Platelet-Derived Transforming Growth Factor-β Down-Regulates NKG2D Thereby Inhibiting Natural Killer Cell Antitumor Reactivity

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Abstract

Natural killer (NK) cells play an important role in cancer immunosurveillance and may prevent tumor progression and metastasis due to their ability to mediate direct cellular cytotoxicity and by releasing immunoregulatory cytokines, which shape adaptive immune responses. Their reactivity is governed by various activating and inhibitory molecules expressed on target cells and reciprocal interactions with other hematopoietic cells such as dendritic cells. In mice, thrombocytopenia inhibits metastasis, and this is reversed by NK cell depletion, suggesting that platelets are an important additional player in NK cell-tumor interaction. Moreover, it has been shown that metastasizing tumor cells do not travel through the blood alone but are rapidly coated by platelets. However, the knowledge about the molecular mechanisms by which platelets influence NK cells is fragmentary at best. Here we show that platelet-derived soluble factors, secreted on coating of tumor cells or after stimulation with classic platelet agonists, impair NK cell antitumor reactivity resulting in diminished granule mobilization, cytotoxicity, and IFN-γ production. The impaired NK cell reactivity was not due to induction of apoptosis but mediated by down-regulation of the activating immunoreceptor natural killer group 2, member D (NKG2D) on NK cells by platelet-derived transforming growth factor β (TGF-β). Neutralization of TGF-β in platelet releasate not only prevented NKG2D down-regulation but also restored NK cell antitumor reactivity. Thus, our data elucidate the molecular basis of the previously described influence of platelets on NK cell antitumor reactivity and suggest that therapeutic intervention in tumor cell-platelet interaction and the resulting TGF-β release by platelets may serve to enhance antitumor immunity. [Cancer Res 2009;69(19):7775–83]

Introduction

Metastasized cancer caused by disseminating tumor cells is, with very few exceptions, an incurable disease. Therefore, a better understanding of the mechanisms that influence tumor propagation is key to improve therapeutic options for cancer patients.

Natural killer (NK) cells have initially been identified as lymphoid components of the innate immune system capable of lysing target cells without prior sensitization (1, 2). They play an important role in the immunosurveillance of tumors by recognizing and eliminating malignant cells, thereby preventing both local tumor progression and metastatic spread (3). NK cell reactivity is guided by the principles of "missing-self" and "induced-self," which imply that cells with low or absent expression of MHC class I (missing-self) and/or a stress-induced expression of ligands for activating NK receptors (induced-self) are preferentially recognized and eliminated (4–7). Thus, a balance of activating and inhibitory signals mediated by various receptors determines whether NK cell responses are initiated or not.

Beyond the direct interaction with their target cells, NK cell activity is further influenced by the reciprocal interplay with various other hematopoietic cells such as dendritic cells, monocytes/macrophages, and lymphocytes (8). The complex nature of this cross talk has been exemplified by numerous studies showing that interaction of NK cells and dendritic cells causes multiple and potentially opposite effects on the activity of both involved cell types (8). Yet, no study, especially in humans, has addressed the molecular mechanisms and consequences of the cross talk of NK cells with platelets, another central component of the blood being physiologically in direct proximity of NK cells. This is surprising because murine tumor models document a strong dependence of tumor progression and metastasis on quantitatively and qualitatively normal thrombocytopoiesis (9–11). Of note, inhibition of metastasis caused by antibody-induced thrombocytopenia is reversed by additional depletion of NK cells. Thus, thrombocytopenia may "indirectly" reduce tumor dissemination by allowing NK cells to better exert their antitumor effector functions (9, 12–14). There is convincing evidence that disseminating tumor cells do not travel through the blood alone but surround themselves by coating platelets, which may cause platelet activation (15). Beyond mechanistic hypotheses proposing a "tumor-protective effect" of platelets by preventing immune cells from accessing tumor cells, nothing is yet known about the molecular mechanisms underlying NK cell-platelet interaction.

In this study, we report that platelets secrete soluble factors including transforming growth factor β (TGF-β) on interaction with tumor cells, which down-regulates expression of the activating immunoreceptor natural killer group 2, member D (NKG2D) on NK cells and causes impaired antitumor immunity. Our data provide functional insights into platelet-tumor-NK cell interaction and broaden our understanding of NK cell tumor immunosurveillance.

Materials and Methods

Reagents. The monoclonal antibodies (mAb) AMO1 (anti-MICA), BAMO1 (anti-MICA/B), BMO2 (anti-MICB), and BAMO3 (anti-MICA/B) and recombinant sMICA*04 and sMICB*02 were produced as previously described (16). Anti-CD3-FITC, anti-CD45-phycoerythrin (PE), anti-NKG2D-PE, anti-CD107a-PE, CD56-PECy5, Annexin V-FITC, anti-CD61-conjugates (clone VI-PL2), anti-CD62P-PECy5, as well as the corresponding isotype controls were from BD PharMingen. The anti–pan-cytokeratin polyclonal

Note: H.-G. Kopp and T. Placke contributed equally to this work. T. Placke performed the majority of the experiments. H-G. Kopp contributed to some of the experiments. H.R. Salih and H-G. Kopp designed the project and wrote the article.

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antibody was from DakoCytomation; the Alexa488-conjugated antirabbit IgG was from Invitrogen. Antibodies against TGF-β, human IgG1, and interleukin-2 were from R&D Systems and PromoKine. The goat anti-mouse-PE, goat anti-mouse IgG2a-horseradish peroxidase, and the Cy3-conjugated antimouse IgG were from Jackson ImmunoResearch. All other reagents were obtained from Carl Roth GmbH.

Transfectants and cell lines. C1R-MICA and C1R-neo mock transfectants were previously described (16). The tumor cell lines NCCIT (germ cell) and PC3 and HCT116 (colon) were obtained internally at Eberhard Karls University Tuebingen.

Preparation of NK cells and platelets. Polyclonal NK cells were generated as previously described (17). Alternatively, NK cells were isolated from peripheral blood using the NK Cell Isolation Kit II and MACS columns (Miltenyi Biotech). Experiments were done when purity of NK cells was above 90% as determined by flow cytometry. Platelets were obtained and processed for aggregation as previously described.

