy of certain hematologic malignancies, but have failed so far in solid tumors (1–5). The frustrating results on solid tumors can be explained, at least in part, by the presence at the tumor site of a specific microenvironment capable of interacting with the immune effectors in a complex and still partially unknown cross-talk, which may have unpredictable effects on immune-based therapeutic approaches. The tumor microenvironment can profoundly influence the availability and the function of different immune cell types, including NK cells (6, 7). Indeed, various elements present in the tumor site have been shown to induce downregulation of different activating NK receptors involved in tumor cell recognition (6, 7), including NKG2D, DNAM-1, and the natural cytotoxicity receptors (NCR) NKp30, NKp46, and NKp44 (8).

Moreover, immune cells can participate in the tumor cell editing, favoring the selection/induction of tumor cells abnormally expressing immune-receptor ligands involved in either tumor cell recognition (HLA-I molecules and ligands for NKG2D, DNAM-1, and NCR) or immunoregulatory checkpoints (CTLA-4 and PDL-1; 5, 6, 9–13). Recently, it has also been proposed that certain immune cells present in the tumor microenvironment (namely MDSCs, macrophages, neutrophils, and $CD4^+$ T cells) could even favor tumor progression by promoting epithelial–mesenchymal transition (EMT; refs. 14–18).

According to recent viewpoints, in different tumors, including melanomas, transformed cells would be characterized by a

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remarkable phenotypic and functional plasticity. This plasticity would enable tumor cells to "switch" from a differentiated, proliferative, and poorly invasive state to an undifferentiated, poorly proliferative, and invasive (prometastatic) state (and vice versa; refs. 19, 20). These views unify in a comprehensive phenomenon, which is termed "phenotype switching," different aspects and concepts of the tumor cell biology including the EMT, the generation of cancer stem cells, and the acquisition of drug or radiotherapy resistance (20, 21). Indeed, depending on the tumor cell type, the "phenotype switching" often involves (i) transition toward an undifferentiated/mesenchymal morphology, accompanied by E- and N-cadherin expression switch and cytoskeletal modifications; (ii) acquisition of stemness markers (such as CD166, CD133, and CD271); (iii) proliferation decrease; and (iv) modulation of key transcription factors such as SLUG, SNAIL, and SPARC (19, 22–24). In particular, in melanoma cells, modulation of microphthalmia-associated transcription factor (MITF) appears to play a key role (20).

Various stimuli can favor tumor cell transition including TGFβ and WNT signaling, alteration of receptor Tyr-kinase pathways, hypoxia, and, as mentioned above, the interaction with certain immune cell types (14–19). To our knowledge, no information is available on a possible NK:tumor cell cross-talk in the context of the tumor cell phenotype switching, although the EMT could preferentially occur at the tumor borders, where NK cells have been more frequently localized (6, 18). In this study, we describe and characterize such NK:tumor cell cross-talk and provide evidence of the possible paradoxical activity that NK cells could play in the tumor progression, at least in certain conditions.

Materials and Methods

Generation of polyclonal NK-cell lines

NK cells from healthy donors were purified from peripheral blood and cultured on irradiated feeder cells and rhIL2 for 15 to 20 days (see Supplementary Materials and Methods).

Melanoma cell lines, EMT induction, and cocultures

MeDeBO, MeCoP, MeTU, MePA, and MeTA cell lines were derived from metastatic melanoma resections provided by the local Cancer Surgery Unit and were described previously (25). Once established, the primary cell lines were initially expanded for few passages, phenotypically characterized by FACS, and frozen in multiple aliquots. For the experiments described in this study, cells were thawed, tested for mycoplasma by specific PCR, and used within 30 days. The melanoma cell cultures were phenotypically characterized and assessed for purity by the analysis of informative markers including Mel-CAM/CD146, GD2, and HLA-I. To evaluate EMT/phenotype switch, melanoma cells were cultured in RPMI1640 10% FCS in 6-well plates to 75% confluence, washed, and cultured in complete medium plus 100 IU/mL IL2 either in the absence or in the presence of polyclonal NK cell lines, or in complete medium containing 5 ng/mL TGFβ1 + 10 ng/mL TNFα (EMT cytokines; PeproTech). After 96 hours, melanoma cells were evaluated by FACS/microscopy, or in functional assays, or in proteomic analysis. Coculture melanoma:NK cell ratios were 5:1 (MeDeBO, MeTU, MePA, MeTA) or 2:1 (MeCoP). When indicated, cocultures were performed in transwell devices (Corning Incorporated) maintaining the same ratios and culture times.

Flow cytometry and cytolytic assays

Cells were stained with appropriate mAbs, followed, when needed, by PE-conjugated isotype-specific second reagent (Southern Biotechnology Associated). For intracellular staining of fibronectin, MITF, and Ki67, melanoma cells were fixed and permeabilized using Permeabilization/Fixation Kit (for cytoplasmic proteins) or Foxp3/Transcription Factor Fixation/Permeabilization Kit (for nuclear proteins; eBioscience). Cells were analyzed by FACSCalibur (Becton Dickinson). For antibodies and soluble NCRs, see Supplementary Materials and Methods.

Melanoma cell susceptibility and NK-cell killing capability were evaluated in 4-hour ^{51}Cr-release assays.

Microscopy

Microphotographs were taken using Olympus IX70 microscope equipped with the Hamamatsu ORCA-ER digital camera (images analyzed by Cell^{R} 1.2 Olympus).

Confocal microscopy

Melanoma cells were seeded on glass coverslips and cultured under the indicated conditions. After 4 days, melanoma cells were washed, fixed, permeabilized, and stained with Alexa Fluor 488 phalloidin (Molecular Probes) and DAPI (see Supplementary Materials and Methods).

Matrigel invasion assays

High-density Matrigel (20 μL; 10 mg/mL) with reduced growth factor content (BD Biosciences) were dropped in 12-well plates. Melanoma cells alone, or pretreated for 72 hours with EMT cytokines, or cocultured for 72 hours with NK cells were added in the wells after drop solidification. Melanoma cells were added to obtain 80% confluence (final volume: 1 mL). NK cells and cytokine stimuli were maintained in (co-)cultures for 7 days before taking microphotographs.

Statistical analyses

Statistical analyses were performed by Wilcoxon t test or Mann–Whitney test as indicated in the figure legends (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

Mass spectrometry–based proteomics

Samples were processed by in-Stage Tip protocol (26). Each digested sample was analyzed by high-resolution LC/MS-MS) based on Orbitrap technology. The quantification strategy is based on a label-free approach (LFQ) available in MaxQuant suite. The proteomics data are subjected to a statistical validation applying tools developed in Perseus Software (See Supplementary Materials and Methods; ref. 27).

Bioinformatics analysis

The methodology used in this study combines machine learning approaches (28, 29). In each comparison, proteins are ranked according to their discriminatory power of the corresponding phenotype and measured by a combination of fold change and Fisher ratio. Their predictive accuracy is established using a series of cross-validation experiments. Then, using GeneAnalytics software, we identified the pathways or the biological processes associated with the protein list (see Supplementary Materials and Methods).

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IHC analysis

Sections were obtained from formalin-fixed paraffin-embedded tissue blocks of human primary cutaneous melanomas. Briefly, for IHC, the reaction was revealed using Novolink Polymer (Leica) or Labelled Polymer HRP (Dako) followed by DAB (single stainings of E-cadherin, N-cadherin, ZEB1, ZEB2). For double staining, sections were stained with two consecutive reactions with primary antibodies, using OptiView DAB IHC Detection Kit and ultraView Universal Alkaline Phosphatase RED Detection Kit (to assess E- or N-cadherin expression in areas close to or far from $CD56^+$ NK cells) or Mach 4 MR-AP (Biocare Medical), followed by Ferangi Blue (to assess CD56 and SOX5). The quantification of marker expression was evaluated as percentage of positive cells or as "H-score." The H-score was assigned in each area as the sum of the products of the intensity (0 for negative, 1 for weakly positive, 2 for moderately positive, and 3 for strongly positive) and the extent of immunoexpression (0%–100%).

