Elevated TGF-β1 Secretion and Down-Modulation of NKG2D Underlies Impaired NK Cytotoxicity in Cancer Patients[1]

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NK cell function in cancer patients is severely impaired, but the mechanism underlying this impairment is not clearly understood. In this study we show evidence that TGF-β1 secreted by tumors is responsible for the poor NK lytic activity via down-regulating an NK-activating receptor, NKG2D. The plasma level of TGF-β1 in human lung cancer or colorectal cancer patients was elevated compared with that in normal volunteers, and this elevation was inversely correlated with surface expression of NKG2D on NK cells in these patients. Incubation of NK cells with plasma obtained from cancer patients specifically down-modulated surface NKG2D expression, whereas addition of neutralizing anti-TGF-β1 mAbs completely restored surface NKG2D expression. Likewise, incubation of NK cells and lymphokine-activated killer cells with TGF-β1 resulted in dramatic reduction of surface NKG2D expression associated with impaired NK cytotoxicity. Modulation of NKG2D by TGF-β1 was specific, as expression of other NK receptors, CD94/NKG2A, CD44, CD16, 2B4, or CD56, was not affected by TGF-β1. Impaired NK cytotoxicity by TGF-β1 was not due to alteration of lytic moieties, such as perforin or Fas, or apoptotic pathway, but, rather, appeared to be due to lack of NKG2D expression. Taken together, our data suggest that impaired NK function in cancer patients can be attributed to down-modulation of activating receptors, such as NKG2D, via secretion of TGF-β1.   *The Journal of Immunology,* 2004, 172: 7335–7340.

N atural killer cells mediate lysis of tumor cells as well as pathogen-infected cells (1, 2). NK cell activity is regulated by functionally opposing receptors, inhibitory receptors that bind class I MHC molecules and activating receptors that bind ligands on tumors and/or virus-infected cells. In the resting state, the function of activating receptors is inhibited due to ligation of inhibitory receptors with MHC class I molecules. Loss of MHC class I molecules due to infection or tumor formation relieves this inhibition and thus confers NK activation. In humans, inhibitory receptors include killer Ig-like receptor 2DL, killer Ig-like receptor 3DL1, and CD94/NKG2A (3, 4), whereas activating receptors include natural cytotoxicity receptors NKp30, NKp44, and NKp46 and a recently characterized lectin-like molecule, NKG2D (5, 6).

NKG2D is expressed on NK cells, CD8 $\alpha\beta$ T cells, and $\gamma\delta$ T cells. Ligands of NKG2D, MHC class I chain-related molecules (MIC) A and MICB in humans and the Rae1 and H60 families in mice (7–9), are not detected on normal cells, but are induced upon physical stress or tumor formation (10, 11). Up-regulation of MICA/B and Rae1 in tumor cells suggests that NKG2D may play a critical role in regulating tumor development and growth. Indeed, tumor cells expressing Rae1 or H60 were shown to be efficiently lysed in vitro and completely rejected by syngeneic mice (12, 13).

However, despite the presence of MIC ligands on many progressing tumors, including breast, lung, gastric, renal, colon, and ovarian carcinomas (14), these tumors still grow, suggesting that the MIC/NKG2D signaling is functionally impaired.

TGF-β1 is a potent immunosuppressive molecule produced by many cancer cells. TGF-β1 has been shown to stimulate tumor growth while inhibiting expansion, cytotoxicity, and cytokine production of purified NK cells and IL-2- or IL-12-activated killer cells in vitro (15–18). Consistent with this, TGF-β1 has been reported to suppress NK activity in a mouse model (19, 20). In patients with lung or colorectal cancer, the plasma concentration of TGF-β1 was found to be elevated, which correlated with the degree of tumor progression (21–23). As impaired NK activity was widely observed in advanced cancer patients (24–26), we hypothesize that TGF-β1 produced in cancer patients may affect the expression and function of NK receptors involved in the lysis of tumor cells. We now provide evidence that TGF-β1 present in plasma of advanced cancer patients can modulate NK responses by down-regulating NKG2D expression.

Materials and Methods

Preparation of human PBL and NK cells

Human PBMCs were derived from 36 cancer patients (27 lung cancer and nine colorectal cancer). Tumors were diagnosed by histopathological criteria. Normal PBMCs were obtained from random 20 healthy volunteers. These activities were approved by institutional review boards, and all subjects gave written informed consent. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). After removing plastic adherent monocytes, PBLs were collected, and NK cells were isolated via negative selection. Briefly, PBLs were incubated with anti-CD3 and anti-CD20 mAbs for 30 min at 4°C, and subsequently incubated with goat anti-mouse IgG-coated Dynabeads (Dynal Biotech, Oslo, Norway) for 30 min at 4°C. After immunomagnetic depletion, CD3$^-$CD20$^-$CD56$^+$ cells (>95%, confirmed by FACS analysis) were used directly or were cultured in the presence of rIL-2 or rIL-15 to obtain activated NK cell populations.

