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## TGF- $\beta$ Utilizes SMAD3 to Inhibit CD16-Mediated IFN- $\gamma$ Production and Antibody-Dependent Cellular Cytotoxicity in Human NK Cells<sup>1</sup>

Rossana Trotta<sup>2, \*</sup>, Jessica Dal Col<sup>\*</sup>, Jianhua Yu<sup>\*</sup>, David Ciarlariello<sup>\*</sup>, Brittany Thomas<sup>\*</sup>, Xiaoli Zhang<sup>†</sup>, Jeffrey Allard II<sup>\*</sup>, Min Wei<sup>\*</sup>, Hsiaoyin Mao<sup>\*</sup>, John C. Byrd<sup>‡, §, ¶</sup>, Danilo Perrotti<sup>\*, ¶</sup>, and Michael A. Caligiuri<sup>2, \*, ‡, ¶</sup>

<sup>\*</sup>Department of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University, Columbus, OH 43210

<sup>†</sup>The Center for Biostatistics, The Ohio State University, Columbus, OH 43210

<sup>‡</sup>The Division of Hematology/Oncology, Department of Internal Medicine, The Ohio State University, Columbus, OH 43210

<sup>§</sup>The Department of Medical Chemistry, College of Pharmacy, The Ohio State University, Columbus, OH 43210

<sup>¶</sup>The Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210

### Abstract

TGF- $\beta$  can be a potent suppressor of lymphocyte effector cell functions and can mediate these effects via distinct molecular pathways. The role of TGF- $\beta$  in regulating CD16-mediated NK cell IFN- $\gamma$  production and antibody-dependent cellular cytotoxicity (ADCC) is unclear, as are the signaling pathways that may be utilized. Treatment of primary human NK cells with TGF- $\beta$  inhibited IFN- $\gamma$  production induced by CD16 activation with or without IL-12 or IL-2, and it did so without affecting the phosphorylation/activation of MAP kinases ERK and p38, as well as STAT4. TGF- $\beta$  treatment induced SMAD3 phosphorylation, and ectopic overexpression of SMAD3 resulted in a significant decrease in IFN- $\gamma$  gene expression following CD16 activation with or without IL-12 or IL-2. Likewise, NK cells obtained from *smad3*<sup>-/-</sup> mice produced more IFN- $\gamma$  in response to CD16 activation plus IL-12 when compared with NK cells obtained from wild-type mice. Coactivation of human NK cells via CD16 and IL-12 induced expression of *T-BET*, the positive regulator of IFN- $\gamma$ , and *T-BET* was suppressed by TGF- $\beta$  and by SMAD3 overexpression. An extended treatment of primary NK cells with TGF- $\beta$  was required to inhibit ADCC, and it did so by inhibiting granzyme A and granzyme B expression. This effect was accentuated in cells overexpressing SMAD3. Collectively, our results indicate that TGF- $\beta$  inhibits CD16-mediated human NK cell IFN- $\gamma$  production and ADCC, and these effects are mediated via SMAD3.

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<sup>2</sup>Address correspondence and reprint requests to Drs. Rossana Trotta and Michael A. Caligiuri, The Ohio State University Comprehensive Cancer Center, 884 OSU Biomedical Research Tower, 460 West 12th Avenue, Columbus, OH 43210. rossana.trotta@osumc.edu and michael.caligiuri@osumc.edu.

### Disclosures

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Natural killer cells are large granular lymphocytes and critical components of the innate immune system (1). They produce immunoregulatory cytokines and chemokines and mediate cytotoxicity against a variety of malignant and infected target cells that lack cognate MHC class I ligands (2,3). Most NK cells express the low-affinity receptor for the Fc fragment of IgG (Fc $\gamma$ RIIA, CD16) (4). The more abundant CD56<sup>dim</sup> NK subset has high surface density expression of CD16, whereas the minority CD56<sup>bright</sup> NK subset has low to absent CD16 expression (5).

CD16 is an activating receptor characterized by an  $\alpha$ -chain that binds IgG and associated  $\zeta$ - and  $\gamma$ -chains containing cytoplasmic immune receptor tyrosine-based activation motifs (ITAM) required for triggering cell activation (6). Crosslinking of CD16 on NK cells results in the sequential activation of the Lck src kinase and members of Syk family, Syk and ZAP70. Subsequent signaling events include tyrosine phosphorylation and activation of phospholipase C (PLC)<sup>3</sup>  $\gamma$ 1 and PLC $\gamma$ 2, followed by an increase in intracellular Ca<sup>2+</sup> concentration (7,8), PI3K, and then ras activation (9,10). Downstream signaling events include activation of the MAP kinases ERK, p38, and the JNK kinases (11–13). CD16 is the activating NK cell receptor required for triggering antibody-dependent cellular cytotoxicity (ADCC), and it can also mediate IFN- $\gamma$ , TNF- $\alpha$ , and chemokine production (12–14). IFN- $\gamma$  production and ADCC can be enhanced when CD16-activated NK cells are costimulated with either IL-12 or IL-2 (15,16). Finally, IL-21 enhances the efficacy of an antitumor mAb in a murine solid tumor model, and this effect depends on the presence of IFN- $\gamma$  (17).