Platelets were obtained from donors not taking any medication for at least 10 d before blood collection. Citrated blood was centrifuged for 20 min at $120 \times g$ and the upper layer was harvested as platelet-rich plasma. Platelets were washed twice with citrate wash buffer (128 mmol/L NaCl, 11 mmol/L glucose, 7.5 mmol/L Na_2HPO_4, 4.8 mmol/L sodium citrate, 4.3 mmol/L NaH_2PO_4, 2.4 citric acid, 0.35% bovine serum albumin, and 50 ng/mL prostaglandin E_1) and resuspended in RPMI containing 10% FCS to a final concentration of 3×10^5/μL.

Platelets were activated by adding 0.075 IU/mL thrombin (Dade Behring), 10 μg/mL collagen (Mascia Brunelli), or 3×10^5 tumor cells/mL with 5% platelet-poor plasma to washed platelets in an aggregometer (Chronolog). All platelet suspensions were stirred for 10 min at 1,000 rpm and subsequently centrifuged for 15 min at 13,000 rpm. The supernatant was used as platelet releasate (PR).

Flow cytometry. Cells were incubated with the indicated specific mAb or isotype control (all at 10 μg/mL) followed by goat anti-mouse-PE conjugate (1:100) as secondary reagent and then analyzed on a FACSCalibur (Becton Dickinson). Conjugated mAb and the respective isotype controls were used at 2 μL/100,000 cells. Where indicated, specific fluorescence indices were calculated by dividing median fluorescence obtained with specific mAb by median fluorescence obtained with isotype control.

Immunofluorescence. HCT116 tumor cells (1×10^3/mL) were added to platelet-rich plasma at 300,000/μL under shear stress. Macroscopically and on transillumination, no clot formation was observed. Cytospin preparations of the cell suspension were either stained according to May-Gruenwald-Giemsa or processed for immunofluorescence as follows: After nonspecific protein block, the indicated primary antibodies were incubated overnight at 4°C. After successive PBS washes, sections were incubated in appropriate secondary antibodies. Anti-CD62P-PECy5 was directly labeled (BD PharMingen). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

ELISA. Detection of soluble MICA and MICB was done using our previously described sandwich ELISA with a detection limit of 80 pg/mL (16). IFN-γ and TGF-β levels were analyzed using OptEIA sets from PharMingen and DuoSet ELISA development system from R&D Systems, respectively, according to the manufacturer's instructions. All concentrations are expressed as mean ± SEM of triplicates.

Cytotoxicity assay. Cytotoxicity of NK cells was analyzed by a standard chromium release assay as previously described (17). Percentage of lysis was calculated as follows: $100 \times$ [(experimental release − spontaneous release) / (maximum release − spontaneous release)].

Determination of apoptosis in NK cells. NK cells (5×10^5) were cultured in the presence or absence of differentially induced PR for the indicated times. Subsequently, apoptosis was determined by staining NK cells in 50-μL buffer containing 1 μL of both Annexin V-FITC and propidium iodide (PI) followed by flow cytometry.

Results

Tumor cells are rapidly coated by activated platelets. In initial experiments, we coincubated several different epithelial tumor cells (PC3, NCCIT, and HCT116) with platelet-rich plasma under shear stress, as described previously (18). Each single tumor cell was efficiently coated by a layer of platelets even at minimal tumor cell concentrations in the absence of detectable platelet aggregation as documented by light microscopy and immunofluorescence analysis (Fig. 1*A* and *B*). These data show that even low amounts of tumor cells rapidly and efficiently get coated with platelets, and this occurs also in the absence of plasma. Tumor cell–coating platelets were found to express the activation marker CD62P (P-selectin; ref. 19). This shows that platelet adhesion to tumor cells initiates platelet activation, thereby causing degranulation and subsequent release of granule contents (Fig. 1*C*). Coating was also observed using flow cytometry, which resulted in pseudoexpression of platelet markers on tumor cells after coincubation. Of note, tumor cells themselves did not express CD61 or CD62P (Fig. 1*D*). We therefore hypothesized that platelets may modulate NK cell reactivity through soluble factors, which we subsumed under the term "platelet releasate" (PR).

Platelet-derived soluble factors impair NK cell antitumor reactivity. To establish the influence of PR on the antitumor reactivity of cytotoxic lymphocytes, peripheral blood mononuclear cells (PBMC) were incubated overnight in the presence or absence of PR obtained by activation of platelets with several different platelet agonists including collagen, thrombin, and tumor cells, respectively, or the appropriate controls. Subsequently, we performed degranulation assays in cultures with HCT116 tumor cells and used counterstaining for CD3 and CD56 to discern the different cytotoxic lymphocyte subpopulations. The presence of tumor cells substantially induced CD107a expression as surrogate marker for granule mobilization on $CD3^-CD56^+$ NK cells, whereas no relevant up-regulation was observed on $CD3^+CD8^+$ T cells and $CD3^+CD56^+$ NKT cells, indicating that solely NK cells mediated antitumor reactivity in our setting. The presence of either PR markedly reduced the detectable levels of CD107a on NK cells in cultures with tumor cells, whereas the controls did not substantially alter NK cell degranulation. Of note, similar effects were observed using PR obtained after stimulation with "classic" platelet agonists, such as collagen or thrombin, compared with tumor cell–induced PR. Likewise, the effects were reproducible when various different tumor cell lines were used as targets (Fig. 2*A* and data not shown).

To ascertain that the observed effects were caused by a direct effect of PR on NK cells and not due to indirect effects via modulation of other immune effector cells, we performed cytotoxicity assays using polyclonal NK cells incubated overnight in the presence or absence of differentially induced PR or the respective controls. Preincubation with PR caused a statistically significant (all $P < 0.05$, Student's t test) reduction of NK cell cytotoxicity against HCT116 and NCCIT tumor cells (up to 60% reduction, effector-to-target ratio of 40:1), whereas the controls did not substantially alter NK cell cytotoxicity (Fig. 2*B*). Again, similar results were observed with the various differentially induced PR.