Reagents

FITC-, PE-, or CyChrome-conjugated anti-human CD3, CD4, CD8, CD16, CD20, CD44, CD56, CD94, 2B4, and perforin mAbs were purchased from BD PharMingen (San Diego, CA). Anti-human CD178 (FAS ligand) mAb

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[3] Abbreviations used in this paper: MIC, MHC class I chain-related molecule; MFI, mean fluorescent intensity; PI, propidium iodide; DAP, DNAX-activating protein.

were purchased from Ancell (Bayport, MN). Human rIL-2, rIL-15, and rTGF-β1 and anti-NKG2D and anti-TGF-β1 mAb were purchased from R&D Systems (Minneapolis, MN).

Cell cultures

Purified NK cells and PBLs were cultured in RPMI 1640 containing 10% FBS. Where indicated, cells were cultured in the presence of various concentrations of cytokines or TGF-β1 for 1–3 days. Cells were harvested and stained with appropriate Abs for FACS. To generate the active form of TGF-β1, TGF-β1 was dissolved in 4 mM HCl containing 1 mg/ml BSA and was further diluted with RPMI 1640 before addition into NK cells. The final concentration of HCl had no effect on NK cell function. The human T lymphoblast CEM cell line, used as a target in the cytotoxicity assay, was maintained in RPMI 1640 containing 10% FBS.

Cytotoxicity assay

Cytotoxicity mediated by NK cells was determined by a ^{51}Cr release assay. Target cells (CEM) were labeled with 100 μCi of Na$_2$^{51}CrO$_4$ (1 mCi; NEN, Boston, MA) for 1 h at 37°C, washed three times, and adjusted to 1 × 10^5 cells/ml. Serially diluted PBLs or purified NK cells were mixed with ^{51}Cr-labeled CEM cells at various E:T cell ratios and incubated for 4 h at 37°C. Supernatants were harvested, and ^{51}Cr released was measured using a gamma counter. Spontaneous release, always <10% of maximal release for CEM, was measured after incubation of target cells with medium only. Maximal release was determined in wells containing target cells only after addition of 5% Triton X-100. The percentage of specific lysis was determined as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Flow cytometry and apoptosis assay

Cells (5 × 10^5) were resuspended in FACS binding solution (1% FBS and 0.1% sodium azide in PBS) and incubated with various combinations of FITC- and PE-labeled mAbs for 30 min at 4°C. Cells were then washed and resuspended in PBS containing 2% paraformaldehyde. For analysis of NKG2D expression on NK cells from normal volunteers and cancer patients, three-color FACS staining methods were used. Isolated PBMCs were incubated with anti-CD3, -CD56, and -NKG2D mAb, and NKG2D expression was determined by mean fluorescence intensity (MFI) on CD3$^-$CD56$^+$ gated cells. Analysis of perforin and Fas ligand expression was conducted using Cytofix/Cytoperm intracellular staining kits (BD PharMingen). All stained cells were detected by FACSCalibur (BD Biosciences, San Jose, CA) and analyzed by CellQuest software. For flow cytometric apoptosis assay, 1 × 10^6 cells were stained with FITC-labeled annexin V and propidium iodide (PI; Molecular Probes, Eugene, OR) as suggested by the manufacturer and were analyzed by FACS. Data are shown from a representative experiment with five different healthy donors.

Plasma preparation and ELISA

For detection of plasma cytokines, blood samples were taken from each individual, immediately transferred to tubes containing EDTA, and centrifuged for 30 min at 1500 × g at 4°C. The resulting plasma was transferred to polypropylene microtubes and stored at −80°C. Samples were thawed at room temperature at the time of cytokine measurement. For measurement of the total TGF-β1 concentration, 0.1 ml of 2.5 N acetic acid/10 M urea was added to 0.1 ml of plasma, followed by mixing to activate the TGF-β1 at room temperature. After 10 min, the resulting plasma was neutralized with 0.1 ml of 2.7 N NaOH/1 M HEPES and diluted before analysis. Concentrations of IL-4, IL-10, or TGF-β1 were determined by ELISA using commercially available Ab pairs and recombinant standards (R&D Systems). The lower detection limits of these cytokines were as follows: IL-4, <30 pg/ml; IL-10, <30 pg/ml; and TGF-β1, <30 pg/ml.

Statistics

Statistical analysis was conducted using Student's *t* test. The correlation between groups was evaluated by Pearson's correlation coefficient (*r*). Statistical significance was accepted at $p < 0.05$.