TGF- $\beta$  is a pleiotropic cytokine with potent immunosuppressive effects in mammals (18). TGF- $\beta$  can suppress NK spontaneous killing and NK cytokine production, as well as the expression of activating NK receptors such as NKp30 and NKG2D (18–22). TGF- $\beta$  has multiple pathways by which it can signal, including MAPK and PP2A, as well as the SMADs, a family of structurally related proteins (23). In general, the binding of an active TGF- $\beta$  molecule to the TGF- $\beta$  receptor induces the phosphorylation of the type I receptor by the type II receptor kinase. The activated type I receptor in turn phosphorylates selected SMAD (i.e., SMAD2 and SMAD3), and these receptor-activated SMAD (R-SMADs) then form a complex with a common SMAD (Co-SMAD) i.e., SMAD4. Activated SMAD complexes translocate to the nucleus where they regulate the transcription of target genes.

It is currently unknown if TGF- $\beta$  has suppressive effects on NK effector functions mediated via CD16 and, if so, what signaling intermediates are used to carry out these functions. In this report we investigated the role of TGF- $\beta$  and its mediator SMAD3 in regulating IFN- $\gamma$  production and ADCC in CD16-activated human NK cells.

## Materials and Methods

### Cell lines and NK cell preparations

The human IL-2-dependent NK cell line NK-92 (gift of Dr. H. Klingemann, Tufts New England Medical Center, Boston, MA) was maintained in culture in RPMI 1640 medium (Invitrogen) supplemented with 20% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine, and 15 ng/ml recombinant human IL-2 (Hoffman-LaRoche). The NK-92 PINCO and NK-92 PINCO-SMAD3 cell lines have been previously generated and characterized in our laboratory (22). The amphotropic-packaging cell line Phoenix (gift of Dr. G. P. Nolan, Stanford University, Stanford, CA) was maintained in culture in DMEM (Invitrogen)/10% FBS medium and grown for 16–18 h to 80% confluence before transfection by calcium phosphate-DNA precipitation (ProFection system from Promega). Human NK cells were

<sup>3</sup>Abbreviations used in this paper: PLC, phospholipase C; ADCC, antibody-dependent cellular cytotoxicity; hIgG, human IgG; wt, wild type.

isolated from peripheral blood leukopacks of healthy individuals (American Red Cross, Columbus, OH) by incubation for 30 min with RosetteSep NK cell Ab mixture (StemCell Technologies), followed by Ficoll-Hypaque density gradient centrifugation. The fresh NK cell preparations were >85% CD56<sup>+</sup>, as determined by direct immunofluorescence using an anti-CD56 PE-conjugated mAb (Immunotech). NK cell preparations containing >98% CD56<sup>+</sup> NK cells were obtained by positive selection using CD56 MicroBeads and MACS separation columns from Miltenyi Biotec. All work with human materials was approved by the Cancer Institutional Review Board of The Ohio State University.

### Retroviral infection of the NK-92 cell line

To generate PINCO-CD16 retroviral vector, the pCMV6-XL4 plasmid containing the CD16A human cDNA clone was obtained from OriGene. PCR amplification of CD16A cDNA was performed by using a 5' primer containing a *Bam*HI restriction site and the first six codons of CD16A gene (5'-CGC GGA TCC ATG GGT GGA GGG GCT GGG-3') and a 3' primer containing an *Eco*RI restriction site followed by the last 22 nucleotides of CD16A cDNA (5'-CCG GAA TTC TCA TTT GTC TTG AGG GTC CTT T-3'). PCR amplification of CD16A cDNA was carried by using Expand High Fidelity PCR system (Roche Applied Science), and the following amplification conditions: 94°C for 5 min, followed by 35 cycles of 94°C (35 s), 55°C (35 s), and 72°C (1 min). The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen) and analyzed by sequencing. After *Bam*HI/*Eco*RI digestion, the sequenced CD16A cDNA was directionally subcloned into the *Bam*HI/*Eco*RI-digested PINCO retroviral vector (24,25). For infection, the NK-92 cell line or NK-92-PINCO and NK-92-PINCO-SMAD3 cell lines were first incubated for 2 or 3 days in the presence of 900 IU/ml IL-2 followed by methods that have been previously published (26). Briefly, infectious supernatant from PINCO and PINCO-CD16 retrovirally transfected Phoenix cells were collected after 48 h and used for three cycles of infections. Upon infection, NK-92 cells were stained with an anti-CD16 allophycocyanin-conjugated mAb (Immunotech) and sorted (FACSVantage, BD Biosciences). CD16 surface expression was confirmed by a biotinylated anti-CD16 Ab and streptavidin-PE conjugate (BD Biosciences), an allophycocyanin-Cy7-conjugated anti-CD16 Ab (BD Biosciences), or a PE-conjugated anti-CD16 Ab (Immunotech) using a FACSCalibur and CellQuest software (BD Biosciences).

### Mice

C57BL/6 wild-type (wt) and T-bet<sup>-/-</sup> female mice were purchased from The Jackson Laboratory. Smad3<sup>-/-</sup> and wt C57BL/6 were female littermates and were a kind gift of Dr. Michael Weinstein (The Ohio State University, Columbus, OH). Mouse NK cells were purified by DX5/NK1.1-positive selection from single-cell suspensions of splenocytes (Miltenyi Biotec). All animal work was approved by The Ohio State University Animal Care and Use Committee, and mice were treated in accordance with the institutional guidelines for animal care.