Because release of IFN-γ is a second major mechanism by which NK cells participate in antitumor immunity, we analyzed whether PR also altered NK cell cytokine production. NK cells were again cultured overnight in the presence or absence of differentially induced PR or the respective controls. Subsequently, the indicated tumor cells were added and culture was done for additional 24 h before analysis of culture supernatants by ELISA. In the absence of tumor cells, NK cells produced low levels of IFN-γ, which were not markedly altered by PR. The presence of HCT116

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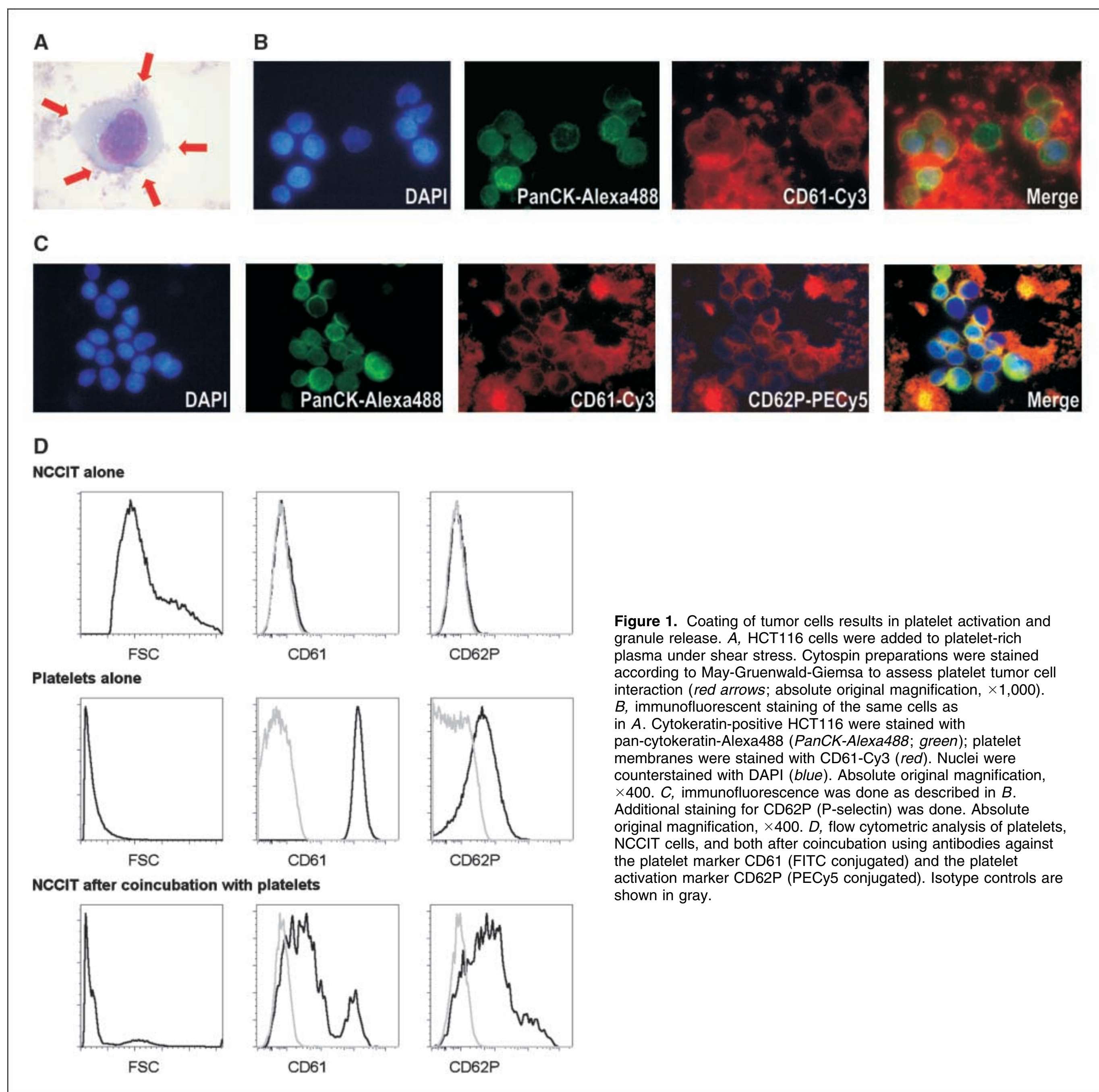

Figure 1. Coating of tumor cells results in platelet activation and granule release. *A,* HCT116 cells were added to platelet-rich plasma under shear stress. Cytospin preparations were stained according to May-Gruenwald-Giemsa to assess platelet tumor cell interaction (*red arrows*; absolute original magnification, ×1,000). *B,* immunofluorescent staining of the same cells as in *A*. Cytokeratin-positive HCT116 were stained with pan-cytokeratin-Alexa488 (*PanCK-Alexa488*; *green*); platelet membranes were stained with CD61-Cy3 (*red*). Nuclei were counterstained with DAPI (*blue*). Absolute original magnification, ×400. *C,* immunofluorescence was done as described in *B*. Additional staining for CD62P (P-selectin) was done. Absolute original magnification, ×400. *D,* flow cytometric analysis of platelets, NCCIT cells, and both after coincubation using antibodies against the platelet marker CD61 (FITC conjugated) and the platelet activation marker CD62P (PECy5 conjugated). Isotype controls are shown in gray.

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and NCCIT tumor cells potently stimulated NK cell IFN-γ production, and this was significantly (all $P < 0.05$, Mann-Whitney U test) reduced by the various PR (up to 67%), whereas the respective controls had no relevant effect (Fig. 2*C*). Together, these data show that, regardless of the agonist used for activation, platelets release soluble factors that impair the antitumor reactivity of NK cells.

Platelet-derived soluble factors do not induce NK cell death. Because platelet-derived factors have been reported to exert cytotoxic effects on both blood and tumor cells (20–22), we investigated whether induction of cell death in NK cells by PR was responsible for reduced NK cell reactivity. NK cells were incubated in the presence or absence of PR or the corresponding controls for 24 h, and subsequently, we determined the percentages of apoptotic NK cells. Fluorescence-activated cell sorting (FACS) analyses using Annexin V/PI and the Nicoletti method revealed that PR did not induce NK cell apoptosis, whereas high amounts of apoptotic NK cells were detected in the presence of staurosporin serving as positive control (Fig. 3 and data not shown). Thus, the inhibitory effects of PR are not a consequence of cell death induction in NK cells.