Results

Elevated plasma TGF-β1 concentrations and decreased NKG2D surface expression on NK cells in patients with lung or colorectal cancers

To determine whether there is any correlation between the level of plasma TGF-β1 and NKG2D surface expression on NK cells, we obtained blood samples from 37 cancer patients (28 lung cancer

and nine colorectal cancer) and measured the levels of TGF-β1 and NKG2D by ELISA and FACS, respectively. In line with previous reports (21–23), cancer patients showed elevated levels of TGF-β1 compared with healthy volunteers (Fig. 1*A*; $n = 20$; $p < 0.01$). In contrast, surface NKG2D expression on CD3$^-$CD56$^+$ NK cells in these cancer patients was reduced (Fig. 1*B*, *top panel*). Interestingly, reduction of surface NKG2D level was variable among patients (Fig. 1*B*, *bottom panel*), presumably reflecting the distinct status of tumor progression in individual patients. To determine whether the variable level of NKG2D down-regulation
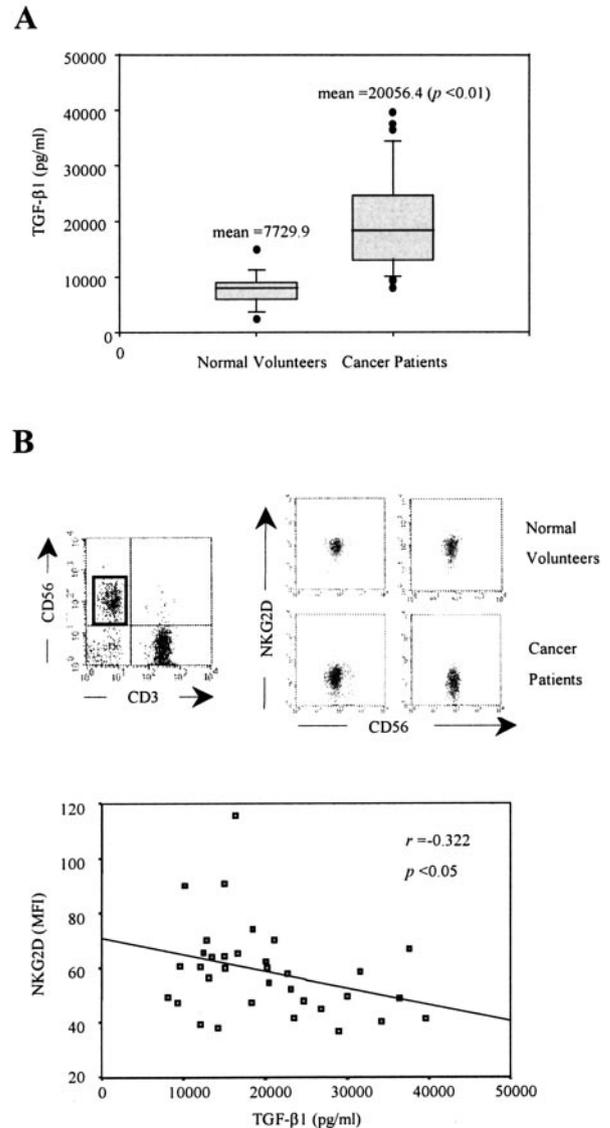

FIGURE 1. Correlation between the level of plasma TGF-β1 and NKG2D expression on human NK cells prepared from cancer patients. *A*, Plasma concentrations of total TGF-β1 in cancer patients (lung cancer, $n = 28$; colorectal cancer, $n = 9$) and normal volunteers ($n = 20$) were measured by ELISA. *B*, Surface expression of NKG2D was analyzed by three-color flow cytometry on CD3$^-$CD56$^+$ NK cells from cancer patients ($n = 37$) and normal volunteers ($n = 20$). Dot plots showing surface NKG2D obtained from two cancer patients (shown as samples in the plot below) and two normal volunteers (not shown below) were made to visually detect MFI changes in cancer patients. There was individual variation in the MFI level of surface NKG2D expression; nevertheless, these two samples represent the MFI from the major population in each group. Below, the correlation between plasma TGF-β1 and NKG2D expression level in cancer patients was determined by Pearson's correlation coefficient (*r*), and the associated probability (*p*) were calculated for each combination.

was associated with different levels of plasma TGF-β1 in these patients, statistical analysis was performed. As shown in Fig. 1*B*, an inversely linear relationship exists between plasma TGF-β1 and the level of NKG2D on NK cells ($r = -0.322$). These data suggest that the systemic impairment of NKG2D expression may be linked to the aberrant secretion of TGF-β1 in these cancer patients.

TGF-β1 present in plasma of cancer patients is responsible for down-regulation of NKG2D