Patient studies

For the use of material from the patients, written informed consent was obtained. The studies were conducted in accordance with the Declaration of Helsinki and were approved by the local Institutional Review Boards to WV (WW-IMMUNOCANCER^{hum}, code NP-906) and to MCM (PR023REG2013).

Results

NK cells promote EMT and phenotype switching in melanoma cell lines

In order to study the possible effect of NK cells on the tumor cell phenotype switch, we set up mixed cell coculture experiments using primary polyclonal NK cell lines derived from healthy donors and 5 melanoma cell lines (MeDeBO, MeCoP, MeTU, MePA, and MeTA; ref. 25). These melanoma cell lines were selected on the basis of their different EMT characteristics at baseline and differential ability to undergo modulation of their phenotype and/or function in response to EMT-inducing cytokines (TNFα + TGFβ1; EMT cytokines) (23). Cocultures were done at low NK:melanoma cell ratios, that is, cell-to-cell proportions coherent with the generally low NK-cell infiltrate observed in tumors (6, 9). As controls, melanoma cells were cultured alone either in the absence of additional stimuli or in the presence of EMT cytokines. After 72 hours of coculture, melanoma cells were analyzed by FACS or microscopy for the expression and/or the distribution of informative markers and typical EMT proteins.

Thus, we first analyzed the stemness markers CD271 and CD166, the transcription factor MITF, and the G protein–coupled receptor GPR56. As also mentioned above, MITF expression decrease is a hallmark of the phenotype switch in melanoma, while GPR56 expression has been inversely correlated with the acquisition of prometastatic properties (30). As shown in Fig. 1A, upon exposure to NK cells, most melanoma cell lines significantly increased the expression of CD271 and CD166 and reduced the expression of MITF and GPR56. Specifically, MeDeBO, MeCoP, and MeTU modified, at various extents, all the analyzed markers, whereas MePA and MeTA showed changes only in CD271 and MITF, respectively. Noteworthy, the effect of coculture with NK cells was similar to (or even more pronounced than) that of EMT cytokines in all the analyzed melanoma cell lines.

We then evaluated typical features of the EMT such as cadherin switch (concomitant downregulation of E-cadherin and upregulation of N-cadherin expression), fibronectin expression, F-actin filament reorganization, and morphologic changes. Among the cell lines analyzed, MeDeBO and MeCoP cells showed clearest EMT-related changes in response to both NK and cytokine stimulation, whereas MePA and MeTA cells, expressing EMT-related features at baseline, poorly modified their phenotype (Fig. 1B; Supplementary Fig. S1). MeDeBO cells (which highly expressed E-cadherin at baseline) showed cadherin switch, fibronectin expression increases, and cytoskeleton reorganization, as indicated by the generation of F-actin stress fibers, whereas MeCoP cells, besides increasing F-actin reorganization, acquired a clear spindle-shaped morphology typical of EMT.

We next analyzed Ki67 expression, a reliable marker to evaluate cell growth. As shown in Fig. 1C, inhibition of proliferation in MeDeBO and MeCoP occurred after coculture with NK cells, further supporting the notion that NK cells may induce phenotype switching.

Finally, we analyzed whether NK cells had any effect on the invasive capability of melanoma cells. To this end, melanoma cells were assessed for the capacity to enter and move through Matrigel spheres. Among the analyzed cell lines, MeCoP cells showed invasive capabilities after induction of EMT by NK coculture or EMT cytokines. Thus, spindle-shaped MeCoP cells acquired a bundle organization that favored the invasion of the Matrigel sphere (Fig. 1D; Supplementary Fig. S2).

Because the process of EMT, and in particular the downregulation of E-cadherin, can be regulated by specific transcriptional factors, we assessed the effects of NK cells on SNAIL, SLUG, TWIST, and ZEB2 expression in MeDeBo cells. As shown in Supplementary Fig. S3A, NK cells induced increased expression of SLUG and ZEB2 in MeDeBO cells after 24 and 48 hours of coculture. This finding is in line with the progressive decrease of E-cadherin expression in MeDeBO cells (Supplementary Fig. S3B).

Altogether, our experiments indicate that NK cells are capable of inducing different changes in melanoma cells compatible with EMT and phenotype switching.

NK-induced melanoma phenotype switching/EMT depends on cell-to-cell interaction and release of IFNγ and TNFα

In order to define the mechanisms underlying the NK cell–mediated induction of the phenotype switching in melanoma cells, we performed coculture experiments in transwells (outlined in Supplementary Fig. S4). We chose the representative cell lines, MeDeBO and MeCoP, and analyzed parameters suitable for evaluating the effect of NK cells. In particular, MeDeBO cells were analyzed for the expression of E-cadherin, CD271, MITF, GPR56, and Ki67, whereas MeCoP cells were assessed essentially for their morphology. As shown in Fig. 2A and B, MeDeBo and MeCoP cells underwent phenotypic or morphologic changes when NK and melanoma cells were cultured in contact but not when cocultures were performed in transwell (i.e., NK and melanoma cells were cultured in the upper and lower chamber, respectively, TW). On the other hand, the phenotype switching (or the EMT) was induced in melanoma cells alone in the lower chamber when NK:melanoma cocultures were set up in the upper chamber of the transwell (TW/w). These experiments indicate that NK–tumor cell contact