PR impairs NK cell NKG2D expression. In search of the molecular mechanism by which PR impaired NK cell functions, we studied whether preincubation with PR altered the expression of NK cell receptors, among them NKG2D. NK cells were incubated overnight in the presence or absence of PR generated on

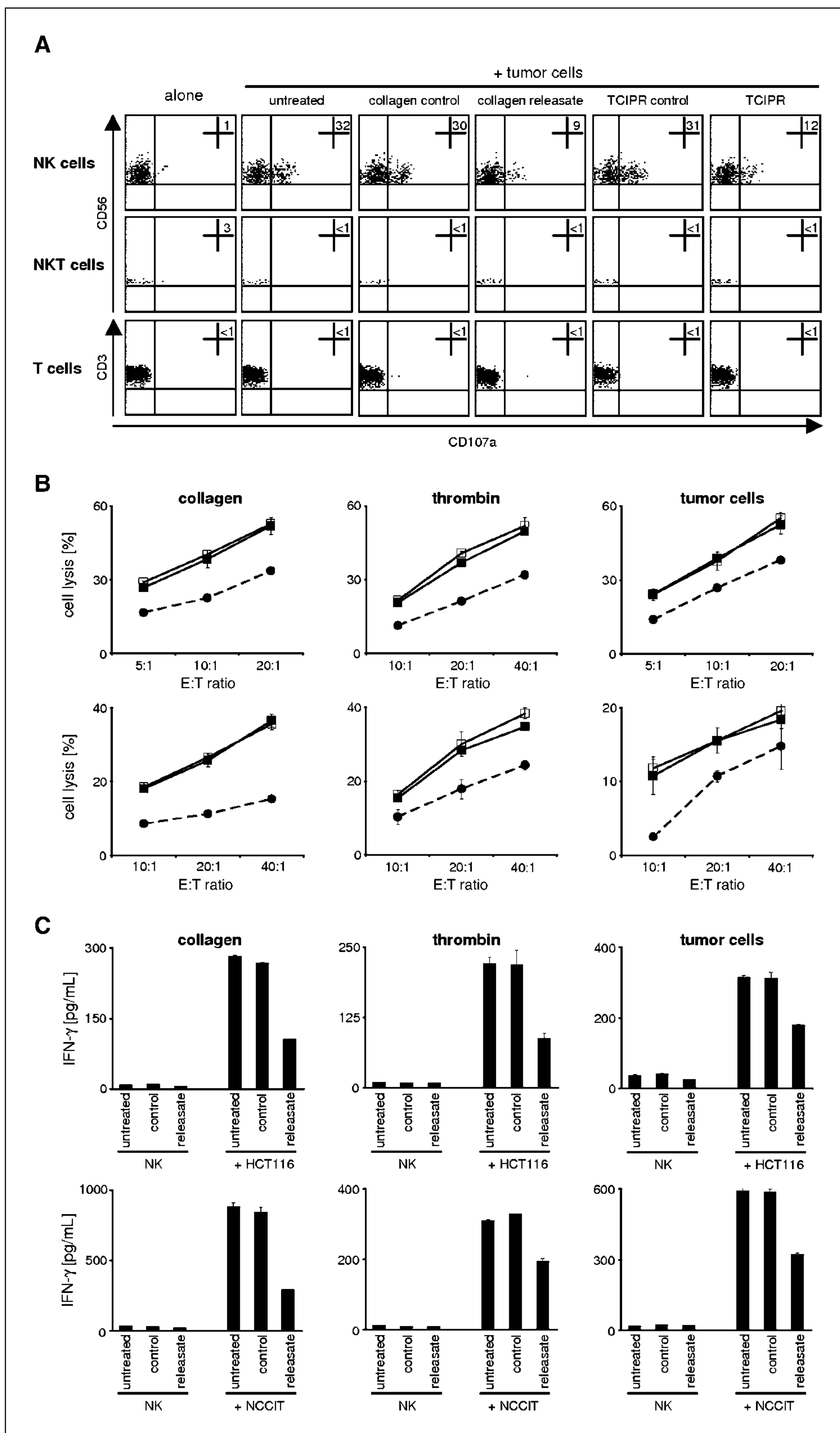

Figure 2. PR inhibits NK cell antitumor reactivity. PBMC (*A*) and polyclonal NK cells (*B* and *C*) were cultured overnight in the presence or absence of the indicated PR or the respective agonist controls. *A,* granule mobilization after 3 h was analyzed by FACS for CD107a expression and counterstaining for CD3 and CD56. Numbers in top right quadrants indicate the percentage of CD107a-positive cells. *TCIPR,* tumor cell–induced PR. *B,* cytotoxicity of NK cells was evaluated by 4 h chromium release assays. Results with HCT116 (*top*) and NCCIT tumor cells (*bottom*) as targets. *Filled squares,* untreated; *open squares,* control; *circles,* PR-treated NK cells. *E:T ratio,* effector-to-target ratio. *C,* IFN-γ levels in cultures of tumor cells and NK cells were determined after 24 h by ELISA. One representative experiment each of a total of at least three with similar results is shown.

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stimulation of platelets with different agonists or in the presence of controls, and subsequently, expression of NK cell receptors was determined by FACS. The differentially induced PR, but not the respective controls, were found to cause a substantial down-regulation of NKG2D expression on NK cells, whereas no relevant effect on expression of CD45, CD56, CD86, CD178, and CD253 was observed, which confirmed the specificity of the observed effect. PR also influenced the expression of the NK cell receptors NKp30,

NKp44, NKp46, and NKp80, but to a lesser extent and less consistently (Fig. 4*A* and data not shown). Of note, a similar reduction of NKG2D expression was observed using PR obtained after stimulation of platelets with either classic agonists or tumor cell–induced PR (Fig. 4*A, right*).

NKG2D expression on cytotoxic lymphocytes is down-regulated after interaction with its ligands (NKG2DL), as well as by the cytokine TGF-β(23). We first determined whether PR contained soluble MIC molecules using supernatants of C1R-MICA, C1R-MICB, and C1R-neo (mock) transfectants as positive controls. As expected, high levels of sMIC molecules were detected in supernatants of C1R-MIC transfectants but not in supernatants of the C1R-mock controls. No relevant levels of sMIC molecules were detectable in the different investigated PR. This excluded that NKG2D expression on NK cells was diminished on interaction with soluble NKG2DL (Fig. 4*B*). However, we found high levels of total and active TGF-β in the various PR but not in the respective controls (Fig. 4*C*), which is in line with the notion that platelets contain and may secrete large quantities of active TGF-β (24). To ascertain that, in fact, TGF-β was responsible for NKG2D down-regulation, we cultured NK cells overnight in PR with and without blocking mAb against TGF-β. Whereas isotype control mAb did not alter the PR-induced reduction of NKG2D expression on NK cells, the presence of anti–TGF-β largely prevented NKG2D down-regulation. This confirmed that the TGF-β contained in PR was, at least in part, responsible for the observed reduction of NKG2D expression levels (Fig. 4*D*). Thus, platelet-derived TGF-β diminishes the expression of a potent activating immunoreceptor on NK cells.