To investigate if TGF-β1 present in plasma of cancer patients was responsible for down-modulation of NKG2D, we incubated freshly isolated NK cells obtained from healthy volunteers with plasma obtained from cancer patients. As shown in Fig. 2*A*, incubation of NK cells with plasma containing high levels of TGF-β1 obtained from cancer patients significantly inhibited surface NKG2D expression (compare *lane 1* vs *lane 2*). Neutralizing TGF-β1 by adding anti-TGF-β1 mAbs to the culture restored the level of surface NKG2D (*lane 3*), indicating that TGF-β1 present in plasma was responsible for a reduction in NKG2D expression. Neutralizing other cytokines, IL-4 or IL-10, did not prevent down-modulation of NKG2D (*lanes 4* and *5*), suggesting that down-modulation of NKG2D was specific to TGF-β1. In contrast, incubation of NK cells with plasma obtained from healthy volunteers did not modulate surface NKG2D level (Fig. 2*B*). Furthermore, neither anti-TGF-β1 mAbs nor anti-IL-4 or anti-IL-10 mAbs affected surface expression of NKG2D when cells were incubated with normal plasma (Fig. 2*B*). Together, our data strongly suggest that secretion
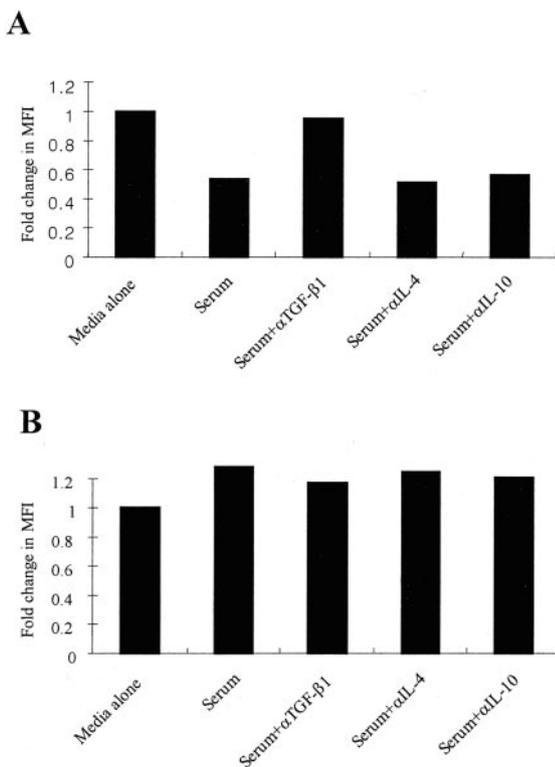
of TGF-β1 in cancer patients can down-modulate NKG2D expression on NK cells.

Effect of TGF-β1 on the NKG2D expression of freshly isolated or lymphokine-activated human NK cells

Human NK cells express NKG2D in the resting state, and the level of NKG2D is up-regulated upon activation (27). To further confirm that TGF-β1 mediates down-regulation of NKG2D on resting NK cells, we cultured purified NK cells with various doses of TGF-β1 and analyzed NKG2D expression by FACS. As shown in Fig. 3 (*left panel*), addition of TGF-β1 reduced surface NKG2D expression in a dose-dependent manner. Down-modulation of NKG2D was evident at 0.1 μg/ml TGF-β1 and reached a maximal level at 5 ng/ml TGF-β1. Increasing the TGF-β1 concentration up to 20 ng/ml did not further inhibit NKG2D expression (data not shown). To determine whether TGF-β1 can also affect the up-regulation of NKG2D upon activation, NK cells were cultured with either IL-2 (100 U/ml) or IL-15 (100 ng/ml) for 2 days to generate lymphokine-activated NK cells. As shown in Fig. 3, IL-2 (*middle panel*) and IL-15 (*right panel*) significantly up-regulated surface NKG2D; however, addition of TGF-β1 dose-dependently reduced the level of surface NKG2D. These data demonstrate that TGF-β1 can down-regulate surface NKG2D expression on resting NK cells and inhibit up-regulation of NKG2D upon IL-2 or IL-15 stimulation. In contrast, TGF-β1 did not alter the level of other NK receptors, including MHC class I-specific inhibitory receptors, CD94/NKG2A, or the activation/memory marker, CD44 (Fig. 4). Similarly, surface expression of other activating receptors, CD16, 2B4, and CD56, was not affected by treatment with TGF-β1 (data not shown). These data suggest that TGF-β1 specifically down-modulates NKG2D without affecting other NK receptors.

Effect of TGF-β1 on NK cytotoxicity

We next examined whether TGF-β1-treated NK cells showed impaired cytotoxicity. CEM cells were chosen as a target because they can be lysed through a NKG2D-sensitive pathway (28). Treatment of freshly isolated NK cells with TGF-β1 suppressed NK-dependent lysis of CEM cells as shown in Fig. 5. The basal level of lysis against CEM targets in freshly isolated NK cells (Fig. 5,
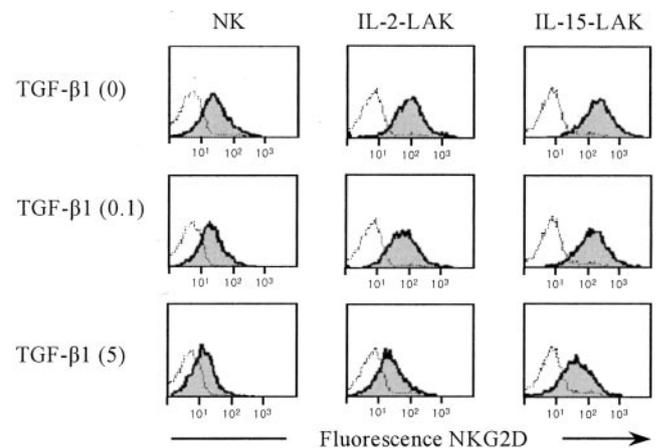

FIGURE 2. Incubation of NK cells with plasma obtained from cancer patients inhibits surface NKG2D expression in a TGF-β1-dependent manner. Purified NK cells were cultured with 100 U/ml IL-2 in the presence of a 1/5 dilution of plasma from a cancer patient (*A*) or a normal volunteer (*B*). Cells were harvested after 24 h and stained with anti-NKG2D mAb. Single-color flow cytometry was performed, and results are presented as the fold change in MFI. For neutralization experiments, 10 μg/ml mAb against TGF-β1, IL-4, or IL-10 was added to the culture.