### Cell stimulation

Before CD16 stimulation, NK-92 cells were cultured in IL-2-free medium containing 10% FBS for 36 h. For experiments using immobilized Abs, wells of flat-bottom plates were coated with PBS-diluted Ab overnight at 4°C. Abs as indicated were: 100 µg/ml human IgG (Sigma-Aldrich), 10 µg/ml 3G8, a mouse anti-human CD16 mAb (kindly provided by the late Dr. Bice Perussia), and 10 µg/ml of rat anti-mouse CD16 mAb (R&D Systems). Plates were then washed with cold PBS, and NK cells (1 × 10<sup>6</sup>/ml) were plated and cultured overnight with cytokines as indicated. Cytokines used were IL-12 (10 ng/ml, kindly provided by Genetics Institute), IL-2 (15 ng/ml), and TGF-β (10 ng/ml, R&D Systems). All cytokines were added to cell cultures simultaneously with commencement of CD16

activation, that is, there was no preincubation with TGF- $\beta$  before CD16 or cytokine activation. Supernatants were analyzed for IFN- $\gamma$  or TNF- $\alpha$  protein by ELISA (see below), and cell pellets were analyzed for IFN- $\gamma$  or *T-BET* transcript by real-time PCR (see below). For signaling experiments, primary human CD56<sup>+</sup> NK cells were incubated in RPMI 1640 medium containing 1% BSA ( $5 \times 10^6$ /ml; 37°C) for 5 or 15 min with or without TGF- $\beta$  (10 ng/ml), 10  $\mu$ g/ml 3G8 anti-CD16 mAb plus 50  $\mu$ g/ml of goat anti-mouse F(ab')<sub>2</sub> (Sigma-Aldrich) and IL-12 (10 ng/ml) or IL-2 (15 ng/ml), as indicated. Where noted, the specific inhibitor of TGF- $\beta$  receptor kinase SB 431542 (Sigma-Aldrich) was added at the indicated concentrations for 40 min at 37°C before stimulation. The inhibitor was present throughout stimulation.

### Western blot analysis

Cells were harvested, washed once with ice-cold PBS, and lysed ( $10^8$  cells/ml RIPA buffer: 0.15 M NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris (pH 8.0), supplemented with protease and phosphatase inhibitors, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 10 mM  $\beta$ -glycerol-phosphate, 1 mM EDTA, and a protease inhibitor cocktail tablet from Roche Applied Science), as described (26). Alternatively, cells were directly lysed in Laemmli buffer ( $2 \times 10^5$  cells/20  $\mu$ l). The preparation of cytoplasmic and nuclear extracts was performed using a kit from Pierce. Western blotting was performed according to previously published protocols (12), and Ab-reactive proteins were detected with HRP-labeled sheep anti-rabbit, mouse, and/or goat Ig sera and ECL (Amersham). Proteins were analyzed in 4–15% SDS-PAGE (Bio-Rad Laboratories) using reducing conditions. Monoclonal and polyclonal Abs used were: polyclonal rabbit sera anti-phospho-ERK<sup>Thr202Tyr204</sup>, anti-phospho-p38<sup>Thr180Tyr182</sup>, anti-phospho-STAT5<sup>Tyr694</sup>, anti-phospho-SMAD3<sup>Ser423/425</sup>, anti-PLC $\gamma$ 2, anti-SyK, anti-Vav, anti-granzyme A, anti-granzyme B, and mAb anti-ERK from Cell Signaling Technology; polyclonal rabbit sera anti-phospho-STAT4<sup>Tyr693</sup> and anti-SMAD3 from Zymed Laboratories; monoclonal anti-GRB2 Ab from Transduction Laboratories; and monoclonal anti-histone H1 and polyclonal rabbit anti-perforin 1 Abs from Santa Cruz Biotechnology.

### ELISA assays

Quantification of human IFN- $\gamma$  was performed as previously described (27) using commercially available mAb pairs (Endogen). Cell-free supernatants were collected after 18 h of incubation at 37°C. For the detection of mouse IFN- $\gamma$ , an ELISA kit from Pierce was used. Quantification of human TNF- $\alpha$  protein was performed using a kit from R&D Systems. Results are shown as the means of triplicate wells  $\pm$  SEM.

### Real-time RT-PCR

Total mRNA was extracted using RNeasy Mini or Micro kits (Qiagen), and cDNA was generated according to the manufacturer's recommendations (Invitrogen). Real-time RT-PCR reactions for IFN- $\gamma$  and human *T-BET* transcripts were performed as described (22). The reactions for human granzyme A (forward primer 5'-TCCTATAGATTTCTGG CATCCTCTC-3', reverse primer 5'-TTCCTCCAATAATTTTTTCACA GACA-3', and probe 5'-FAM-CAGTTGTCGTTTCTCTCCTGCTAATT CCTGAAG-3'TAMRA), human granzyme B (forward primer 5'-TCCT AAGAAGTTCTCCAACGACATC-3', reverse primer 5'-GCACAGCT CTGGTCCGCT-3', and probe 5'-FAM-TGCTACTGCAGCTGGAGA GAAAGGCC-3'TAMRA), and perforin 1 (forward primer 5'-CAGCAC TGACACGGTGGAGT-3', reverse primer 5'-GTCAGGGTGCAGCGGG-3', and probe 5'-FAM-CCGCTTCTACAGTTTCCATGTGGTACACAC TC-3'TAMRA) were performed similarly. cDNA from PHA-activated human lymphocytes served as positive controls for cytokine transcripts, and water (no template) was used as a negative control. Reactions were performed using an ABI Prism 7700 Sequence Detector (TaqMan; PE

Applied Biosystems), and data were analyzed with the Sequence Detector version 1.6 software to establish the PCR cycle at which the fluorescence exceeded a set threshold,  $C_t$ , for each sample. Data were analyzed according to the comparative  $C_t$  method (3), using the internal control 18S transcript levels to normalize differences in sample loading and preparation. Results represent the  $n$ -fold difference of transcript levels in a particular sample compared with calibrator cDNA (cDNA samples of unstimulated primary NK cells or PINCO-infected unstimulated NK-92 cells), which was arbitrarily assigned a value of 1. Results are expressed as the means  $\pm$  SEM of triplicate reaction wells.