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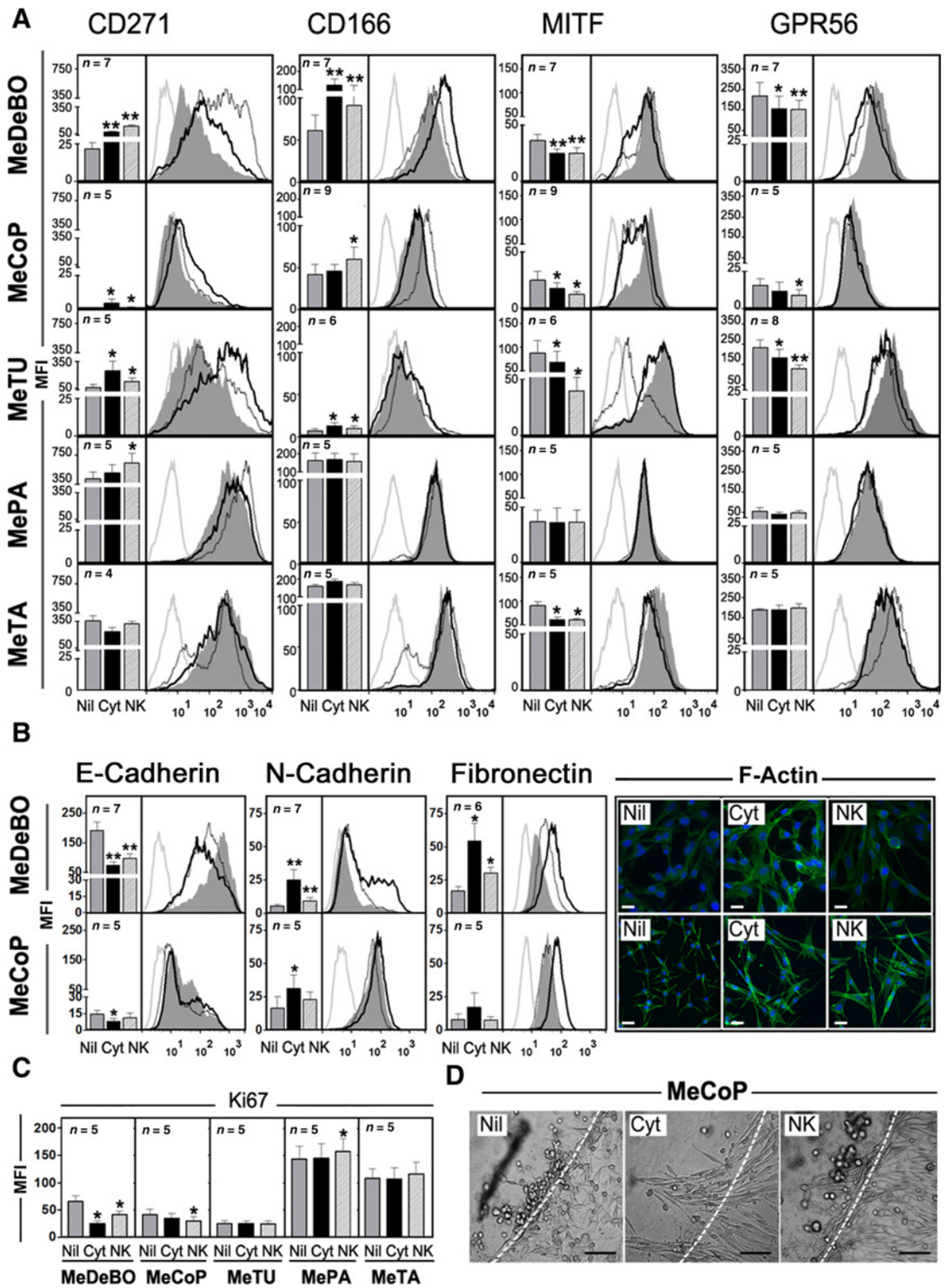

Figure 1.

NK cells can induce traits of the phenotype switch in melanoma cells. The indicated melanoma cell lines were cultured alone (Nil), exposed to EMT cytokines, or cocultured with NK cells, and analyzed by FACS or microscopy to evaluate. **A,** Informative markers of malignancy and/or stemness. **B,** EMT markers and morphology, and F-actin distribution (green). Scale bars, 50 μm. **C,** Ki67 expression. **D,** Capability of invading a Matrigel droplet (the border is indicated by the white dashed line). Scale bars, 200 μm. Data from FACS analyses are represented by histograms as mean fluorescence intensity (MFI) mean + SEM of at least four independent experiments. FACS profiles from a representative experiment are also shown in **A** and **B**. Isotype control (gray profiles), melanoma cells cultured alone (gray-filled profiles), with cytokines (black profiles), with NK cells (dashed profiles). Statistical analyses were performed by Wilcoxon *t* test.

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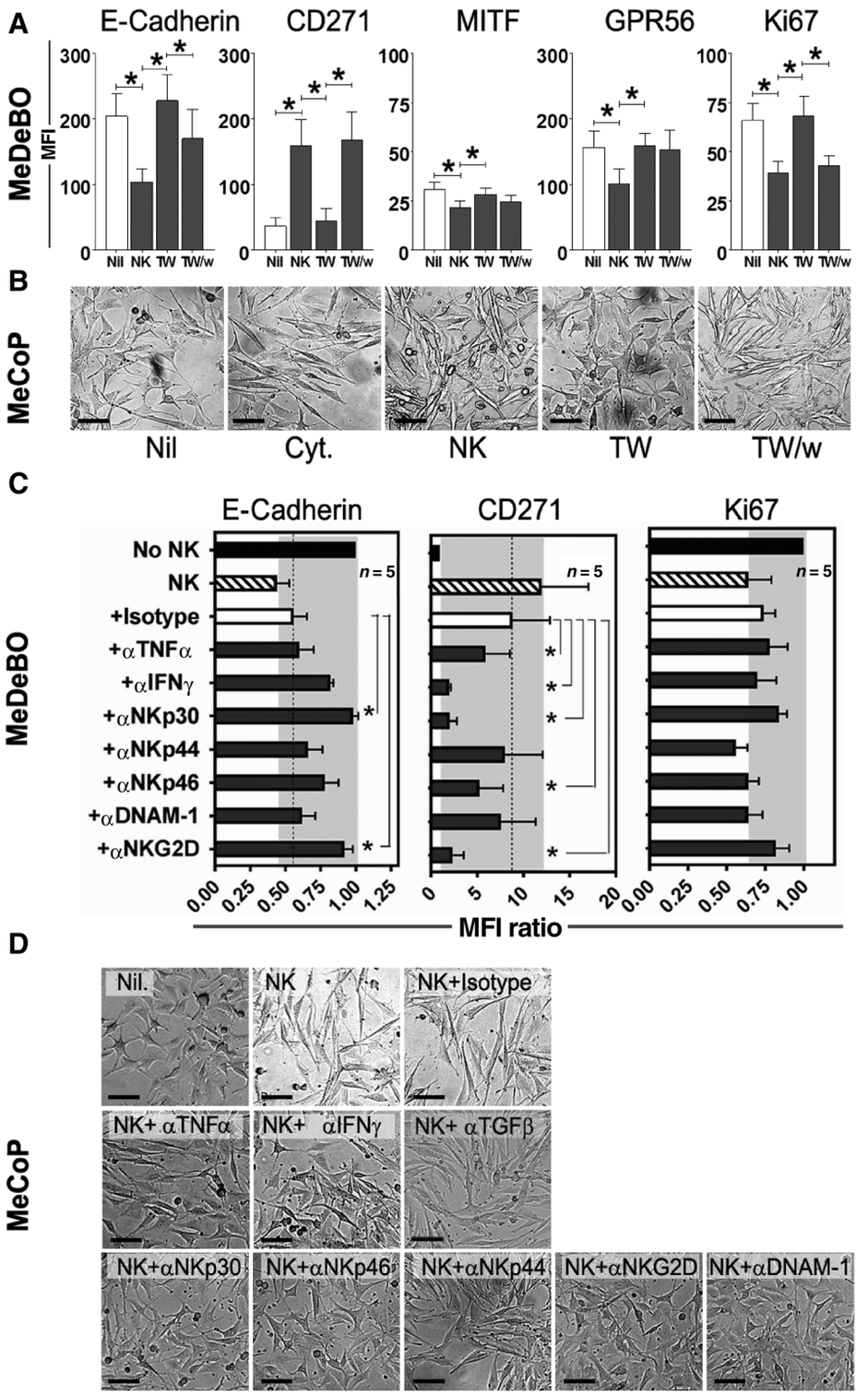

Figure 2.

NK-induced phenotype switch involves cell-to-cell interactions, engagement of NK triggering receptors, and cytokine release. **A** and **B,** Melanoma cells were cultured alone (Nil), with NK cells (NK), with NK cells in transwell devices. The upper chamber of transwell was loaded with NK cells (TW) or with NK + melanoma cells (TW/w). Informative markers (MeDeBO) or morphology changes (MeCoP) were analyzed by FACS or microscopy. Scale bars, 100 μm. The control MeCoP cells were also cultured in the presence of EMT cytokines. Histograms represent mean fluorescence intensity (MFI) mean + SEM of 6 independent experiments. **C** and **D,** Melanoma cells were cultured alone (Nil), with NK cells (NK), with NK cells in the presence of blocking mAbs to the indicated receptors or cytokines. Informative markers (MeDeBO) or morphology changes (MeCoP) were analyzed by FACS or microscopy. Scale bars, 100 μm. In **C**, the expression changes induced in the various conditions are calculated as mean fluorescence intensity ratio to Nil condition (mean + SEM of at least 5 experiments is shown). Statistically significant differences to the control Ab "isotype" condition are indicated. The shady areas highlight the extent of NK-induced changes and potential phenotype restoration by blocking mAbs. Statistical analyses were performed by Wilcoxon *t* test.

is required for inducing melanoma cell modification and that most of the effects induced by NK cells are mediated by soluble factors released following such cell-to-cell interaction.