FIGURE 3. Effect of TGF-β1 on NKG2D expression of freshly isolated or lymphokine-activated human NK cells. Purified NK cells were cultured with or without 100 U/ml IL-2 or 100 ng/ml IL-15 in the presence of the indicated concentrations (nanograms per milliliter) of TGF-β1. Cells were harvested after 2 days and were stained with specific mAb for NKG2D. Single-color flow cytometry was performed (*x*-axis, $\log_{10}$ fluorescent intensity; *y*-axis, cell count). Solid and dotted lines indicate staining with anti-NKG2D mAb and its isotype-matched control, respectively.
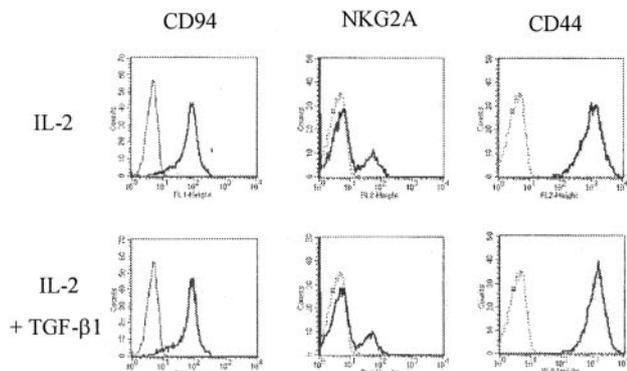
FIGURE 4. TGF-β1 does not affect surface expression of CD94/NKG2A or CD44 on IL-2-activated human NK cells. Purified NK cells were cultured with 100 U/ml IL-2 in the presence or the absence of 5 ng/ml TGF-β1. Cells were harvested after 2 days and were stained with specific mAbs for each NK receptor. Single-color flow cytometry was performed (x-axis, $\log_{10}$ fluorescent intensity; y-axis, cell count). Solid and dotted lines indicate staining with anti-CD94, anti-NKG2A or anti-CD44 mAb and their isotype-matched controls, respectively.

top panel; 6%) was low compared with that in IL-2-activated (Fig. 5, *middle panel*; 48%) or IL-15-activated (Fig. 5, *bottom panel*; 50%) NK cells, presumably due to the low expression of NKG2D under resting conditions (Fig. 3). Treatment of either resting or activated NK cells with TGF-β1 dose-dependently suppressed cytotoxicity against CEM targets. Maximal inhibition of lysis was observed at 5 ng/ml TGF-β1, similar to the concentration required for NKG2D down-regulation (Fig. 3).

We next investigated whether down-regulation of NK cytotoxicity by TGF-β1 was associated with alteration of lytic moieties or the apoptotic pathway, such as perforin or Fas ligand. As shown in Fig. 6, the level of neither perforin nor Fas ligand was altered by treatment with TGF-β1 in resting or IL-2/IL-15-activated NK cells. Furthermore, TGF-β1 did not appear to induce apoptosis, as the levels of annexin V and PI in TGF-β1-treated cells was comparable to that in untreated cells. Taken together, these results demonstrate that TGF-β1 suppresses NK cytotoxicity mainly through down-modulation of NKG2D, without affecting the lytic or apoptotic pathway.

Discussion

Impaired NK cell responses in advanced cancer patients have been widely observed (24–26), but the molecular mechanism underlying this impairment is still not completely understood. In this study we provide evidence that TGF-β1 secreted by tumors in both lung and colorectal cancer patients can suppress NK lytic activity by down-modulating an NK-activating receptor, NKG2D. Incubation of freshly isolated NK cells with TGF-β1-containing plasma obtained from the cancer patients significantly inhibited the surface expression of NKG2D, whereas addition of blocking anti-TGF-β1 mAbs completely restored surface NKG2D to normal levels. Consistent with this, the level of plasma TGF-β1 was inversely correlated with the level of NKG2D down-regulation; thus, more progressed cancer patients secreted higher levels of TGF-β1 and showed more profound down-regulation of NKG2D.