### Cytotoxicity assays

Primary CD56<sup>+</sup> human NK cells were treated with TGF- $\beta$  for 24 h or 4 days, as indicated. NK-92 effector cells were cultured in IL-2-free medium containing 10% FBS for 36 h before the assay, and TGF- $\beta$  was added 24 h before the assay. The murine mastocytoma P815 cells coated with an anti-mouse lymphocyte rabbit polyclonal Ab (Accurate Chemical & Scientific) were used as target cells in a 3-h <sup>51</sup>Cr-release ADCC assay. A constant number of target cells (10<sup>4</sup>/well) and serial dilution of effector cells were used in triplicates. Spontaneous release was always <10%.

### Statistics

For data analysis, an unpaired  $t$  test was used if two independent group comparisons were involved. A paired  $t$  test or a simple random effects model was used to take account of the dependency between observations if the data were correlated, that is, the observations are from the same donor cells. The Bonferroni method was used to correct for multiple tests.

## Results

### TGF- $\beta$ inhibits NK cell IFN- $\gamma$ production following CD16 activation

To determine whether TGF- $\beta$  suppresses IFN- $\gamma$  production induced by CD16 activation, primary CD56<sup>+</sup> human NK cells were activated overnight with immobilized human IgG (hIgG) with or without IL-12 or IL-2 and in the presence or absence of TGF- $\beta$ . The presence of TGF- $\beta$  in culture resulted in suppression of NK cell IFN- $\gamma$  production following CD16 activation, without or with the synergy in IFN- $\gamma$  production afforded by IL-12 or IL-2 (Fig. 1, A and B). Inhibition of IFN- $\gamma$  was observed at both transcript (Fig. 1A) and protein level (Fig. 1B), and the percentage inhibition by TGF- $\beta$  was higher for CD16 activation with IL-12 costimulation compared with IL-2. Additionally, TGF- $\beta$  treatment of NK cells also inhibited TNF- $\alpha$  induced by CD16 stimulation without or with IL-12 or IL-2 (Fig. 1C).

TGF- $\beta$  did not decrease surface density expression of CD16 as assessed by flow cytometry (Fig. 2A), regardless of culture in IL-12 or IL-2 (data not shown). To elucidate the molecular mechanism by which TGF- $\beta$  inhibited CD16-mediated IFN- $\gamma$  production, we assessed the phosphorylation/activation of signaling molecules known to be downstream of CD16, IL-12, and TGF- $\beta$ . TGF- $\beta$  treatment of primary human NK cells did not affect early phosphorylation/activation of STAT4, ERK, or p38 kinases following CD16 activation in the presence or absence of IL-12 (Fig. 2B). Identical results were obtained for phosphorylation of STAT5, ERK, or p38 when primary human NK cells were activated by CD16 in the presence of IL-2 (data not shown).

We did observe a strong induction of SMAD3 phosphorylation in primary human NK cells as a consequence of TGF- $\beta$  treatment (Fig. 2B). Because of this, we hypothesized a possible role for SMAD3 in the TGF- $\beta$ -mediated inhibition of CD16-induced IFN- $\gamma$ . To test our hypothesis, we generated an NK-92 cell line expressing CD16 following retroviral infection of the NK-92 cell line with a GFP-expressing PINCO vector containing the CD16A  $\alpha$ -chain



cDNA. After confirming CD16 surface expression in the GFP- and CD16-sorted NK-92 cells (Fig. 3A), we showed that CD16 activation in the presence of IL-12 induced IFN- $\gamma$  production (Fig. 3B), and that the CD16<sup>+</sup> NK-92-CD16 cell line could mediate ADCC against P815 Ab-coated target cells (Fig. 3C).

A NK-92 cell line overexpressing SMAD3 (NK-92-SMAD3) was previously generated in our laboratory (22). We next used retroviral infection to express the CD16A  $\alpha$ -chain in the NK-92-SMAD3 cell line, and sorted for the CD16<sup>+</sup> fraction of cells (Fig. 4A). NK-92-SMAD3-CD16<sup>+</sup> cells and NK-92-CD16<sup>+</sup> mock-infected cells were then activated overnight with immobilized anti-CD16 Ab in the presence or absence of IL-12 or IL-2, and in the presence or absence of TGF- $\beta$ . Ectopic over-expression of SMAD3 inhibited IFN- $\gamma$  production following CD16 activation without or with coactivation by IL-12 or IL-2. The addition of TGF- $\beta$  further enhanced this inhibition of IFN- $\gamma$  production at both transcript and protein levels (Fig. 4B, *left and right panels*, respectively). Similar results were obtained with CD16 activation in the presence of IL-15 or IL-18 (data not shown).