In order to molecularly characterize the nature of the NK:melanoma cell interactions, we performed coculture experiments in the presence of blocking antibodies directed to major activating NK receptors or NK-derived cytokines. As shown in Fig. 2C, anti-IFNγ Abs and, to a minor extent, anti-TNFα Abs inhibited the effect of NK cells on CD271 expression in MeDeBO cells. In addition, anti-IFNγ Abs also showed an

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inhibitory trend toward the NK-induced effect on E-cadherin. Significant inhibitory effects were obtained also by blocking different activating receptors, with maximal effects achieved upon NKp30 and NKG2D blockage. NKp30 and NKG2D blockage also appeared to inhibit the NK-mediated effect on melanoma proliferation (although without reaching statistical significance; Fig. 2C, see Ki67 expression). Nevertheless, also NKp44, DNAM-1, and NKp46 receptors could contribute to the NK:melanoma interaction as their combined blockage resulted in an inhibitory effect comparable with that obtained by NKp30 masking (Supplementary Fig. S5A). Similarly, various blocking Abs also showed an effect in the MeCoP:NK cell coculture. Thus, anti-IFNγ, anti-NKp30, anti-NKp46, anti-NKG2D, as well as anti–DNAM-1 Abs efficiently counteracted the NK-induced morphologic transition of MeCoP cells, whereas anti-NKp44 and anti-TNFα Abs showed minor effects (Fig. 2D). Finally, we also analyzed whether TGFβ could be involved in the NK-induced effect on melanoma cells, because such EMT-inducing cytokine may be either produced by tumor cells or induced in cocultures. As shown in Supplementary Fig. S5B and in Fig. 2D, anti-TGFβ–blocking Abs did not interfere with the phenotypic or morphologic changes induced by NK cells in MeDeBO and MeCoP cells.

In conclusion, our results indicate that the induction of tumor phenotype switching/EMT by NK cells is primarily sustained by IFNγ (and partly by TNFα), that is, cytokines that are released upon NK:melanoma cell interaction and engagement of activating receptors.

Effect of phenotype switching/EMT on the NK:melanoma cell cross-talk

We then analyzed whether undergoing a phenotype switch modifies melanoma cell susceptibility to NK cell–mediated attack, or their ability to suppress NK-cell activity.

The phenotype switching/EMT on MeDeBO and MeCoP cells, induced either by exposure to EMT cytokines or by cell coculture with NK cells, did not substantially modify the surface expression of NKG2D or DNAM-1 ligands, nor did it modify the binding of the soluble NCRs (to their ligands at the tumor cell surface; Supplementary Fig. S6). We also assessed the expression of PD-L1, as this suppressive molecule can be induced during EMT on breast cancer cells (13). As shown in Supplementary Fig. S6, exposure to both cytokine and NK cells resulted in a slight induction of PD-L1 expression in MeCoP cells, whereas it was ineffective in MeDeBO cells. On the other hand, MeCoP cells evidencing phenotype switch showed a marked HLA-I surface expression increase accompanied by increased resistance to NK-cell killing activity (Fig. 3A and B). HLA-I masking by specific mAbs completely restored the susceptibility of "cocultured" MeCoP cells to NK cell–mediated killing, an effect that was incomplete on cytokine-treated melanoma cells (Fig. 3C), suggesting that, besides HLA-I upregulation, additional mechanism of resistance may be induced by EMT cytokines. MeDeBO cells lacked surface HLA-I molecules. Accordingly, they did not show any modification of HLA-I expression and did not modify their susceptibility to NK cell–mediated killing (Fig. 3D and E). Exposure to EMT cytokines or coculture with NK cells led to HLA-I upregulation also on MePA, MeTU, and MeTA cells, despite in these cases, the effects were less statistically significant (Supplementary Fig. S7). We also assessed whether exposure to NK cells could increase resistance of MeCoP and MeDeBO cells to therapeutic treatments such as cytotoxic drugs or γ-radiation. As shown in Supplementary Fig. S8, NK cells did not modify the susceptibility of melanoma cells to taxol, while increased resistance to γ-radiation in MeCoP (although without reaching statistical significance).

In order to assess the effect of EMT on the immunosuppressive capability of melanoma cells, MeCoP and MeDeBO cells were exposed to EMT cytokines to induce phenotype switching (EMT melanoma cells), and, afterwards, cocultured with NK cells for 3 days. As TGFβ can affect both NK-cell function and phenotype, EMT melanoma cells were extensively washed before starting coculture. NK cells from cocultures were then analyzed for the expression of major activating receptors. As shown in Fig. 4A, melanoma cells were able to induce downregulation of NKp30, NKG2D, and DNAM-1 on NK cells. The difference between untreated and EMT melanoma cells in decreasing NCR expression was statistically significant in the case of MeDeBo cells. Thus, NK cells exposed to MeDeBO cells were also tested in a cytolytic assay. As shown in Fig. 4B, NK cells conditioned by EMT-MeDeBO cells showed maximal inhibition of their ability to kill melanoma cells as compared with NK cells exposed to MeDeBO cells or cultured alone. Because induction of immune checkpoint receptors on NK cells has been associated with tumor progression (12, 31), we also assessed whether EMT melanoma cells could influence TIM-3 and PD-1 expression on NK cells. As shown in Fig. 4A, both melanoma cell lines induced little fluctuations of TIM-3 expression on NK cells. In particular, exposure to EMT-MeDeBO cells resulted in slight, but not significant increase of TIM-3 expression. PD-1 expression was not induced in either the analyzed conditions.

Thus, the phenotype switch/EMT can favor the escape to NK-cell antitumor activity by enabling tumor cells to express higher levels of HLA-I molecules and to increase their ability to target activating receptors on NK cells.

Analysis of proteomic profiles induced by NK cells on MeCoP and MeDeBO cells

The above functional and phenotypic data indicate that NK cells can induce, in melanoma cells, traits of increased malignancy that are hallmarks of phenotype switch or EMT. To gain further insight on this activity and acquire a more comprehensive characterization of the NK cell–mediated effect on melanoma cells, we analyzed the proteomic changes induced by NK cells or by EMT cytokines on MeCoP and MeDeBO cell lines.