Tumors can produce various immunosuppressive molecules, including TGF-β1, IL-4, IL-10, and PGE$_2$ (29, 30). TGF-β1 is secreted by tumors of different histotypes, including melanomas, neuroblastomas, carcinomas, and leukemias, and can allow escape from immune surveillance by inhibiting T and NK cell function (15–20, 31). It was hypothesized that TGF-β1-mediated suppres-
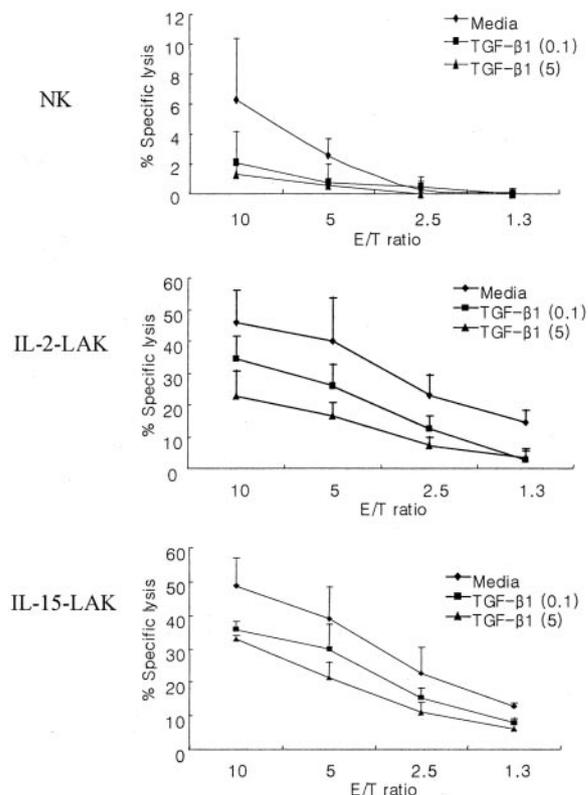

FIGURE 5. TGF-β1 inhibits the cytotoxicity of freshly isolated or lymphokine-activated human NK cells. Purified NK cells were cultured in the presence or the absence of 100 U/ml IL-2, 100 ng/ml IL-15, or the indicated concentrations (nanograms per milliliter) of TGF-β1. Cells were harvested after 2 days and subjected to 4-h ^{51}Cr release assays using CEM target cells. Results are expressed as the percentage of specific lysis.

sion of cytotoxic T cell activity was partially mediated by upregulation of inhibitory receptors, CD94/NKG2A (32). However, our data from NK cells show that TGF-β1 does not affect the expression of CD94/NKG2A. Instead, TGF-β1 reduces the surface expression of an activating receptor, NKG2D. Therefore, it appears that NK cells respond to TGF-β1 differently from T cells. While preparing this study, Castriconi et al. (33) reported that TGF-β1 can down-modulate surface expression of NK-activating receptors, NKp30 and NKG2D. Down-modulation of NKp30 was directly associated with reduced NK killing of immature dendritic cells. Although it was proposed that down-modulation of NKG2D might reflect the poor NK cytotoxicity against tumor targets in their study, no direct evidence was provided. Our data shown in this study confirm their in vitro finding and further extended this to the in vivo situation by showing that TGF-β1 present in human cancer patients mediates NKG2D down-modulation of NK cells and, in turn, is responsible for poor NK cytotoxicity.

Ligands of human NKG2D, MICA/B, become expressed when cells receive physical stress or undergo transformation due to genetic changes (10, 11). Upon binding to its ligand, NKG2D stimulates the cell's lytic pathway, resulting in lysis of altered/transformed cells. NKG2D engagement was also shown to induce the proliferation of NK cells and the secretion of cytokines and chemokines, including IFN-γ, GM-CSF, TNF-α, macrophage inflammatory protein-α and -β, and I-309 (9, 27). NKG2D transduces its activating signals through coassociated adaptor molecules, DNAX-activating protein, (DAP) 12, and DAP10. In murine NK cells, association with DAP12 recruits and activates SYK and ZAP70 tyrosine kinases, whereas association with DAP10 recruits
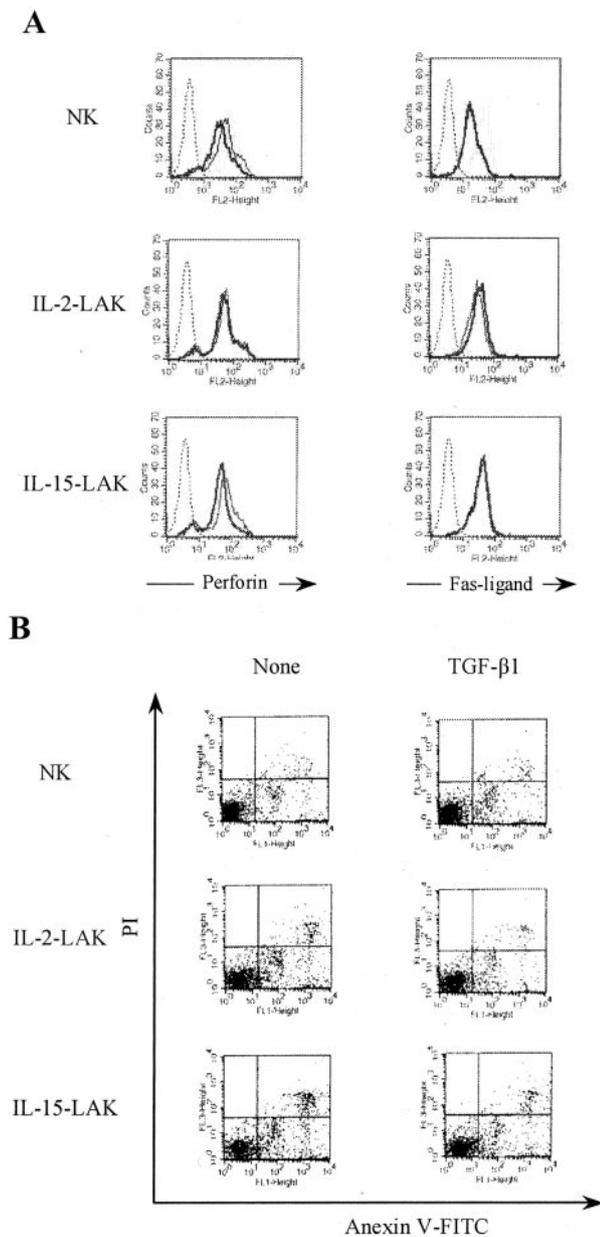
FIGURE 6. TGF-β1 does not affect the level of perforin, Fas ligand, annexin V, or PI in freshly isolated or lymphokine-activated human NK cells. *A*, Purified NK cells were cultured in the presence or the absence of 100 U/ml IL-2, 100 ng/ml IL-15, or 5 ng/ml TGF-β1 for 2 days. Cells were harvested, permeabilized, stained with anti-perforin or anti-Fas ligand mAb, and analyzed by FACS (*x*-axis, $\log_{10}$ fluorescent intensity; *y*-axis, cell count). The dotted lines are negative controls; the solid lines are TGF-β1-untreated cells; the bold lines are TGF-β1-treated cells. *B*, Flow cytometric analysis using annexin V and PI was conducted on NK cells activated as described in *A*.