TGF-β–induced NKG2D down-regulation mediates the inhibitory effect of PR on NK cell reactivity. Finally, we wanted to confirm functionally that the inhibitory effect of PR on NK cell antitumor reactivity was in fact caused by modulation of the NKG2D-NKG2DL system. NK cells were thus cultured overnight in the presence or absence of collagen-induced PR or control medium. Then, we studied cytotoxicity and cytokine production of NK cells in cultures with highly NKG2DL-expressing C1R-MICA transfectants and mock transfectants serving as controls. Cytotoxicity assays revealed that NK cell lysis was not markedly affected by PR or control when mock transfectants (C1R-neo) that constitutively express only low levels of NKG2DL were used as target cells. In contrast, a substantial and statistically significant ($P < 0.05$, Student's t test) reduction of lysis was observed following preincubation of NK cells in PR when our C1R-MICA transfectants, which express high levels of NKG2DL, were used as targets (Fig. 5*A, left*). Similar results were obtained for NK cell cytokine production. The low levels of IFN-γ produced by NK cells alone were not markedly altered by PR or control. The presence of C1R cells substantially induced NK cell cytokine production, and markedly higher levels were detectable in cultures with the C1R-MICA compared with the C1R-neo transfectants. Whereas pretreatment of NK cells with control had no effect, incubation with PR caused a marked and statistically significant ($P < 0.05$, Student's t test) reduction of IFN-γ levels in cultures with C1R-MICA transfectants, and nearly no effect was observed in the cultures with the C1R-neo cells (Fig. 5*A, right*). Thus, reduction of NKG2D expression by PR functionally impairs NKG2D-mediated antitumor reactivity of NK cells.

Next, we preincubated NK cells in the presence or absence of differentially induced PR with or without blocking mAb against TGF-β or isotype control and subsequently studied NK cell cytotoxicity and cytokine production in response to HCT116 tumor cells. Again, PR impaired NK cell cytotoxicity and cytokine production, whereas the control agonists had no effect. Whereas isotype control did not alter the PR-mediated reduction of NK cell reactivity, blocking TGF-β mAb partially restored NK cell cytotoxicity and IFN-γ production (Fig. 5*B* and *C*). Similar results were obtained when other tumor cell lines were used as target cells (data not shown). To further confirm that TGF-β impairs NK cell reactivity in the concentrations contained in the PR, we incubated NK cells overnight in the presence or absence of various concentrations of active recombinant TGF-β1 or vehicle control corresponding in volume to the highest amount contained in the TGF-β preparations. Subsequently, we determined NK cell cytotoxicity and IFN-γ production in cultures with HCT116 tumor cells (Fig. 5*D*). A clear concentration-dependent inhibition of NK cell reactivity after preincubation with TGF-β was observed. Of note, the inhibition of NK cell cytotoxicity and cytokine production observed with PR was comparable to the degree observed with the corresponding concentration of recombinant TGF-β1. Together, these results show that the inhibitory effect of PR on NK cell reactivity is due to TGF-β–mediated impairment of NKG2D expression resulting in reduction of activating stimuli in the delicate balance of signals that govern NK cell antitumor reactivity.

Discussion

Metastatic spread of cancer cells is the basis for the transition of a locally treatable malignancy with relatively good prognosis to a systemic, life-threatening disease, which, with very few exceptions, cannot be cured by presently available treatment options. Tumor

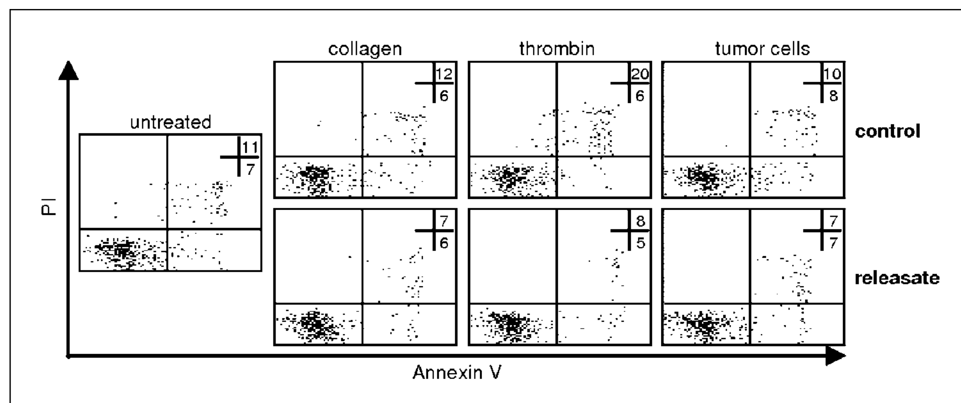

Figure 3. PR does not alter viability of NK cells. NK cells were cultured overnight in the presence or absence of various PR or the respective agonist controls. Subsequently, NK cell viability was analyzed by FACS using PI and Annexin V-FITC. Numbers in dot plots indicate the percentage of Annexin V/PI–positive $CD56^{+}CD3^{-}$ cells. One representative experiment each of a total of three experiments with similar results is shown.