To support these observations in human NK cells, we obtained fresh purified NK cells from sex-matched, sibling pairs of wt and *smad3*<sup>-/-</sup> mice and assessed them for IFN- $\gamma$  production following in vitro coactivation by CD16 and IL-12 in the presence or absence of TGF- $\beta$ . We found that there were higher levels of IFN- $\gamma$  following coactivation via CD16 and IL-12 in the *smad3*<sup>-/-</sup> mice when compared with wt mice. Likewise, the inhibitory effects of TGF- $\beta$  on this process were significantly less, but not absent, in *smad3*<sup>-/-</sup> mice when compared with wt mice (Fig. 4C). The ability of TGF- $\beta$  to modestly suppress IFN- $\gamma$  gene expression in the *smad3*<sup>-/-</sup> NK cells suggests that this inhibitory effect is mediated in a fashion that is partially independent of SMAD3.

We further examined the role of SMAD3 phosphorylation in the inhibition of NK cell IFN- $\gamma$ . We first determined that ectopic expression of SMAD3 in NK-92 cells resulted in a modest degree of SMAD3 phosphorylation within the nucleus (but not in the cytoplasm) even in the absence of exogenous TGF- $\beta$ , which increased dramatically in the presence of TGF- $\beta$  (Fig. 4D, *left panel*). We then used a specific inhibitor of TGF- $\beta$  receptor kinase (SB 431542) and demonstrated that this reagent could inhibit phosphorylation of ectopic SMAD3 in the presence of TGF- $\beta$  (Fig. 4D, *middle panel*) (28). Using the minimal dose of SB 431542 that inhibited SMAD3 phosphorylation, we observed that NK-92 cells had less inhibition of IFN- $\gamma$  secretion when costimulated via CD16 and IL-12. This effect was observed in NK-92 cells that ectopically expressed SMAD3 without and with exogenous TGF- $\beta$  as well as in mock-infected NK-92 cells treated with TGF- $\beta$  (Fig. 4D, *right panel*). Thus, nuclear phosphorylation of SMAD3 is required for the inhibitory effects of TGF- $\beta$  on CD16-activated NK cell production of IFN- $\gamma$ .

T-BET is a master regulator of IFN- $\gamma$  production (29,30). We asked whether human NK cell activation via CD16 induces *T-BET* gene expression and whether TGF- $\beta$ , via SMAD3, can affect this expression. Primary human NK cells were stimulated with immobilized human IgG with or without IL-12 or IL-2, and levels of *T-BET* mRNA were quantified. Interestingly, only CD16 stimulation in combination with IL-12 induced *T-BET* gene expression in primary human NK cells, and this induction was significantly inhibited when NK cells were co-treated with TGF- $\beta$  (Fig. 5A and data not shown). Likewise, CD16<sup>+</sup> NK-92-PINCO-SMAD3 cells coactivated with CD16 and IL-12 had a significantly greater suppression of *T-BET* expression when cultured with TGF- $\beta$ , compared with CD16<sup>+</sup> NK-92-PINCO cells (Fig. 5B). As confirmation, we found that NK cells purified from *tbet*<sup>-/-</sup> mice produced significantly less IFN- $\gamma$  following coactivation via CD16 and IL-12 when compared with wt mice both at the protein (Fig. 5C) and mRNA levels (data not shown). This modest production of IFN- $\gamma$  in CD16-activated *tbet*<sup>-/-</sup> NK cells was further inhibited

in the presence of TGF- $\beta$ , suggesting that this cytokine's suppressive effect is mediated via multiple pathways.

### Effect of TGF- $\beta$ and SMAD3 on ADCC in NK cells

An important function of CD16<sup>+</sup> NK cells is presumably to mediate ADCC (4,31), so we assessed the effects of TGF- $\beta$  on this process. In contrast to CD16-mediated IFN- $\gamma$  production, ADCC was not inhibited by overnight incubation of purified primary human NK cells with TGF- $\beta$  (Fig. 6A, *left*) or with TGF- $\beta$ , IL-12, or IL-2 (data not shown). However, a 4-day treatment of primary human NK cells with TGF- $\beta$  in the presence of IL-2 (to maintain NK cell survival) inhibited both ADCC (Fig. 6A, *right*) and CD16-mediated IFN- $\gamma$  production (data not shown). No diminution of CD16 surface density expression was observed on human NK cells after the 4-day culture (data not shown).

Finally, to determine whether SMAD3 had a role in mediating the effect of TGF- $\beta$  on human NK cell ADCC, CD16<sup>+</sup> NK-92-SMAD3 cells were used as effectors in an ADCC assay and compared with CD16<sup>+</sup> NK-92 PINCO cells. Ectopic SMAD3 expression in CD16<sup>+</sup> NK-92 cells inhibited ADCC, which was further accentuated in the presence of TGF- $\beta$  for 24 h (Fig. 6B).

To elucidate the molecular mechanism(s) by which TGF- $\beta$  inhibited ADCC, we assessed the expression of signaling molecules involved in ADCC and lytic granule protein levels. Treatment of primary NK cells with TGF- $\beta$  for 4 days in presence of IL-2 did not affect the protein expression of PLC $\gamma$ 2, Syk, Vav, and ERK signaling molecules (Fig. 7A) or the protein expression of perforin 1 (Fig. 7B); however, we observed a dramatic inhibition of granzyme A and granzyme B protein expression (Fig. 7B). Utilizing the same cell samples, we also noted a strong inhibition of granzyme A and granzyme B mRNA levels, and a less but significant inhibition of perforin 1 mRNA following TGF- $\beta$  treatment (Fig. 5 C). Likewise, CD16<sup>+</sup> NK-92-SMAD3 cells had less granzyme A, granzyme B, and perforin 1 mRNA compared with CD16<sup>+</sup> NK-92 PINCO cells following culture with TGF- $\beta$  (Fig. 5 D).