both normal and cancer cells. In addition, cells treated with TGF-β1 were shown to express less lytic moieties (18). However, our data show that TGF-β1-mediated impairment of NK cytotoxicity was not due to either increased apoptosis or decreased lytic moieties. These data suggest that TGF-β1 inhibits NK cytotoxicity primarily by inhibiting the expression and signaling of NKG2D without affecting molecules involved in lytic pathway or apoptosis. Recently, it was shown that tumor cells can release the soluble form of MIC ligands, which can inhibit NKG2D function (35, 36). Engagement of soluble MIC with NKG2D resulted in endocytosis and degradation of NKG2D. Our preliminary data also provide evidence that TGF-β1 may partially regulate endocytosis and lysosomal degradation of NKG2D without affecting its mRNA level (data not shown). Therefore, in cancer patients, the function of NKG2D is severely affected by soluble MIC and TGF-β1. It is possible that soluble MIC and TGF-β1 may synergize to downmodulate NKG2D, thus more efficiently suppressing the NKG2D-mediated immune surveillance provided by NK and T cells.

Collectively, our data present the first evidence that secreted TGF-β1 in cancer patients is responsible for impaired NK function by down-modulating surface NKG2D expression. Thus, blocking the function of TGF-β1 and/or soluble MIC may provide the basis for a novel cancer immunotherapy to improve the function of T and NK cells.

References

1. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol. 47:187.*
2. Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol. 17:189.*
3. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol. 16:359.*
4. Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. *Annu. Rev. Immunol. 17:875.*
5. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol. 19:197.*
6. Lanier, L. L. 2003. Natural killer cell receptor signaling. *Curr. Opin. Immunol. 15:308.*
7. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science 285:727.*
8. Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat. Rev. Immunol. 3:781.*
9. Vivier, E., E. Tomasello, and P. Paul. 2002. Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition? *Curr. Opin. Immunol. 14:306.*
10. Groh, V., S. Bahram, S. Bauer, A. Herman, M. Beauchamp, and T. Spies. 1996. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc. Natl. Acad. Sci. USA 93:12445.*
11. Groh, V., R. Rhinehart, J. Randolph-Habecker, M. S. Topp, S. R. Riddell, and T. Spies. 2001. Costimulation of CD8$\alpha\beta$ T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat. Immunol. 2:255.*
12. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci. USA 98:11521.*
13. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature 413:165.*
14. Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein, and T. Spies. 1999. Broad tumor-associated expression and recognition by tumor-derived $\gamma\delta$ T cells of MICA and MICB. *Proc. Natl. Acad. Sci. USA 96:6879.*
15. Rook, A. H., J. H. Kehrl, L. M. Wakefield, A. B. Roberts, M. B. Sporn, D. B. Burlington, H. C. Lane, and A. S. Fauci. 1986. Effects of transforming growth factor β on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *J. Immunol. 136:3916.*
16. Bellone, G., M. Aste-Amezaga, G. Trinchieri, and U. Rodeck. 1995. Regulation of NK cell functions by TGF-β1. *J. Immunol. 155:1066.*
17. Pierson, B. A., K. Gupta, W. S. Hu, and J. S. Miller. 1996. Human natural killer cell expansion is regulated by thrombospondin-mediated activation of transforming growth factor-β1 and independent accessory cell-derived contact and soluble factors. *Blood 87:180.*
18. Malygin, A. M., S. Meri, and T. Timonen. 1993. Regulation of natural killer cell activity by transforming growth factor-β and prostaglandin E$_2$. *Scand. J. Immunol. 37:71.*
19. Arteaga, C. L., S. D. Hurd, A. R. Winnier, M. D. Johnson, B. M. Fendly, and J. T. Forbes. 1993. Anti-transforming growth factor (TGF)-β antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity: implications for a possible role of tumor cell/host TGF-β interactions in human breast cancer progression. *J. Clin. Invest. 92:2569.*