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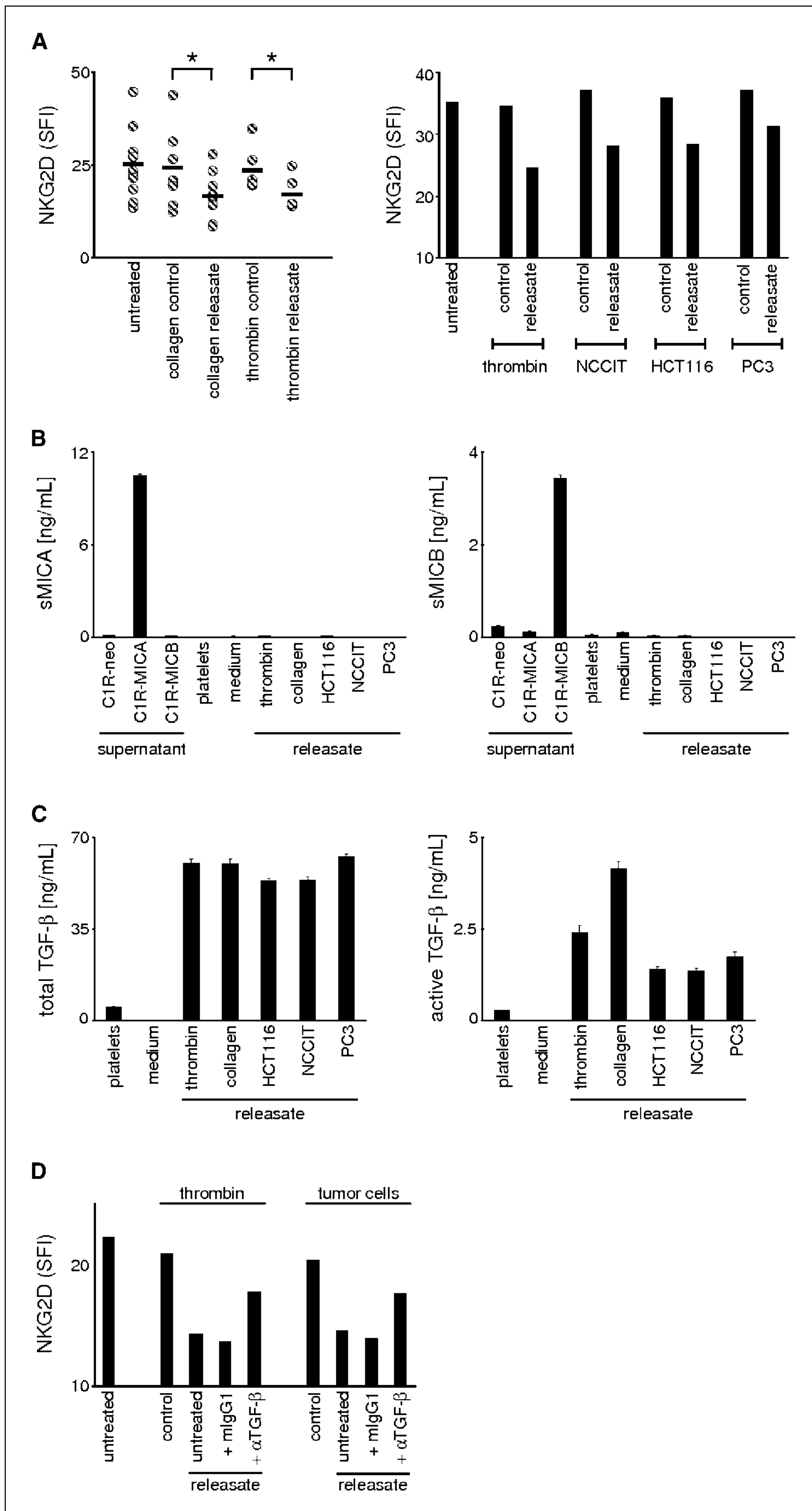

Figure 4. Platelet-derived active TGF-β down-regulates NKG2D expression on NK cells. *A,* NK cells were cultured overnight in the presence or absence of the indicated PR or the respective controls. Subsequently, surface expression of NKG2D on NK cells was determined by FACS and is shown as specific fluorescence index (*SFI*) levels. *Left,* results of 10 investigated platelet donors. *Horizontal lines,* means of the indicated culture conditions. *Right,* exemplary results with NK cells from a single donor. *B,* PR generated with the indicated stimuli and the indicated controls were analyzed for the presence of sMICA and sMICB by ELISA. Supernatants from C1R-transfectants after 48 h of culture served as controls. *C,* levels of total (*left*) and active (*right*) TGF-β within PR generated with the indicated stimuli as well as in the indicated controls were determined by ELISA. *D,* NK cells were cultured overnight in the presence or absence of the indicated PR or the respective controls. Where indicated, 5 μg/mL anti–TGF-β mAb or IgG1 as isotype control was added to the PR. Subsequently, surface expression of NKG2D on NK cells was determined by FACS and is shown as SFI levels. Results of one experiment each of at least four with similar results are shown.

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progression is largely dependent on the interaction of the malignant cells with the immune system and seems to require induction of tolerance of innate and adaptive immune effector cells (25, 26). The mechanisms that enable metastasizing malignant cells traveling through the blood to evade antitumor immunity are still poorly understood. Of note, as early as in the year 1878, Theodor Billroth described viable neoplastic cells in blood clots and suggested that the hemostatic system plays a role in tumor metastasis (27). Recently, it has been shown that thrombocytopenia inhibits tumor metastasis in experimental animals. Indeed, both platelet depletion and inborn thrombocytopenia in $TPO^{-/-}$ or $c\text{-}mpl^{-/-}$ mice reduce formation of metastases by as much as 90% in mouse models (9, 10). Interestingly, concomitant depletion of NK cells resulted in restoration of the metastatic potential of injected tumor cells, which led the investigators to postulate that platelets may somehow "shield" circulating tumor cells from NK cell lysis (9, 12–14). However, the exact molecular mechanisms by which platelets may inhibit NK cell tumor immunosurveillance remain elusive.

In this study, we show that tumor cell–coating platelets display an activated phenotype, which is accompanied by degranulation and release of platelet-contained factors. This PR profoundly