## Discussion

TGF- $\beta$  is a powerful physiological immune suppressant in mammals (18), and several studies have documented this effect on NK cells (19,22,32). In the current study we provide what we think to be several additional novel observations that define TGF- $\beta$  as a negative regulator of Fc $\gamma$  receptor-mediated immune activation in human NK cells. First, we show that TGF- $\beta$  suppresses primary human NK cell IFN- $\gamma$  production that is normally induced following activation by CD16 without or with IL-12 or IL-2. Additionally, we note that while a comparable overnight incubation with TGF- $\beta$  does not affect NK ADCC, prolonged (4-day) incubation with TGF- $\beta$  does inhibit ADCC, and it does so by suppressing granzyme A and granzyme B protein and mRNA expression. Finally, by performing gain-of-function experiments using a human NK cell line engineered to express CD16 with overexpression of SMAD3, as well as loss-of-function experiments using *smad3*<sup>-/-</sup> mice, we have identified SMAD3 as one important transcription factor mediating TGF- $\beta$ 's inhibitory effects on IFN- $\gamma$  production and ADCC following CD16 activation of NK cells. As noted below, we think that these basic discoveries have significant clinical ramifications for the treatment of malignancies such as lymphoma, leukemia, and several epithelial malignancies where therapeutic Abs may rely on ADCC for part of their beneficial action to eliminate tumor cells.

The role of TGF- $\beta$  as an inhibitor of IFN- $\gamma$  production in NK cells has been reported by several groups, including our own (21,22). Yu et al. demonstrated that pro- and

antiinflammatory cytokine signaling reciprocally antagonize each other's effects on human NK cells, possibly in an effort to prevail or to temper the regulation of NK cell IFN- $\gamma$  production (22). In particular, TGF- $\beta$  utilizes SMAD3 to suppress IFN- $\gamma$  directly as well as indirectly via suppression of T-BET, a positive regulator of IFN- $\gamma$ . In contrast, proinflammatory monokines antagonize TGF- $\beta$  signaling by down-modulating the expression of TGF- $\beta$  type II receptors, SMAD2 and SMAD3 (22). Given the role of TGF- $\beta$  and SMAD3 in tempering CD16-induced activation of NK cells elucidated in this report, it is likely, if not certain, that proinflammatory monokines enhance NK cell ADCC and CD16-mediated IFN- $\gamma$  production at least in part by targeting these same mediators of TGF- $\beta$  signaling.

Our results also suggest that the inhibitory effect of TGF- $\beta$  on NK cell IFN- $\gamma$  production following activation via CD16 and IL-12 or IL-2 does not result from an inhibition of early activators such as ERK, p38 kinase, or STAT4/5, which is consistent with some reports but not others (33,34). Indeed, the data presented herein strongly suggest that the inhibition of CD16-mediated IFN- $\gamma$  production mainly depends on the effects of SMAD3, a transcription factor that is activated via the TGF- $\beta$  pathway. SMAD3 directly binds to and represses the proximal promoter of IFN- $\gamma$  and also inhibits the expression of its positive regulator, T-BET (22). However, our experiments that utilized *smad3*<sup>-/-</sup> to assess TGF- $\beta$ -mediated suppression of IFN- $\gamma$  induced via CD16 suggest that a minor component of this suppression occurs independent of SMAD3. The search for these additional mediators is ongoing.

Using the specific inhibitor of TGF- $\beta$ -type I receptor kinase, SB 431542, we also show that phosphorylation of SMAD3 is at least in part responsible for the inhibition of IFN- $\gamma$  induced by TGF- $\beta$  in NK cells costimulated by CD16 and IL-12. As expected, treatment of NK cells with TGF- $\beta$  resulted in SMAD3 phosphorylation and translocation from the cytoplasm to the nuclear compartment. Additionally, overexpression of SMAD3 in NK-92 cells resulted in an unexpected modest level of phosphorylation within the nuclear compartment even in the absence of TGF- $\beta$ , likely the result of endogenous NK cell production of TGF- $\beta$  (35). This autophosphorylation of SMAD3 likely in turn led to the modest inhibition of IFN- $\gamma$  secretion seen in NK cells costimulated by CD16 and IL-12 in absence of TGF- $\beta$ .

We also report that T-BET expression is selectively induced in NK cells following coactivation via CD16 and IL-12, but not via CD16 and IL-2, thus likely accounting for the consistently enhanced IFN- $\gamma$  gene expression under the former costimulators compared with the latter. Likewise, the ability of SMAD3 to suppress induction of T-BET no doubt contributes to TGF- $\beta$ 's greater degree of IFN- $\gamma$  suppression following NK cell coactivation via CD16 and IL-12, compared with CD16 and IL-2. Additionally, NK-92 cells overexpressing SMAD3 cells were observed to produce less IFN- $\gamma$  following costimulation via CD16 and IL-12, but they did not express less T-BET mRNA. These data support the notion that SMAD3 can inhibit IFN- $\gamma$  in a manner that is independent of T-BET, as we have previously reported (22). This also suggests that a quantitatively larger amount of SMAD3 phosphorylation is required to inhibit T-BET expression. In fact, SB 431542 was only able to significantly increase T-BET mRNA expression in NK-92-SMAD3 cells that were costimulated via CD16 and IL-12 and also incubated in TGF- $\beta$  (data not shown).