and activates phosphatidylinositol 3-kinase, similar to the signaling initiated by T cell costimulator molecules, CD28 (6, 34). Indeed, NKG2D was shown to bind DAP-10, not DAP-12, in T cells and to function as a costimulator for T cell activation. Thus, depending on the associated adaptor molecules, NKG2D can function as an activating receptor or costimulatory receptor. By inhibiting NKG2D expression on T cells and NK cells, TGF-β1 can function as a global immune suppressor that inhibits both innate and adaptive immunity.

At present, the molecular mechanism of NKG2D down-regulation is not clear. TGF-β1 has been shown to induce apoptosis of

20. Wallick, S. C., I. S. Figari, R. E. Morris, A. D. Levinson, and M. A. Palladino. 1990. Immunoregulatory role of transforming growth factor β (TGF-β) in development of killer cells: comparison of active and latent TGF-β1. *J. Exp. Med.* *172:1777.*

21. Hasegawa, Y., S. Takanashi, Y. Kanehira, T. Tsushima, T. Imai, and K. Okumura. 2001. Transforming growth factor-β1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. *Cancer 91:964.*

22. Kong, F. M., M. K. Washington, R. L. Jirtle, and M. S. Anscher. 1996. Plasma transforming growth factor-β1 reflects disease status in patients with lung cancer after radiotherapy: a possible tumor marker. *Lung Cancer 16:47.*

23. Narai, S., M. Watanabe, H. Hasegawa, H. Nishibori, T. Endo, T. Kubota, and M. Kitajima. 2002. Significance of transforming growth factor β1 as a new tumor marker for colorectal cancer. *Int. J. Cancer 97:508.*

24. Espi, A., J. Arenas, E. Garcia-Granero, E. Marti, and S. Lledo. 1996. Relationship of curative surgery on natural killer cell activity in colorectal cancer. *Dis. Colon Rectum 39:429.*

25. Kastelan, M., K. Kovacic, R. Tarle, I. Kraljic, and M. Tarle. 1997. Analysis of NK cell activity, lymphocyte reactivity to mitogens and serotest PSA and TPS values in patients with primary and disseminated prostate cancer, PIN and BPH. *Anticancer Res. 17:1671.*

26. Koda, K., N. Saito, N. Takiguchi, K. Oda, M. Nunomura, and N. Nakajima. 1997. Preoperative natural killer cell activity: correlation with distant metastases in curatively research colorectal carcinomas. *Int. Surg. 82:190.*

27. Kubin, M., L. Cassiano, J. Chalupny, W. Chin, D. Cosman, W. Fanslow, J. Mullberg, A. M. Rousseau, D. Ulrich, and R. Armitage. 2001. ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. *Eur. J. Immunol. 31:1428.*

28. Pende, D., P. Rivera, S. Marcenaro, C. C. Chang, R. Biassoni, R. Conte, M. Kubin, D. Cosman, S. Ferrone, L. Moretta, et al. 2002. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Res. 62:6178.*

29. Wojtowicz-Praga, S. 1997. Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. *J. Immunother. 20:165.*

30. Elgert, K. D., D. G. Alleva, and D. W. Mullins. 1998. Tumor-induced immune dysfunction: the macrophage connection. *J. Leukocyte Biol. 64:275.*

31. Letterio, J. J., and A. B. Roberts. 1998. Regulation of immune responses by TGF-β. *Annu. Rev. Immunol. 16:137.*

32. Bertone, S., F. Schiavetti, R. Bellomo, C. Vitale, M. Ponte, L. Moretta, and M. C. Mingari. 1999. Transforming growth factor-β-induced expression of CD94/NKG2A inhibitory receptors in human T lymphocytes. *Eur. J. Immunol. 29:23.*

33. Castriconi, R., C. Cantoni, M. Della Chiesa, M. Vitale, E. Marcenaro, R. Conte, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2003. Transforming growth factor β1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proc. Natl. Acad. Sci. USA 100:4120.*

34. Diefenbach, A., E. Tomasello, M. Lucas, A. M. Jamieson, J. K. Hsia, E. Vivier, and D. H. Raulet. 2002. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat. Immunol. 3:1142.*

35. Salih, H. R., H. G. Rammensee, and A. Steinle. 2002. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. *J. Immunol. 169:4098.*

36. Groh, V., J. Wu, C. Yee, and T. Spies. 2002. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature 419:734.*