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Thrombocytopenia inhibits tumor growth and especially metastasis in mice, whereas additional depletion of NK cells reverts this antimetastatic phenotype. It has therefore been speculated that platelets may protect hematogenously disseminating tumor cells from NK-dependent antitumor immunity. Tumor cells do not travel through the blood alone, but are rapidly coated by platelets, and this phenomenon has been proposed to shield disseminating tumor cells from NK-mediated lysis. However, the underlying mechanisms remain largely unclear. In this study, we show that megakaryocytes acquire expression of the TNF family member glucocorticoid-induced TNF-related ligand (GITRL) during differentiation, resulting in GITRL expression by platelets. Upon platelet activation, GITRL is upregulated on the platelet surface in parallel with the α-granular activation marker P-selectin. GITRL is also rapidly mobilized to the platelet surface following interaction with tumor cells, which results in platelet coating. Whereas GITRL, in the fashion of several other TNF family members, is capable of transducing reverse signals, no influence on platelet activation and function was observed upon GITRL triggering. However, platelet coating of tumor cells inhibited NK cell cytotoxicity and IFN-γ production that could partially be restored by blocking GITR on NK cells, thus indicating that platelet-derived GITRL mediates NK-inhibitory forward signaling via GITR. These data identify conferment of GITRL pseudoexpression to tumor cells by platelets as a mechanism by which platelets may alter tumor cell immunogenicity. Our data thus provide further evidence for the involvement of platelets in facilitating evasion of tumor cells from NK cell immune surveillance. The Journal of Immunology, 2012, 189: 154–160.
Materials and Methods

Reagents
Anti–CD3-FITC, CD56-PeCy5, anti-CD61 conjugates (clone V1-PL2), anti-CD41a conjugates (clone H1P8), anti–CD62P-PeCy5, as well as the corresponding isotype controls were from BD Pharmingen (San Diego, CA). The anti–pan-cytokertatin polyclonal Ab was from DakoCytomation; the Alexa 488-conjugated anti-rabbit IgG was from Invitrogen (Karlsruhe, Germany). Human IgG1, anti–GITR (clone 130416), GITR-Fc, recombinant human GITRL, and anti-GITR (clone 109101 and goat polyclonal) were from R&D Systems (Minneapolis, MN). Cytokines were purchased from Immunotools (Friesoythe, Germany). The goat anti–mouse PE and the Cy3-conjugated anti-mouse IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). BATDA and europium solution were obtained from PerkinElmer (Waltham, MA). All other reagents were obtained from Carl Roth (Karlsruhe, Germany). Transwell plates (96 wells) for cytotoxicity assays with a pore size of 0.4 μm were from Corning (Lowell, MA); transwell inserts (for 24-well plates) with a pore size of 0.4 μm for analysis of cytokine release by NK cells were from BD Biosciences (San Diego, CA).

Cell lines
The tumor cell lines SK-Mel, SK-BR-3, and P3C and the NK cell line NK92 were obtained from DSMZ (Braunschweig, Germany) and the American Type Culture Collection (Manassas, VA). Authenticity was determined by validating the respective immunophenotype described by the provider using FACS every 6 mo and specifically prior to use in experiments.

Preparation of NK cells and platelets
Polyclonal NK cells were generated by incubation of non–plastic-adherent PBMCs with irradiated RPMI 8866 feeder cells over 10 d as previously described (20). Experiments were performed when purity of NK cells was >80% as determined by flow cytometry.

Platelets were obtained from donors not taking any medication for at least 10 d prior to blood collection and prepared as previously described (19).

Maturation of megakaryocytes
CD34+ hematopoietic progenitor cells were obtained by magnetic bead separation from G-CSF–mobilized peripheral blood from patients with nonhematological malignancies or healthy donors after informed consent according to the guidelines of the Local Ethics Committee. Mononuclear cells were separated by Ficoll density gradient centrifugation, and CD34+ hematopoietic progenitor cells were obtained utilizing immunomagnetic microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. CD34+ cells were differentiated as described previously (21). In short, cells were incubated in serum-free medium (Invitrogen, Darmstadt, Germany) supplemented with recombinant human thrombopoietin (50 ng/ml; PeproTech, Hamburg, Germany). After 12 d, morphologically typical multinucleated megakaryocytes were harvested at a purity of >90% as assessed by flow cytometric analysis of CD41a+ cells.