High-resolution mass spectrometry analysis of cell lysates led to the definition of about 5,000 proteins for each cell line (in each individual experimental condition). The Spearman rank correlation indicated an acceptable reproducibility among the analyzed biological replicates (average coefficients > 0.92 for each condition in both cell lines; Supplementary Fig. S9A). As shown by the Venn diagrams (Fig. 5A), exposure to NK cells or to EMT cytokines, respectively, induced the modulation of 183 or 268 proteins in MeCoP cells, and 1,466 or 2,957 proteins in MeDeBo cells (T test false discovery rate = 0.05, S0 = 0.1; also see the protein lists in Supplementary Table S1). Thus, the number of differentially expressed proteins greatly differed between the two cell lines, suggesting a potential, marked variability among melanoma cells in the response to stimuli and, specifically, to NK cells. In spite of this variability, a consistent part of the proteins that were

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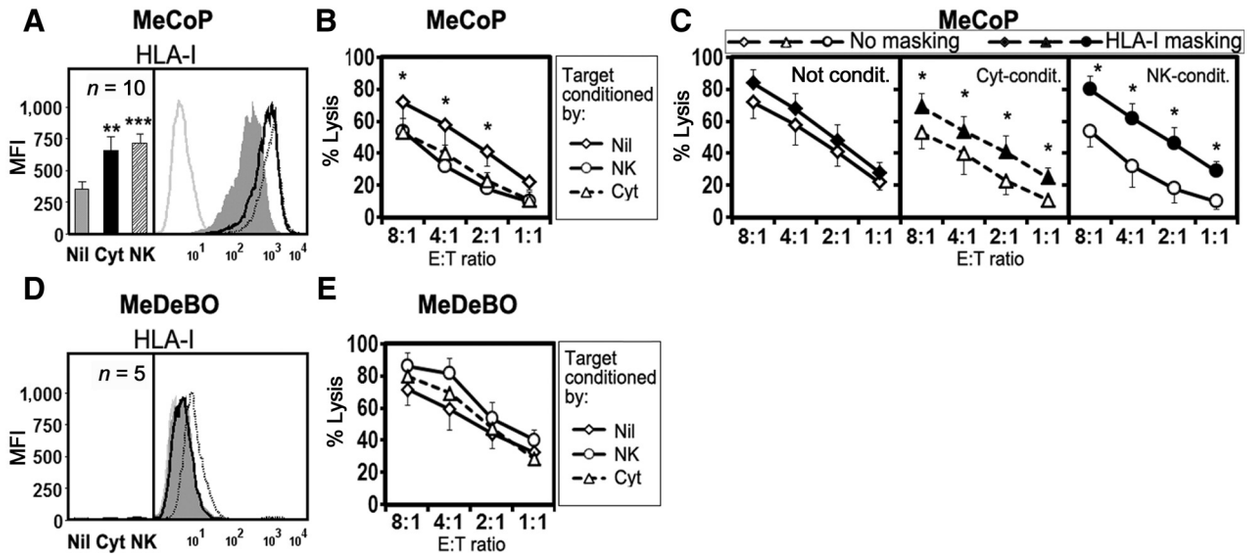

Figure 3.

Effect of EMT on melanoma cell susceptibility to NK lysis. The indicated melanoma cell lines were cultured alone (Nil), with EMT cytokines (cyt), or NK cells (NK), and then analyzed. **A** and **D,** For HLA-I expression by FACS. **B**, **C**, and **E,** For susceptibility to NK-cell lysis in cytolytic assays. In **C**, the effect of HLA-I masking mAbs is also assessed. Data from FACS analyses are represented by histograms as mean fluorescence intensity (MFI) mean + SEM of at least 5 independent experiments. FACS profiles from a representative experiment are also shown. Isotype control (gray profiles), melanoma cells cultured alone (gray-filled profiles), with cytokines (black profiles), with NK cells (dashed profiles). Data from cytolytic assays represent the mean + SEM of 6 samples pooled from three independent experiments. In **B**, statistically significant differences to the "Nil" condition refer to both NK- and cyt-conditioned targets. Statistical analyses were performed by Wilcoxon *t* test (**A** and **D**) and Mann–Whitney test (**B**, **C**, and **E**).

modulated upon NK-cell exposure was also influenced by cytokine treatment in both cell lines analyzed (49% and 72% of NK-modulated proteins in MeCoP and MeDeBo, respectively). In addition, the unsupervised hierarchical clustering analysis of differentially expressed proteins indicated that NK cells and cytokines often induced concordant protein modulation (Fig. 5B; Supplementary Fig. S9B).

To extensively define the functional networks and the potential effects induced by NK cells, we investigated whether the NK-modulated proteins could be enriched in specific biological processes or pathways. Thus, proteins clustered according to their expression values as up- or downregulated were analyzed using GeneAnalytic platform (see Materials and Methods). In either NK-conditioned melanoma cell line, both up- and downregulated protein clusters showed significant enrichment in a sizable number of biological processes or pathways (collectively referred to as entities; Supplementary Tables S2 and S3). As it may be expected, in both cell lines, the upregulated protein clusters showed a great enrichment in entities related to cell death and IFNγ-dependent response, confirming the major role of classical NK-cell functions in the NK:melanoma cell cross-talk (Fig. 5C; Supplementary Tables S2 and S3). On the other hand, upregulated protein clusters also showed enrichment in a large number of entities related to tumor cell invasiveness and dissemination (i.e., cell adhesion, morphology, and motility), induction of classic oncogenic pathways or EMT (including SMAD and ERK signaling, TGFβ pathway, VEGF signaling, developmental biology), or response to stress. Moreover, downregulated protein clusters were enriched in entities related to oncogene suppression, cell proliferation, and metabolism (Fig. 5C; Supplementary Tables S2 and S3).

In conclusion, the proteomic analysis indicates that NK cells can activate at least part of the EMT cytokine–induced programs in melanoma cells. Importantly, they can induce melanoma cells to modify the expression of a wide range of proteins involved in diverse aspects of tumor cell biology, and to acquire proteomic profiles compatible with the switching toward an undifferentiated, poorly proliferative, and invasive (prometastatic) state.

Effects of NK cells at the tumor site

We next analyzed whether NK cells could induce EMT-related effects also at the tumor site. To this end, we assessed by IHC a panel of primary melanoma lesions characterized by either poor or rich NK-cell infiltrate (NK-poor samples: <10 NK cells/mm^2; NK-rich samples: >30 NK cells/mm^2). In particular, samples were assessed for the expression of E- and N-cadherin, and ZEB1, ZEB2, and SLUG transcription factors. SLUG was excluded from the analysis due to poor staining quality of formalin-fixed sections for this marker. The analysis of the other markers revealed that E-cadherin expression was significantly reduced in the group of NK-rich samples, whereas N-cadherin, ZEB1, and ZEB2 expression showed a trend to increase (Fig. 6A). Remarkably, although only E-cadherin modulation reached statistical significance, the expression changes of the four analyzed markers were all in the specific direction of EMT. A more detailed analysis revealed that cadherin expression could be heterogeneously distributed within a single sample. In Fig. 6B, both an NK-rich and an NK-poor case show areas of complete cadherin switch, near the NK-cell infiltrate, and areas (devoid of NK cells) with an opposite cadherin pattern. On their complex, these data suggest that the presence