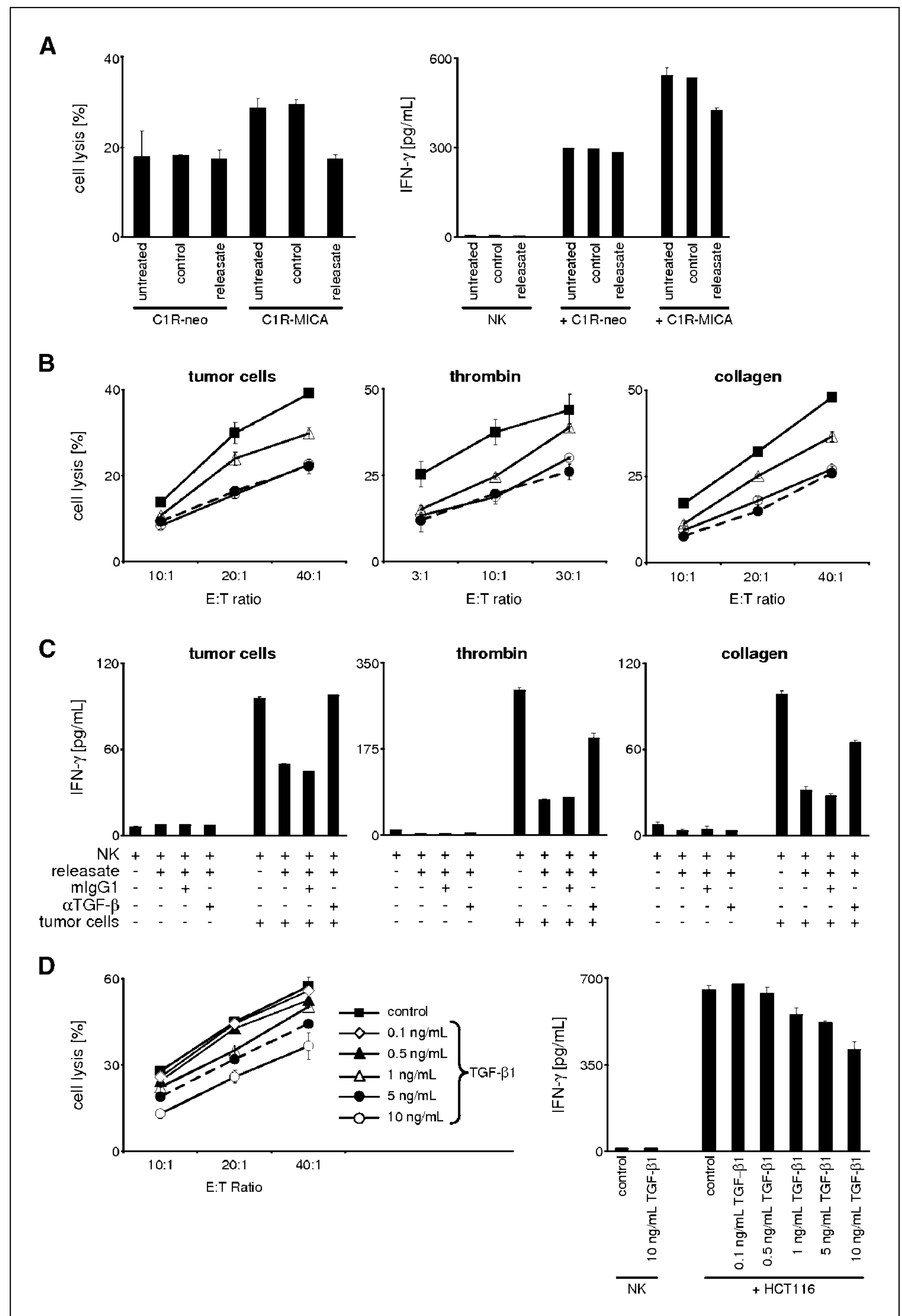

Figure 5. Neutralization of TGF-β in PR restores NK cell antitumor reactivity. NK cells were cultured overnight in the presence or absence of PR, control, or the indicated concentrations of recombinant TGF-β1. *A,* cytotoxicity (*left*, E:T = 10:1) and IFN-γ secretion (*right*) were determined using C1R-MICA and C1R-neo (mock) transfectants as targets. *B* and *C,* anti–TGF-β mAb or IgG1 as isotype control (5 μg/mL) was added to the PR before addition to NK cells where indicated. Cytotoxicity and IFN-γ production were determined in cultures with HCT116 tumor cells. *Squares,* control; *filled circles,* PR; *open circle,* PR + mIgG1; *triangle,* PR + anti–TGF-β. *D,* cytotoxicity (*left*) and IFN-γ secretion (*right*) in cultures with HCT116 tumor cells in the presence of increasing concentrations of TGF-β1 or vehicle control.

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inhibited NK cell degranulation, cytotoxicity, and IFN-γ production in response to different tumor targets. Accordingly, we aimed to identify the factor(s) and mechanisms by which PR impaired NK cell antitumor immunity. NK cell reactivity results from an integrative response to stimulatory and inhibitory signals acting through various NK cell receptors (28). Whereas the function of inhibitory NK cell receptors and their ligands has been analyzed in great detail, the tumor-associated ligands binding to activating NK cell receptors are less well characterized. Exceptions are the MHC class I–related molecules, which bind to the activating, homodimeric C-type lectin-like NKG2D receptor (4). In humans, these NKG2DL consist of two members of the MIC family (MICA and MICB) and six members of the ULBP family of proteins (ULBP1–ULBP4, RAET1G, and RAET1L). NKG2DL are inducibly expressed on cells subjected to genotoxic stress, and expression is associated with malignant transformation, but NKG2DL are generally not expressed in healthy tissues (23, 29). NKG2DL expression renders cells susceptible to NK cell reactivity despite the expression of MHC class I and potently stimulates NK cell cytotoxicity and cytokine secretion (4). Furthermore, NKG2D has been shown to provide protection from spontaneous tumors *in vivo*, and studies in NKG2D-deficient mice confirmed the important role of this NK cell receptor in tumor immunosurveillance (30–32).

We found that tumor cell-platelet interaction causes release of soluble factors, which potently down-regulate NKG2D expression. This is of interest because tumor cells have been shown to escape from NKG2D-mediated antitumor immunity by reduction of NKG2DL expression on their surface and/or by "silencing" NKG2D on cytotoxic lymphocytes (reviewed in ref. 33). The latter is achieved by the release of soluble NKG2DL by tumor cells, which causes systemically diminished NKG2D expression (34, 35). Moreover, the cytokine TGF-β has been found to down-regulate NKG2D expression on NK cells causing impaired antitumor reactivity (36–38). Of note, platelets produce at least 40 times more active TGF-β than any other cell type (24, 39). In our study, we show that platelet-derived TGF-β is, in great part, responsible for platelet-dependent reduction of NKG2D expression and subsequent inhibition of NK cell cytotoxicity and IFN-γ production: Neutralization of TGF-β by a blocking antibody partly restored both the reduced NKG2D expression and the impaired NK cell antitumor reactivity caused by PR.

Of note, different and in part opposite effects of TGF-β have been reported with regard to its influence on progression of malignancies: Depending on disease stage, TGF-β was found to act as tumor suppressor or as pro-oncogenic factor (40). However, animal studies using various approaches document that inhibition of TGF-β signaling prevents tumor progression and metastasis (40, 41). Inhibition of tumor progression by anti–TGF-β strategies in immunocompromised mice was attributed to disinhibition of thrombospondin-1 expression by the tumor stroma, thereby preventing neoangiogenesis (42). Our data indicate that both further cellular sources and further effects of TGF-β may come into play in humans.