RT-PCR
RT-PCR was performed as described previously (22). GITRL primers were 5’-GCTGTGTTGTTCGTAATAAC-3’ and 5’-ACCCCAGTATGTATTATTTT-3’. The PCR product (expected size 546 bp) for human GITRL was separated by electrophoresis on agarose gels and visualized by staining with ethidium bromide.

Western blot
Protein from platelets was isolated with RIPA buffer, and protein concentration was determined by a Bradford assay. Protein (50 μg) from each sample was resolved on a precast 12% NuPAGE gel and transferred on a polyvinylidene difluoride membrane (Invitrogen, Darmstadt, Germany). Membrane was blocked for 1 h at room temperature with Roti-Block, followed by overnight incubation with biotinylated polyclonal GITRL Ab (1:1000; R&D Systems). After 30 min incubation with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA) the proteins were detected by using ECL reagents (GE Healthcare, Freiburg, Germany).

Measurement of platelet aggregation and activation
Aggregation assays were performed in an APACT 4004 aggregometer (Haemochrom Diagnostica, Essen, Germany) equipped with a stirring device, a heated cuvette holder, and time-driven recording of transmission values. Platelets were adjusted to 3 × 10⁹/ml in platelet-rich plasma. GITR-Fc or human IgG was immobilized on the cuvette surface by overnight incubation at 4°C follow by washing. Where indicated, 3 × 10⁹ NK cells were added to platelets. Collagen (5 μg/ml; Mascia Brunelli, Milan, Italy) and ADP (2.5 μM; Sigma-Aldrich, St. Louis, MO) were used as platelet agonists. Platelet aggregation at 1000 rpm was measured for 5 min. For determination of CD62P expression and TGF-β release, platelets were incubated for 30 min with slight shaking (150 rpm).

Coating of tumor cells
Tumor cells were coated with platelets as described previously (19, 23). In brief, platelet-rich plasma (PRP) was obtained from fresh whole blood by centrifugation at 120 × g for 20 min. Tumor cells were incubated in PRP at a tumor cell:platelet ratio of 1:1000 for 30 min under shear stress at 37°C. Tumor cell-induced platelet aggregation was not observed under these conditions. For further investigation, tumor cells were washed afterward to remove surplus platelets and soluble factors. For investigation of IFN-γ production, washed platelets were added to tumor cells instead of PRP.

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 an FCS500 (Beckman Coulter, Krefeld, Germany). Conjugated mAb and the respective isotype controls were used at 2 μl/100,000 cells.

Immunofluorescence
Cytospins of megakaryocytes and coated tumor cells were prepared and processed for immunofluorescence with the indicated Abs as previously described (19). In brief, after nonspecific protein block, primary Abs were incubated overnight at 4°C. After successive PBS washes, sections were incubated in secondary Abs and, subsequently, stained with directly labeled CD61-PeCy5. Sections were counterstained with DAPI.

Cytotoxicity assay
Cytotoxicity of NK cells was analyzed by 2 h BATDA europium release assays as previously described (24). Percentage of lysis was calculated as follows: 100 × [(experimental release – spontaneous release)/maximum release – spontaneous release].

Measurement of cytokines by ELISA
IFN-γ and TGF-β levels were analyzed using OptEIA sets from BD Pharmingen and DuoSet ELISA development system from R&D Systems, respectively, according to the manufacturer’s instructions. All concentrations are expressed as means ± SEM of triplicates.