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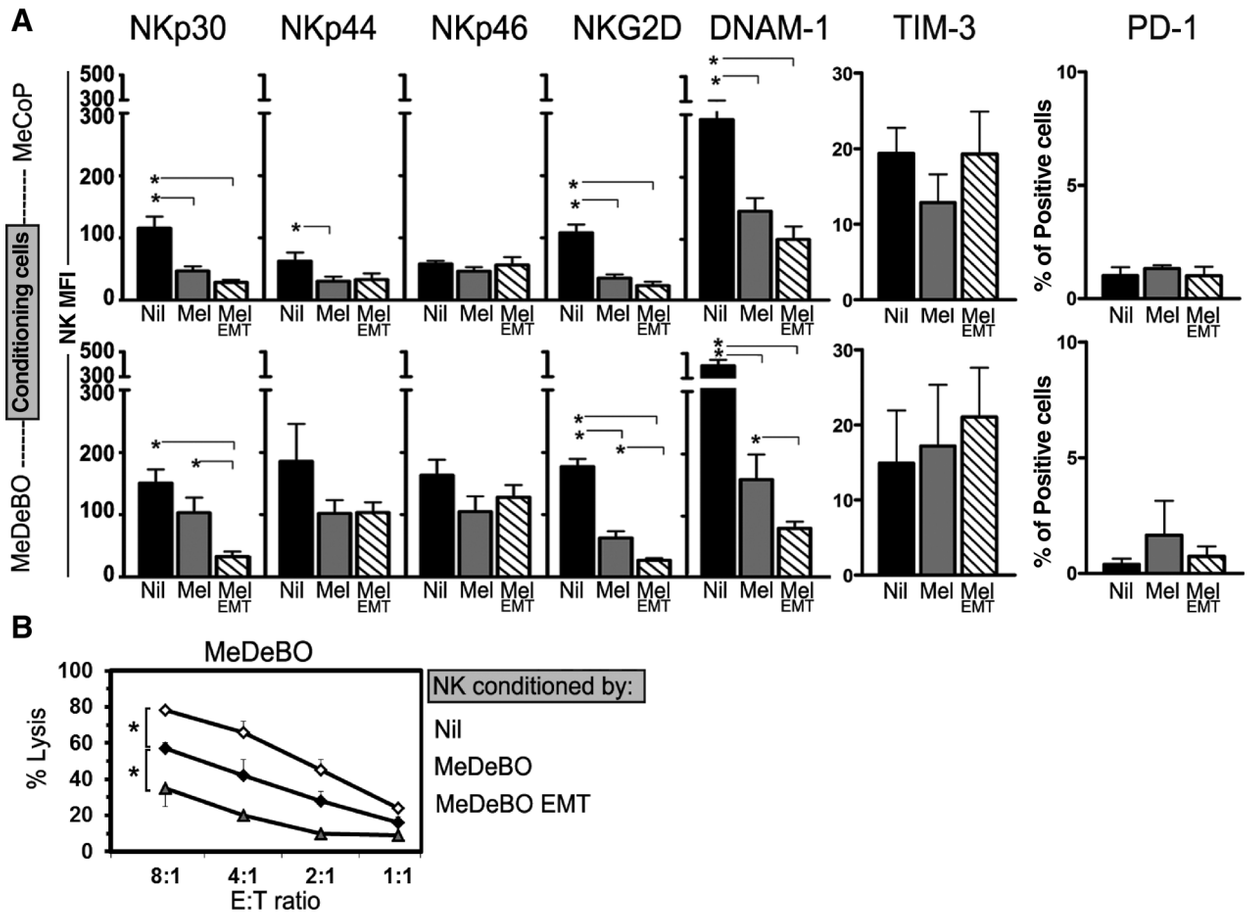

Figure 4.

EMT can increase the suppressive capability of melanoma cells. **A,** NK cells unconditioned (Nil), conditioned with melanoma cells (Mel), or conditioned with melanoma cells exposed to EMT cytokines (Mel EMT) were analyzed by FACS for surface expression of the indicated NK receptors. The conditioning melanoma cell lines are indicated to the left. Histograms represent mean fluorescence intensity (MFI) mean + SEM of at least four independent experiments. **B,** NK cells conditioned as indicated were analyzed for their ability to kill MeDeBO cells in a cytolytic assay. Data represent the mean + SEM of 6 samples pooled from three independent experiments. Statistical analyses were performed by Wilcoxon *t* test (**A**) and Mann–Whitney test (**B**). Before phenotypic or functional analyses, NK cells were counted using trypan blue staining to evaluate dead cells. In all conditions analyzed, NK-cell viability was approximately 90%.

and the proximity of the NK-cell infiltrate may favor the EMT. We also found samples showing rather homogeneous N- or E-cadherin expression, independent on the NK-cell localization. In these cases, the prevalence of various factors known to affect EMT (i.e., cytokines, hypoxia, stromal cells, or other immune cells) may have masked the specific effect of NK cells.

To further dissect the effect of NK cells on EMT, we selected a sample showing heterogeneous distribution of NK cells and cadherins and quantified E- and N-cadherin expression in the areas close to or distant from NK-cell infiltrate (see an example in Supplementary Fig. S10). This quantification was done by calculating the H-score for each of the areas included in the "close" or "far" group (see Supplementary Materials and Methods). As shown in Fig. 6C, the areas close to NK cells showed a statistically significant lower expression of E-cadherin and higher expression of N-cadherin as compared with areas distant from NK cells. These data definitely indicate that, at least in certain cases, NK cells can influence the EMT at the tumor site.

Discussion

This study provides the first experimental evidence that NK cells can induce the phenotype switching of melanoma cells to an undifferentiated, poorly proliferative, proinvasive state. We show that the presence of NK cells in coculture experiments can induce on melanoma cells increased expression of stemness markers, E- and N-cadherin switch, cytoskeletal and morphologic rearrangements, reduced tumor proliferation rates, and also, in one melanoma cell line, increased invasiveness. Moreover, the analysis of the NK-induced proteomic profiles indicates that NK cells may profoundly intervene in the overall biology of melanoma cells, not only by affecting important pathways related to tumorigenesis, cell survival/death, and response to stress, but also by influencing various metabolic processes.

Transwell experiments indicate that at least some of the NK-mediated effects are triggered by cell-to-cell interactions but can be extended to melanoma cells that are not in close contact with

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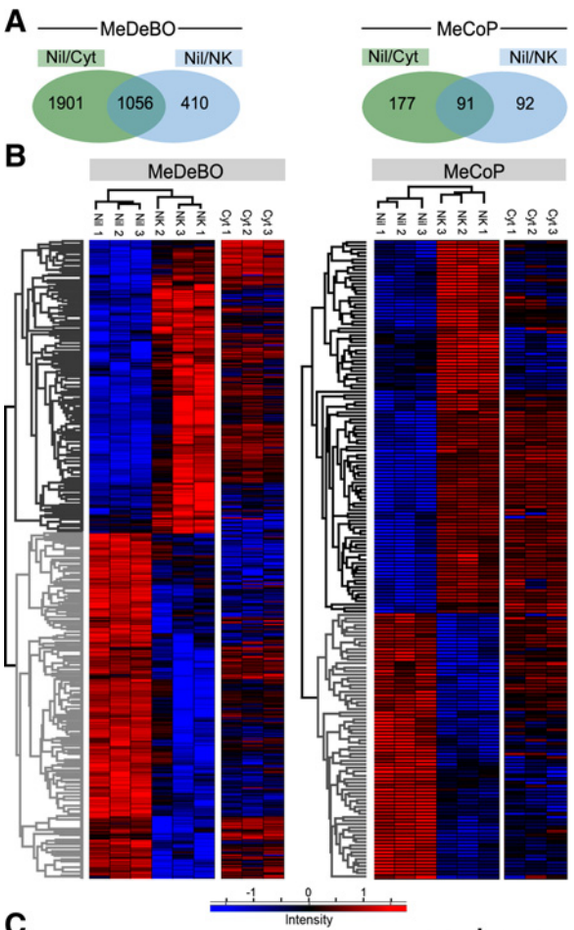

C

Selected groups of entities	MeDeBO Number of entities Up-	MeDeBO Down-	MeCoP Up-	MeCoP Down-
Immune response				
Antigen presentation	9		15	
Cytokine signaling pathway	2			
IFNb/a pathway	2		3	
IFNg pathway	3		4	
Susceptibility to NK cell killing	1		4	
Cell death				
Apoptosis	6			
Negative regulation of apoptosis	1			
Non-apoptotic programmed cell death	2		1	
Adhesion/morphology/motility				
Cell adhesion	3	1	1	
Morphology/motility	6	4	3	
Oncogenic pathways and tumor progression				
Oncogenic pathways and tumor progression	14	6	4	4
Proliferation				
Cell cycle	5	6		13
Response to stress				
UPR response	4		1	
DNA damage		4		
Response to acidic stress			1	
Metabolism				
Generic metabolism		4		4
Metabolism of carbohydrates		10		
Metabolism of cofactors and vitamins		4		
Metabolism of lipids		11		13
Metabolism of nucleotides		3		
Metabolism of proteins	3	10		1
Energy metabolism		7		1
Metabolism of terpenoids and polyketides		1		2
Total selected enriched entities	61	71	37	38
Total enriched entities	103	82	51	42

NK cells, suggesting that NK cells can edit bystander melanoma cells (i.e., cells that cannot be directly reached and killed). This effect could be important at the tumor site, where melanoma cells typically outnumber NK cells.