Our data and previously published data (20) show that TGF- $\beta$  does not exert its immune suppressive effects via the down-modulation of CD16 surface expression on human NK cells. Other activating NK receptors such as NKp30 and NKG2D are down-modulated by TGF- $\beta$  (20), and TGF- $\beta$  down-modulates the CD16-associated  $\gamma$ -chain, which consequently results in lower surface density expression of CD16 in monocytes (36).



A relatively short-term treatment of primary NK cells with TGF- $\beta$  does not modulate ADCC, which is consistent with results reported for natural cytotoxicity (19). However, we did see a relatively profound reduction in ADCC following a prolonged incubation of primary human NK cells with TGF- $\beta$ . Based on previously published work with NK cells (37), it would be reasonable to speculate that one component likely contributing to this reduction in NK cell ADCC is a TGF- $\beta$ 1-mediated diminution in proteins responsible for cytotoxicity, such as perforin and granzyme A. TGF- $\beta$  is also known to suppress these same molecules in CD8 CTLs (38,39). Indeed, we observed a significant inhibition of granzyme A and granzyme B mRNA and protein expression in primary human NK cells treated with TGF- $\beta$ , and this effect was enhanced in NK-92 cells by overexpression of SMAD3. We also observed an inhibition of perforin 1 mRNA, but this was not seen at the protein level. This could possibly be explained by a relatively high degree of perforin 1 protein stability. Lee et al. have reported that TGF- $\beta$  does not affect perforin 1 protein levels in cultured primary human NK cells (40). Thus, it appears that TGF- $\beta$  inhibits human NK cell ADCC at least in part by suppressing granzyme A and granzyme B protein expression, and it does so via SMAD3.

The suppression of ADCC observed following prolonged exposure of IL-2-stimulated primary human NK cells to TGF- $\beta$  is not without clinical relevance. Several clinical studies that have assessed the antitumor efficacy of the anti-CD20 mAb rituximab for the treatment of low-grade lymphoma strongly suggest that polymorphisms that enhance CD16 engagement of the IgG1 Fc-binding domain are important for the mediation of ADCC in vivo (31,41–43). Given this, and the ability of proinflammatory cytokines to enhance ADCC in vivo, a phase II study of rituximab infusion with concomitant administration of intermediate-dose IL-2 to activate CD16<sup>+</sup> NK cells was undertaken in an effort to determine whether enhanced ADCC could improve clinical outcome. All patients had previously failed therapy with rituximab alone. Despite in vivo expansion of NK cells and achievement of IL-2 concentrations that activate NK cells, no clinical benefit was observed (44). One potential explanation for this finding was the observation that there was a concomitant expansion of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in this study, as has been noted in other studies using low-intermediate doses of IL-2 (44–46). Regulatory T cells are known to constitutively express TGF- $\beta$  (47), so it is conceivable, if not likely, that the chronic exposure of IL-2-activated NK cells to TGF- $\beta$  via the regulatory T cell expansion contributed at least in part to an in vivo suppression of NK ADCC. From these data one might surmise that neutralization of TGF- $\beta$  (48,49) or elimination of regulatory T cell expansion (50,51) would result in enhanced efficacy of activated NK cell ADCC over that of rituximab Ab therapy alone in this patient population. Another alternative might be to activate NK ADCC with a cytokine that does not appear to expand regulatory T cells (52). A clinical trial testing this approach is currently underway with IL-21.

## Acknowledgments

R.T. dedicates this manuscript to the memory of Dr. Bice Perussia (1950–2006) for her mentorship and for her substantial contributions to the understanding of Fc $\gamma$ RIIIA biology in human NK cells. We thank Don M. Benson for helpful discussion.