Results
Thrombopoietic cells express GITRL
Previous data show expression of several TNF family members on platelets (25–33). In this study we set out to determine whether GITRL was expressed upon in vitro differentiation of megakaryocytes from CD34+ stem cells. Cell purity of megakaryocytes as determined by flow cytometric evaluation of CD41a expression was >90% (data not shown). RNA was isolated at days 6, 9, and 12 during differentiation and RT-PCR was performed for semi-quantitative analysis of GITRL transcript levels, which revealed that GITRL expression is upregulated in the course of megakaryocytic differentiation (Fig. 1A). Morphologically, megakaryocytes could be identified as typical large, multinucleated cells. Immunofluorescent staining of mature megakaryocytes revealed expression of typical markers of thrombopoiesis such as CD41a in parallel with cytoplasmic expression of GITRL (Fig. 1B). To demonstrate that GITRL is passed on by megakaryocytes to their platelet progeny in humans, we analyzed peripheral blood platelets for the expression of GITRL utilizing flow cytometry. The α-granular protein P-selectin (CD62P) served as marker for platelet activation. CD62P+ platelets displayed no relevant GITRL surface expression, whereas substantial GITRL levels were detected on the surface of the CD62P+ fraction. To determine whether GITRL, similar to other TNF family members (25–27,
peaks, isotype control. (Counterstaining for CD62P. Shaded peaks, staining with specific Ab; open analyzed by flow cytometry after fixation with 1% paraformaldehyde and resting and activated (exposure to collagen or ADP for 5 min) platelets was 3 with DAPI (blue). Original magnification using CD41a (green) and GITRL (red) Abs. Nuclei were counterstained generated megakaryocytes were analyzed by immunofluorescent staining for 5 min, substantial GITRL expression could be detected on the platelet surface upon activation (Fig. 1D and data not shown). Taken together, these data demonstrate that GITRL protein content in platelets from different donors and cancer patients varied substantially. Notably, the total GITRL protein content did not differ in untreated or resting platelets from the same donor, indicating that GITRL is translocated to the platelet surface upon activation (Fig. 1D and data not shown). Taken together, these data demonstrate that GITRL is expressed on megakaryocytes and platelets and appears on the platelet surface following activation.

**GITRL does not influence platelet activity**

Comparable with multiple other members of the TNF receptor/ligand family, bidirectional signaling has been reported after GITR–GITRL interaction, and GITRL “reverse signaling” occurs both in malignant and nonmalignant cells (20, 24, 34, 35). We therefore analyzed whether engagement of GITR on platelets by GITRL would result in measurable responses. To this end, platelet aggregation in response to standard agonists was measured in the absence or presence of immobilized GITR-Fc (which induces GITRL crosslinking and thus signaling) or isotype control as well as in the presence of GITR-expressing NK cells. Neither incubation on immobilized GITR-Fc nor presence of NK cells that constitutively express GITR (20, 36) induced changes in collagen- or ADP-induced platelet aggregation under shear stress (Fig. 2A). Additionally, platelet aggregation was not induced by immobilized GITR-Fc or NK cell-expressed GITR in the absence of agonists. In line with these results, analysis of CD62P expression on platelets revealed no response to GITRL stimulation, whereas collagen as positive control strongly induced CD62P upregulation (Fig. 2B). Furthermore, measurement of TGF-β in platelet supernatants did not reveal an effect of GITR–GITRL interaction as induced by the above-described treatment conditions (Fig. 2C). We thus concluded that platelet GITRL does not transduce a reverse signal capable of substantially altering platelet aggregation or degranulation.

**Tumor cell coating by platelets results in pseudoexpression of GITRL**

Upon entering the blood stream, tumor cells activate both the plasmatic coagulation cascade and platelets, which rapidly adhere to the tumor cell surface (6, 7). The functional consequence of this finding may be an immunoprotective effect, shielding hema-