The NK-mediated tumor cell editing appears to depend, at least in part, on the engagement of various activating NK receptors and the release of cytokines, TNFα and, most notably, IFNγ. Along this line, IFNγ has been previously suggested to play a role in several mechanisms of tumor evasion from NK cells as it enhances the expression of "protective" HLA-I molecules and induces immunosuppressive mediators such as IDO (6, 9, 25). In addition, IFNγ released by NK cells may induce autophagy, an event that can protect tumor cells from both NK- and T-cell attack (32–34). We have analyzed whether NK cells could promote conversion of cytosolic light chain 3I (LC3I) form in the "autophagic" LC3II form. We did not observe significant effects in MeDeBO and MeCoP cells (Supplementary Fig. S11), suggesting that in our experimental system, NK cells may not influence the process of autophagy. Both cell lines, however, expressed LC3II at baseline. Further studies on different tumor cells should definitely address this issue.

Nevertheless, a possible targeting of IFNγ in the context of NK-based therapies must be considered with caution, because IFNγ has also been implicated in different antitumor immune responses and, notably, in NK-mediated antitumor activity (35, 36). Further studies aimed at the fine dissection of the NK:melanoma molecular cross-talk would help to find out suitable therapeutic targets in this context.

Whatever would be the underlying mechanisms, the NK-mediated effect on melanoma cells appears to largely overlap that of the EMT cytokines. Indeed, both NK cells and EMT cytokines can modify the phenotype of MeDeBO, MeCoP, and (in part) MeTU while having little effects on MePA and MeTA cell lines. In line with these findings, MePA and MeTA cells show no detectable E-cadherin protein expression at baseline and express N-cadherin, CD271, and CD166 at highest levels (Fig. 1A; Supplementary Fig. S1), suggesting that they might have already undergone the phenotype switch to some extent. The proteomic analysis of two melanoma cell lines exposed to NK cells or to EMT cytokines confirms that NK cells can specifically favor the EMT or the tumor phenotype switching,

Figure 5.

Analysis of proteomic profiles induced by NK cells on MeCoP and MeDeBO cells. **A,** Venn diagrams indicating the number of proteins modulated by EMT cytokines, by NK cells or by both stimuli. Differentially expressed proteins were selected by *t* test analysis (false discovery rate = 0.05; S0 = 0.1) of stimulated and unstimulated melanoma cells from three independent experiments. **B,** Hierarchical clustering analysis of differentially expressed proteins in melanoma cells unstimulated (Nil1, 2, 3) or exposed to NK cells (NK1, 2, 3) in three independent experiments. For these proteins, the relative expression levels in the EMT cytokine–stimulated melanoma cells for comparison are also shown. **C,** Analysis of up- and downregulated protein enrichment in selected biological processes or pathways (entities). The enriched entities are grouped according to their affiliation to specific cellular functions or behaviors. The groups are assigned to wide functional categories identified by a color code. For each group, the number of entities enriched in up- or downregulated proteins is indicated in pink and pale blue (red and blue highlight number of entities >5). The number of selected or total enriched entities is reported at the bottom.

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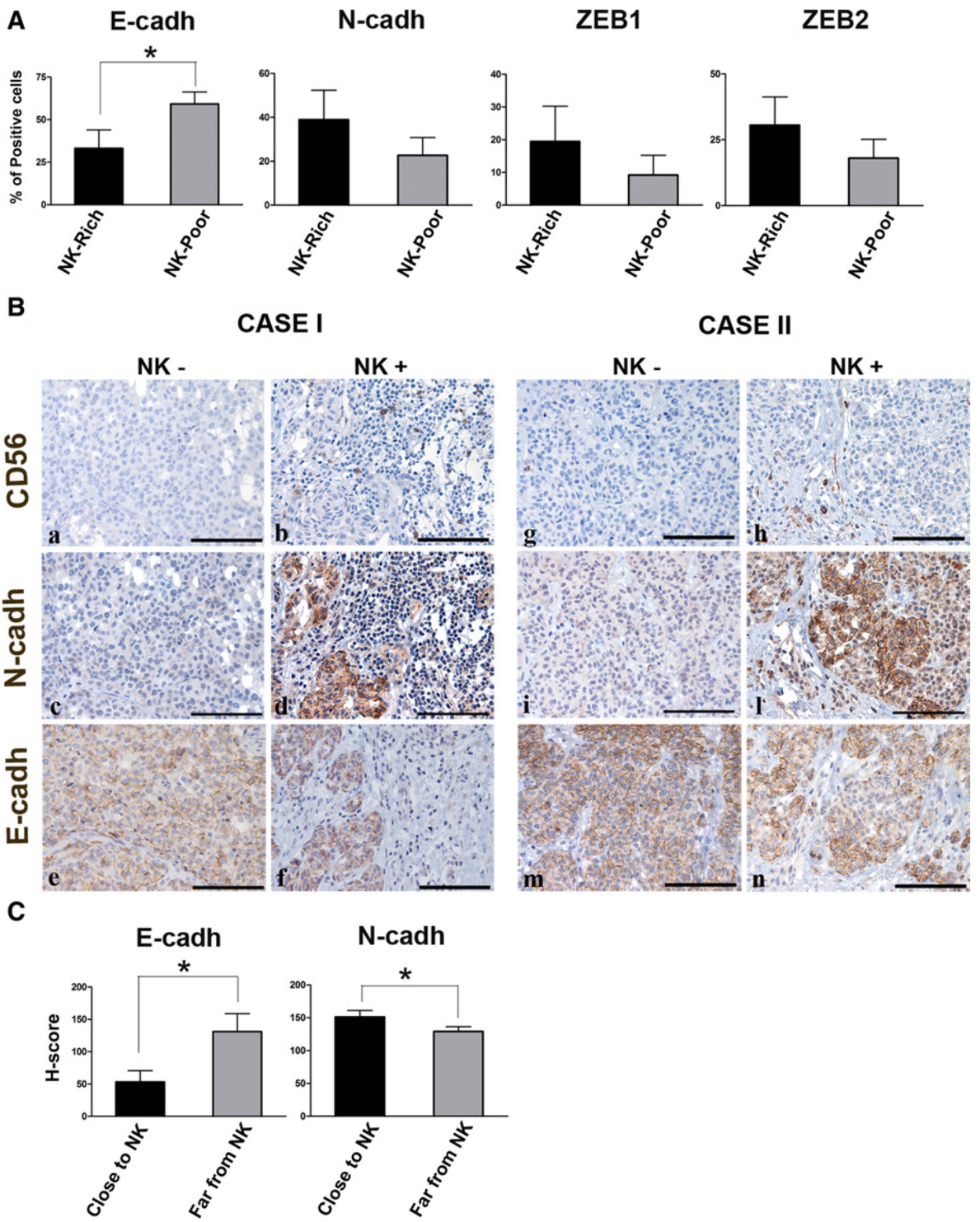

Figure 6.