Taken together, although an inhibiting effect of platelets on NK cell tumor immunosurveillance has been postulated for many years, our study for the first time provides insights into the molecular mechanism of tumor-platelet-NK interaction. Numerous attempts have been made to engraft NK cells in the treatment of cancer by approaches or interventions that prevent suppression or stimulate the reactivity of autologous or allogeneic NK cells (3, 5). Based on our results, we postulate that tumor cell-platelet-NK interaction plays an important role in the immune escape of circulating tumor cells and thus in the pathophysiology of metastatic tumor spread. We predict that anti-platelet and/or anti–TGF-β strategies may serve well to reinforce NK cell reactivity and to make NK-enabling strategies work against cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol 1975;5:112–7.

2. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer 1975;16:216–29.

3. Terme M, Ullrich E, Delahaye NF, Chaput N, Zitvogel L. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. Nat Immunol 2008;9:486–94.

4. Lanier LL. NK cell recognition. Annu Rev Immunol 2005;23:225–74.

5. Ljunggren HG and Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol 2007;7:329–39.

6. Farag SS and Caligiuri MA. Human natural killer cell development and biology. Blood Rev 2006;20:123–37.

7. Moretta L and Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J 2004;23:255–9.

8. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9:503–10.

9. Nieswandt B, Hafner M, Echtenacher B, Männel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Res 1999;59:1295–300.

10. Jin DK, Shido K, Kopp HG et al. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4^{+} hemangiocytes. Nat Med 2006;12:557–67.

11. Gasic GJ, Gasic TB, Stewart CC. Antimetastatic effects associated with platelet reduction. Proc Natl Acad Sci U S A 1968;61:46–52.

12. Palumbo JS, Talmage KE, Massari JV et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. Blood 2005;105:178–85.

13. Palumbo JS, Talmage KE, Massari JV et al. Tumor cell-associated tissue factor and circulating hemostatic factors cooperate to increase metastatic potential through natural killer cell-dependent and-independent mechanisms. Blood 2007;110:133–41.

14. Palumbo JS, Barney KA, Blevins EA et al. Factor XIII transglutaminase supports hematogenous tumor cell metastasis through a mechanism dependent on natural killer cell function. J Thromb Haemost 2008;6:812–9.

15. Borsig L, Wong R, Feramisco J et al. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci U S A 2001;98:3352–7.

16. Salih HR, Antropius H, Gieseke F et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood 2003;102:1389–96.

17. Baltz KM, Krusch M, Bringmann A et al. Cancer immunoediting by GITR (glucocorticoid-induced TNF-related protein) ligand in humans: NK cell/tumor cell interactions. FASEB J 2007;21:2442–54.

18. Jurasz P, Alonso-Escolano D, Radomski MW. Platelet-cancer interactions: mechanisms and pharmacology of tumour cell-induced platelet aggregation. Br J Pharmacol 2004;143:819–26.

19. Kappelmayer J, Nagy B, Jr., Miszti-Blasius K, Hevessy Z, Setiadi H. The emerging value of P-selectin as a disease marker. Clin Chem Lab Med 2004;42:475–86.

20. Okada M, Kodama T, Tominaga A et al. Cytotoxicity of activated platelets to autologous red blood cells. Br J Haematol 1992;82:142–50.

21. Sagawa T, Kodama T, Tominaga A, Okada M. Human platelets effectively kill K-562 cells, a chronic myelogenic leukemia cell line, *in vitro*. Jpn J Cancer Res 1990; 81:449–53.
22. Sagawa T, Tominaga A, Kodama T, Okada M. Cytotoxicity of unstimulated and thrombin-activated platelets to human tumour cells. Immunology 1993;78: 650–6.
23. Salih HR, Holdenrieder S, Steinle A. Soluble NKG2D ligands: prevalence, release, and functional impact. Front Biosci 2008;13:3448–56.
24. Ahamed J, Burg N, Yoshinaga K et al. *In vitro* and *in vivo* evidence for shear-induced activation of latent transforming growth factor-β1. Blood 2008;112:3650–60.
25. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21:137–48.
26. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol 2006;6:836–48.
27. Billroth T. Lectures on surgical pathology and therapeutics: a handbook for students and practitioners. London: New Sydenham Society; 1878.
28. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008;9:495–502.
29. Eagle RA and Trowsdale J. Promiscuity and the single receptor: NKG2D. Nat Rev Immunol 2007;7: 737–44.
30. Oppenheim DE, Roberts SJ, Clarke SL et al. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity *in vivo* and reduces tumor immunosurveillance. Nat Immunol 2005;6:928–37.
31. Smyth MJ, Swann J, Cretney E et al. NKG2D function protects the host from tumor initiation. J Exp Med 2005; 202:583–8.
32. Guerra N, Tan YX, Joncker NT et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 2008;28:571–80.
33. Waldhauer I and Steinle A. NK cells and cancer immunosurveillance. Oncogene 2008;27:5932–43.
34. Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J Immunol 2002;169:4098–102.
35. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 2002;419:734–8.
36. Castriconi R, Cantoni C, Della CM et al. Transforming growth factor β1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. Proc Natl Acad Sci U S A 2003; 100:4120–5.
37. Eisele G, Wischhusen J, Mittelbronn M et al. TGF-β and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. Brain 2006;129:2416–25.
38. Friese MA, Wischhusen J, Wick W et al. RNA interference targeting transforming growth factor-β enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity *in vivo*. Cancer Res 2004; 64:7596–603.
39. Blakytny R, Ludlow A, Martin GE et al. Latent TGF-β1 activation by platelets. J Cell Physiol 2004;199:67–76.
40. Yang YA, Dukhanina O, Tang B et al. Lifetime exposure to a soluble TGF-β antagonist protects mice against metastasis without adverse side effects. J Clin Invest 2002;109:1607–15.
41. Muraoka RS, Dumont N, Ritter CA et al. Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest 2002;109:1551–9.
42. Komuro A, Yashiro M, Iwata C et al. Diffuse-type gastric carcinoma: progression, angiogenesis, and transforming growth factor β signaling. J Natl Cancer Inst 2009;101:592–604.

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