togenously disseminating tumor cells and leukemic blasts from being recognized and lysed by NK cells. Adhesion of platelets on tumor cell surfaces can be reproduced in vitro by coincubation of both cell types under shear stress in a platelet aggregometer. Low concentrations of tumor cells do not induce visible platelet aggregation, but platelet adherence to tumor cells occurs (19). Flow cytometric analyses with three tumor cell lines revealed no ex-
expression of GITRL or CD41a in the absence of platelets. When tumor cells were incubated with a thousand-fold excess of platelets, substantial levels of GITRL were detected by flow cytometry on the tumor cell surface (Fig. 3A). Similar CD41a expression levels were observed after coincubation of the three different tumor cell lines with platelets, indicating comparable coating efficiency with all three lines. Notably, GITRL expression levels on platelet-coated SK-BR-3 were higher as compared with SK-Mel and PC3 cells. Interestingly, this was correlated with higher expression of tissue factor (Fig. 3B), which may cause more efficient platelet activation (represented by higher CD62P expression) on SK-BR-3 cells. In line with our data on GITRL expression in resting and activated state, CD62P expression correlated with GITRL expression on platelets. Immunofluorescence analysis of PC3 cells with and without coating platelets again revealed that tumor cells alone do not express relevant levels of the platelet marker CD41a or GITRL. After coincubation with platelets, PC3 cells displayed surface expression of both CD41a and significant levels of GITRL (Fig. 3C). Non-tumor cell-related staining among platelet-coated tumor cells represents nonadherent platelets, which also served as positive staining control. Taken together, these data demonstrate that tumor cells are rapidly coated in the presence of platelets, which results in apparent expression of platelet molecules, including immunoregulatory GITRL by the tumor cells. We suggest to refer to this phenomenon as “pseudoexpression” of platelet membrane-bound immunoregulatory molecules by tumor cells. It may also confer a “pseudo-self” immunophenotype to malignant cells from the host antitumor immune system’s perspective (37).

Platelet-derived GITRL inhibits NK cell antitumor activity

To establish the functional significance of platelet-derived GITRL pseudoeexpression on tumor cells, and because GITR inhibits the reactivity of human NK cells (20, 24, 36, 38, 39), SK-Mel, PC3, and SK-BR-3 tumor cells were coincubated with NK cells with and without antecedent coating by platelets. Intentionally, platelets and NK cells were from different donors to exclude potential inhibitory effects by autologous MHC class I, which is also highly expressed on platelets (40). Tumor cells were washed extensively after coating to remove surplus platelets and to exclude effects by immunoregulatory factors contained in platelet releasate such as TGF-β. When tumor cells were coated by platelets, NK cytotoxicity was significantly reduced. Blockade of NK cell expressed GITR largely and significantly (p < 0.05, Student t test) reversed this effect, whereas blocking of GITR–GITRL interaction did not alter NK cell reactivity in the absence of platelets, as the employed tumor cells do not constitutively express GITRL. These results confirmed that platelet-derived GITRL exerts an inhibitory effect on NK cell cytotoxicity (Fig. 4A). A second major effector mechanism by which NK cells contribute to antitumor immunity is their role as an “early source of IFN-γ” (17, 18). Therefore, we examined IFN-γ production by NK cells in response to tumor cells in the presence or absence of coating platelets. Notably, presence of uncoated PC3 and SK-Mel tumor cells resulted in substantial IFN-γ secretion by NK cells, whereas presence of SK-BR-3 cells did not induce any IFN-γ production in analyses with NK cells of 10 different donors, neither in the presence or absence of IL-2. Platelet coating significantly decreased the cytokine production of NK cells in response to PC3 and SK-Mel. Although IFN-γ levels

![FIGURE 3. Platelet coating of tumor cells results in pseudoeexpression of GITRL. (A) The indicated tumor cells were incubated with platelets as described in Materials and Methods. GITRL, CD41a, and CD62P expression was analyzed by flow cytometry. (B) Untreated tumor cells were analyzed for tissue factor expression by flow cytometry. (C) Single-cell suspensions of the indicated tumor cell lines underwent immunofluorescent staining after incubation with or without platelets. Cells were stained with pan-cytokeratin followed by Alexa 488-conjugated secondary Ab (green), CD61-PeCy5 (red), and GITRL followed by PeCy3-conjugated Ab (yellow). Nuclei were counterstained with DAPI (blue). Original magnification ×400.](http://www.jimmunol.org/)

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in cultures with untreated tumor cells were not altered by addition of blocking GITR Ab, prevention of GITR signaling partially but significantly (p < 0.05, Student t test) restored IFN-γ production in cultures with platelet-coated PC3 and SK-Mel tumor cells (Fig. 4B).