IHC analysis of EMT markers and NK-cell infiltrate in melanoma lesions. **A,** Statistical analysis of E-cadherin, N-cadherin, ZEB1, and ZEB2 expression in 9 "NK-rich" and 13 "NK-poor" melanoma specimens. **B,** Subserial sections from two primary cutaneous melanoma cases (CASE I: NK-poor – CASE II: NK-rich). The subserial sections were stained for CD56 (**a**, **b**, **g**, and **h**), N-cadherin (**c**, **d**, **i**, and **l**), and E-cadherin (**e**, **f**, **m**, and **n**) as labeled. For each case, areas with or without NK cells (NK+ or NK−) are shown. Original magnification, ×10 (scale bar, 200 μm). **C,** Statistical analysis of H-score for the expression of E-cadherin and N-cadherin in the areas "far from NK" and "close to NK." Fields analyzed/condition: $n = 10$. Statistical analyses were performed by Mann–Whitney test (**A**) and Wilcoxon t test (**C**).

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but also highlights the complexity of the NK-mediated effects on melanoma cells. As shown in Fig. 5A–C, the NK-induced proteomic changes largely overlap those induced by EMT cytokines and involve biological processes or pathways related to tumor cell transition to aggressive forms. These changes also include the modulation of proteins involved in several metabolic processes or in cell-cycle regulation, suggesting that NK cells can contribute to the phenotype switch also through a general downregulation of tumor cell metabolism and proliferation. Remarkably, these effects may favor the generation of the so-called "dormant tumor cells" (37), which are thought to give rise to tumor relapse and metastasis. Finally, both the analyzed melanoma cell lines show clear increments of proteins involved in the interaction with immune cells and, in particular, in the response to IFNγ, confirming the pivotal role of this cytokine in the NK:melanoma cell cross-talk.

The IHC analysis of NK-rich and NK-poor melanoma specimens shows that in NK-rich samples, four EMT markers, including E-cadherin, N-cadherin, ZEB1, and ZEB2, were all modulated in the direction of the EMT (although only for E-cadherin this modulation reached statistical significance). In addition, in a sample showing heterogeneous cadherin and NK-cell distribution, the modulation of both E- and N-cadherin significantly correlated with the vicinity of melanoma cells to the local NK-cell infiltrate. On their complex, the IHC data indicate that NK cells can effectively participate in the induction of the EMT at the tumor site. In this context, it should also be considered that several microenvironmental factors can influence EMT markers, and veil, in certain circumstances, the effect of NK cells. In search of EMT markers that could be more specifically modulated by NK cells, we reevaluated the proteomic data and noticed that MeDeBO cells exposed to either NK cells or cytokines showed upregulation of SOX5, a transcription factor involved in the negative regulation of MITF and in the induction of EMT (see Supplementary Table S1; refs. 38, 39). We analyzed this molecule in the melanoma lesions and found a clear statistical correlation between the rich NK-cell infiltrate and the high expression of SOX5 (Supplementary Fig. S12A and S12B). In our opinion, this marker deserves further detailed studies to define its possible role in the NK:tumor cell cross-talk.

MITF downregulation is distinctive of phenotype switching in melanoma cells. However, the effects of NK cells may be extended to tumors of epithelial origin, as suggested by the observation that NK cells can promote the upregulation of two master regulators of EMT (Supplementary Fig. S3A) and can induce morphologic changes (compatible with EMT) on the cervix adenocarcinoma HeLa cell line (Supplementary Fig. S13).

Our data indicate that NK cells promote EMT, but also show that EMT profoundly influences NK cells. Indeed, EMT can sharply strengthen the suppressive capability of melanoma cells by enhancing their ability to induce the downregulation of important activating receptors on NK cells (Fig. 4A and B). Some recent studies suggested that the process of EMT plays a role in different mechanisms of tumor escape. Thus, for example, EMT has been shown to induce an immunosuppressive microenvironment in hepatocellular carcinoma (40), or to reduce the ability of breast cancer cells to form immunologic synapses with cytotoxic T lymphocytes (34). On the other hand, it has also been reported that EMT induction could promote NKG2D-L upregulation on colorectal cells and on immortalized keratinocytes, or induce increased NK cell–mediated metastasis-specific immunosurveillance in lung cancer (41, 42). Nevertheless, besides HLA-I molecules, no major NK-receptor ligands were significantly modified by EMT in our experiments on melanoma cells (Supplementary Fig. S6), suggesting that a certain variability among tumor cell types could exist in the response to EMT stimuli.

In summary, the NK:tumor cell cross-talk in the context of EMT may occur in melanoma and affect the progression, and perhaps the fate, of the tumor. This issue opens important questions on how the tumor microenvironment could influence the outcome of such "NK:melanoma:EMT" cross-talk. Indeed, tumor-associated immune and stromal cells, as well as tumor-associated hypoxia, are known to favor EMT (19) and/or modulate NK-cell function (6, 7). Additional questions regard the type and the functional status of the NK cells that participate in the cross-talk at the tumor site. Conventional $CD56^{dim}CD16^{bright}$ NK cells have been reported to infiltrate tumor tissues in several solid tumors (18, 43). Tissue $CD56^{dim}CD16^{bright}$ cells generally lack markers of tissue-resident lymphocytes (i.e., CD69, CD103, or CD49a) and are considered as cells recirculating from peripheral blood (44, 45), where, indeed, $CD56^{dim}CD16^{bright}$ cells are largely represented. Along this line, peripheral blood–derived NK cells have been recently shown to migrate in response to chemotactic stimuli released during NK:melanoma cell interaction (46). Intriguingly, tumor tissues are also frequently infiltrated by cells expressing the $CD56^{bright}CD16^{dim}$ phenotype (47). These cells constitute a small fraction of circulating NK cells but are well represented in certain tissues or secondary lymphoid organs (44, 47). $CD56^{bright}CD16^{dim}$ cells are characterized by low cytotoxicity and high IFNγ production in response to cytokines; hence, they may have a different, even more pronounced, effect on tumor phenotype switch and EMT. Thus, our data on PBNK cells offer reliable hints to characterize the NK:tumor cell cross-talk, at least for tumors showing the $CD56^{dim}CD16^{bright}$ NK-cell infiltrate. Further studies involving specific NK-cell subsets and dissecting the effect of specific components of the tumor microenvironment would integrate and extend our data and provide important clues to design personalized and hopefully effective NK-based therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: L. Huergo-Zapico, J.L. Fernández-Martínez, E.J. DeAndrés-Galiana

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Huergo-Zapico, C. Cantoni, C. Lavarello, A. Petretto, G. Pietra, M. Bugatti, E. Munari, M. Marconi, W. Vermi, L. Moretta

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Huergo-Zapico, M. Parodi, C. Cantoni, C. Lavarello, J.L. Fernández-Martínez, A. Petretto, E.J. DeAndrés-Galiana, M. Balsamo, A. López-Soto, G. Pietra, M. Bugatti, E. Munari, W. Vermi, L. Moretta, S. González, M. Vitale

Writing, review, and/or revision of the manuscript: L. Huergo-Zapico, M. Parodi, C. Cantoni, J.L. Fernández-Martínez, E.J. DeAndrés-Galiana, A. López-Soto, M.C. Mingari, L. Moretta, S. González, M. Vitale

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Huergo-Zapico

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NK-cell Editing Mediates Epithelial-to-Mesenchymal Transition via Phenotypic and Proteomic Changes in Melanoma Cell Lines

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