To further confirm that GITR–GITRL interaction contributed to reduced NK reactivity against platelet-coated tumor cells, we employed the GITR⁺ NK cell line NK92 (24). Tumor cell coating by platelets did not alter cytotoxicity of NK92 cells. Addition of blocking GITR Ab had no influence, thereby providing further evidence that GITR–GITRL interaction was responsible for NK cell inhibition in the experiments with GITR-bearing primary NK cells (Fig. 4C). Notably, the blocking GITR Ab also did not alter IFN-γ production of the NK92 cells (Fig. 4D), even when IFN-γ secretion was slightly diminished by platelet coating. Just as blocking GITR–GITRL interaction was more effective with regard to cytotoxicity than IFN-γ production of primary NK cells, this may be due to the different assay conditions (e.g., 2 h assay time for measurement of cytotoxicity as compared with overnight for IFN-γ production). Furthermore, cytotoxicity and IFN-γ secretion of NK cells are governed by at least partially different mechanisms/signaling pathways (41–44), which is also highlighted by the fact that SK-BR-3 cells induced NK cell cytotoxicity but not IFN-γ secretion. Additionally, it is possible that inhibition of NK cell IFN-γ production may be mediated by yet unidentified membrane-bound molecules or contamination with low levels of platelet releasate. The latter mediates inhibitory effects, for example, by NKG2D downregulation, which requires several hours to occur (19).

To further investigate the potential influence of soluble factors on NK cell reactivity in our setting, we analyzed cytotoxicity and IFN-γ release of NK cells in response to untreated PC3 tumor cells with platelets, tumor cells, or platelet-coated tumor cells being present in the assay, but separated by 0.4-μm transwell inserts (Fig. 4E, 4F). Presence of spatially separated resting platelets did not alter NK cell reactivity. Presence of tumor cells and
washed platelet/tumor cell aggregates both reduced cytotoxicity of NK cells to the same extent. Thus, soluble factors released from tumor cells and platelet-coated tumor cells, but not factors released from platelets, inhibited tumor cell lysis in this experimental system. Again, probably for the same reasons as stated above, results regarding IFN-γ secretion differed from those of cytotoxicity assays. Presence of spatially separated platelet-coated tumor cells reduced IFN-γ secretion of NK cells, whereas untreated tumor cells did not affect cytokine release. Importantly, both with regard to cytotoxicity and IFN-γ secretion, inhibition of NK reactivity was most pronounced when platelet-coated tumor cells were in direct contact with NK cells (Fig. 4E, 4F). These findings provide strong evidence for the protean importance of membrane-bound molecules in inhibiting NK cell reactivity by tumor-coating platelets.

Finally, we analyzed the influence of platelet-expressed GITRL on NK cell IFN-γ production as induced by IL-15 in the absence of tumor targets. NK cells released only low levels of IFN-γ in the absence of IL-15, but substantial IFN-γ production was observed upon cytokine stimulation. IFN-γ production was strongly inhibited in the presence of platelets, which upregulated GITRL within the time of in vitro culture for analysis of IFN-γ production (not shown). The inhibitory effect of the platelets was partially but significantly (p < 0.05, Student t test) restored upon the addition of anti-GITR Ab (Fig. 4G). This excluded that the effects of platelet coating on NK reactivity were solely mediated by effects on the tumor cells and established proof of principle that platelet GITRL does directly influence NK cell reactivity. Taken together, these data clearly demonstrate that platelet-derived GITRL inhibits NK cell reactivity upon coating of (metastasizing) tumor cells, for example, after entering the blood stream.

Discussion

Increasing evidence points to an important role of platelets in the modulation of inflammation and immune responses beyond their function in hemostasis (40). In this study, we describe that GITRL is upregulated during megakaryopoiesis, which results in GITRL expression by platelets. We found further that GITRL expression levels are upregulated upon platelet activation because of rapid translocation of preformed cellular protein to the platelet surface, which is similar to other TNF superfamily members expressed in platelets (25–27, 30). Similar to other platelet-expressed TNF family members, platelet GITRL transduces forward signals into GITR+ cells. No reverse signaling after GITRL stimulation into platelets was detectable, whereas reverse signaling via other platelet-expressed TNF family members has not yet been studied. The only exception is platelet CD40L, which does affect platelet reactivity by reverse signaling (28, 29).

The data presented in this study provide evidence that forward signaling through platelet-expressed GITRL contributes to the tumor-protective, NK inhibiting effects of platelets. We and others have previously reported that GITR is expressed on NK cells and reduces their cytotoxicity and IFN-γ production upon engagement of GITRL (20, 24, 36, 38, 39). In this study, we demonstrate that tumor cells rapidly get coated in the presence of platelets, resulting in impaired NK antitumor reactivity, which is in line with findings of previous studies (6, 7, 11). Several mechanisms contribute to this inhibitory effect, and recently we demonstrated that this can comprise release of NK-inhibitory cytokines from platelets and altered MHC class I expression by tumor cells (19, 37). In this study, we demonstrate that conferment of GITRL to tumor cells by coating platelets contributes to the same. Because platelets upregulate NK-inhibitory GITRL upon activation, their capacity to inhibit NK cell reactivity via GITR–GITRL interaction is relevant in situations leading to their activation, which may occur upon interaction with malignant cells entering the bloodstream. The NK-inhibitory effect of platelet-expressed GITRL was revealed by blocking approaches, in which neutralizing GITR Ab partially restored NK reactivity against platelet-coated tumor cells. Cytotoxicity and cytokine release assays in transwell systems indicated that in our experimental setting membrane-bound platelet molecules were more relevant for NK cell inhibition than were soluble factors. Analysis of activation-induced NK cell cytokine production in the absence of tumor targets confirmed that platelets can in fact reduce NK effector function upon GITRL–GITR interaction and confirmed that the observed inhibitory effect was due to induction of inhibitory GITR signals in NK cells. Notably, neither NK cytotoxicity nor cytokine production was completely restored by GITR blockade, which clearly indicates that other platelet-expressed NK modulatory molecules beyond GITRL contribute to the inhibitory effect. Identification of these additional molecules is the subject of ongoing studies.

The influence of GITR–GITRL interaction in tumor immune surveillance was characterized in multiple studies. Results obtained in different mouse models indicate that stimulation of GITR potently induces T cell antitumor immunity against various malignancies. However, data by us and others regarding the role of GITR in human NK cells indicate that stimulation of GITR inhibits NK antitumor reactivity (20, 24, 36, 38). The available data indicate that the consequences of GITR–GITRL interaction may vary between mice and humans, between T cells and NK cells, and, upon treatment with agonistic Abs, they seem to be dependent on the time of intervention, the biological environment, and the level of the ongoing immune response (34, 35, 45–48). Our data demonstrate that platelets may also contribute to the interplay of GITR/GITRL-expressing components of the hematopoietic system and add another level of complexity to the picture of GITR and its ligand in immunity. As of now, the role of platelets not only in the crosstalk via GITR–GITRL, but rather during immune responses in general, remains incompletely defined, but increasing evidence indicates that platelets, beyond their hemostatic function, are important modulators of immune responses, especially of NK cells. Much further work is required to delineate the influence of platelets in general and as third players when it comes to tumor–NK cell interactions. In this context it is important to consider that discrimination of prometastatic effects of platelets and the plasmatic coagulation system, let alone the specific underlying mechanisms, is difficult in animal models, because both may promote metastasis on multiple different levels (5). Additionally, the influence of a single molecule within the multitude of inhibitory and activating receptors governing NK reactivity may also more easily be elucidated by in vitro studies. Knockout or inhibition of GITR in vivo would not only influence NK cells, but also other GITR-bearing cells of the immune system. Additionally, not only GITR but also GITRL transduces immunomodulatory signals. Thus, interfering with GITR in mouse models may cause various effects that influence multiple cell types that interact via GITR–GITRL. Perhaps most importantly, results by us and others revealed that the consequences of GITR triggering may differ in human and mouse NK cells (20, 24, 35, 36, 38, 39). Our in vitro study in turn enabled the detailed dissection of the mechanisms by which GITR and its platelet-expressed ligand may contribute to the evasion of tumor cells from NK-mediated immune surveillance.

Tumor cells and platelets both express inhibitory and stimulatory ligands for NK cells, and, in the end, it will be the interplay of all of them that dictates upon tumor cell lysis or immune escape. Suitable future studies are required to elucidate the complex in-
terplay of NK cells, platelets, and tumor cells, which then would hold promise to establish novel therapeutic targets to possibly help us overcome obstacles in tumor immunotherapy.

Disclosures

The authors have no financial conflicts of interest.

References