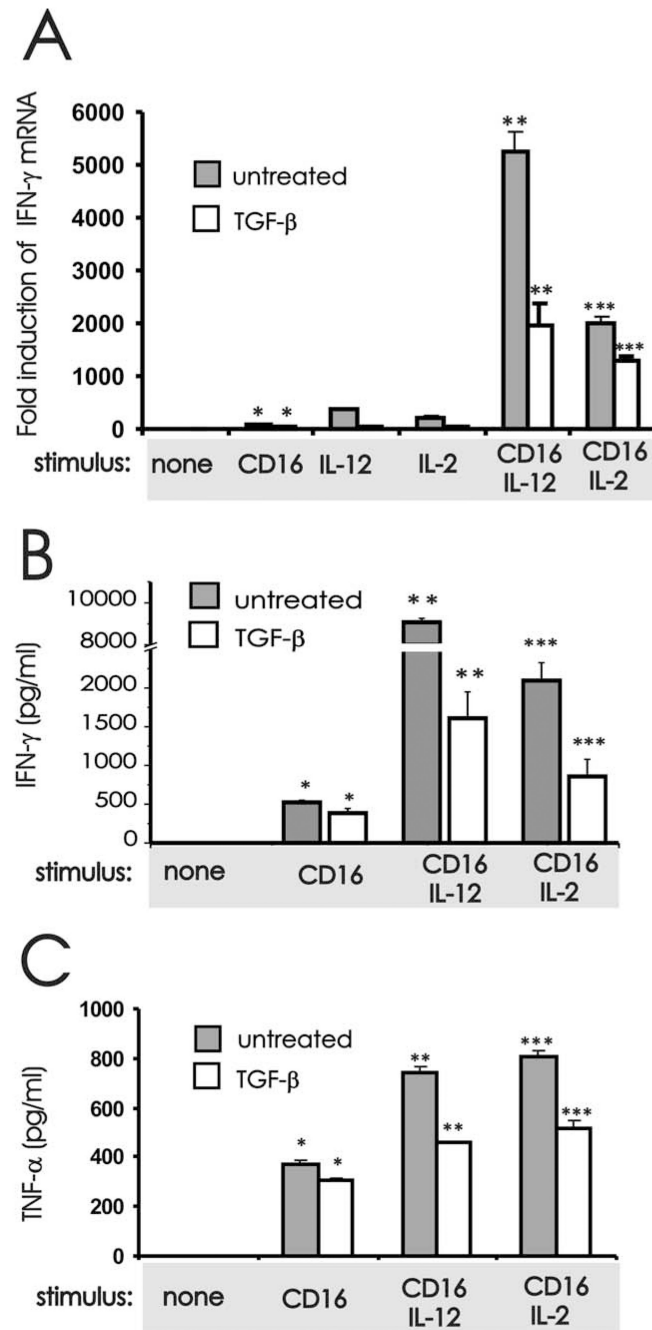
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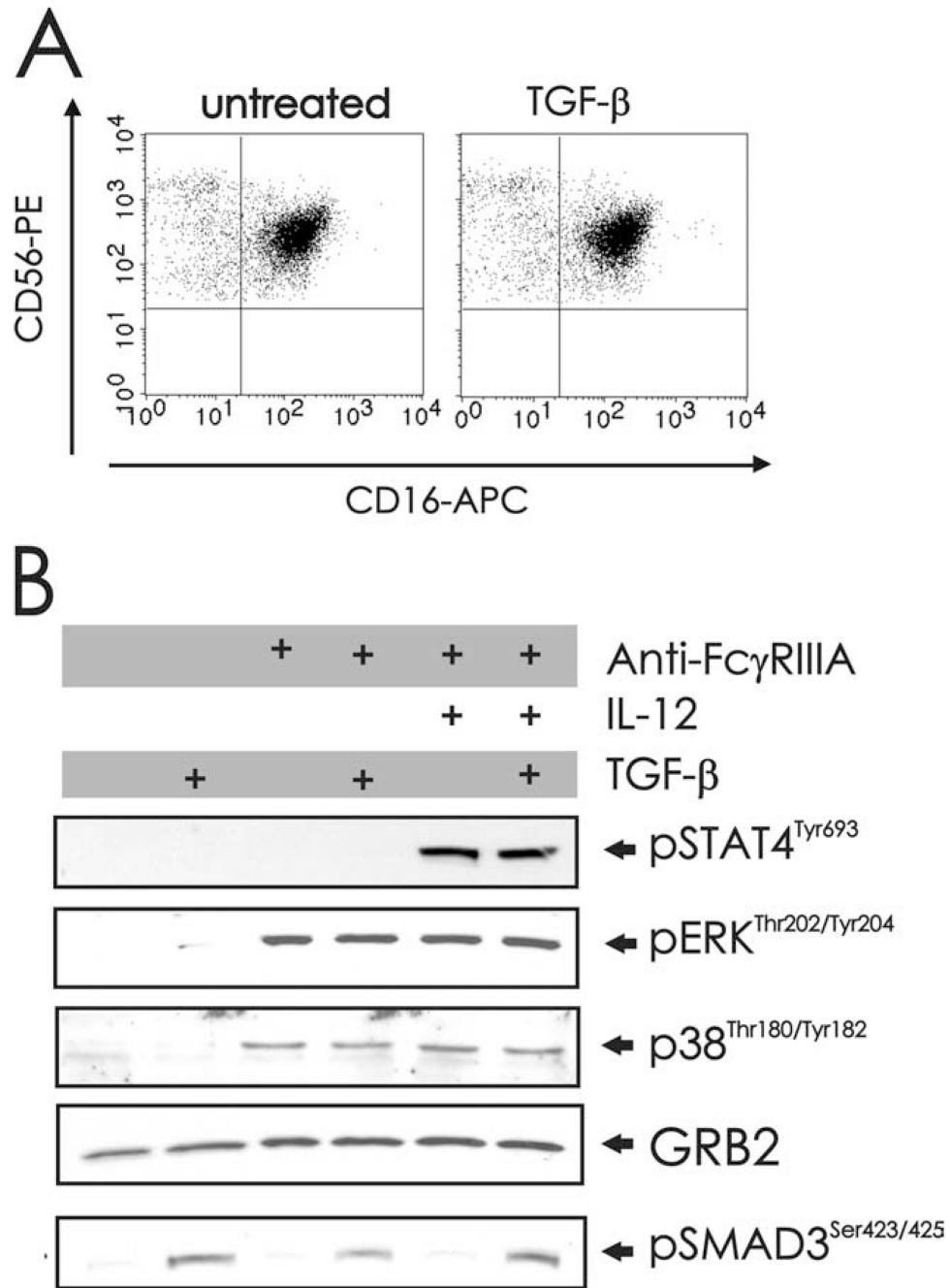
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**FIGURE 1.**

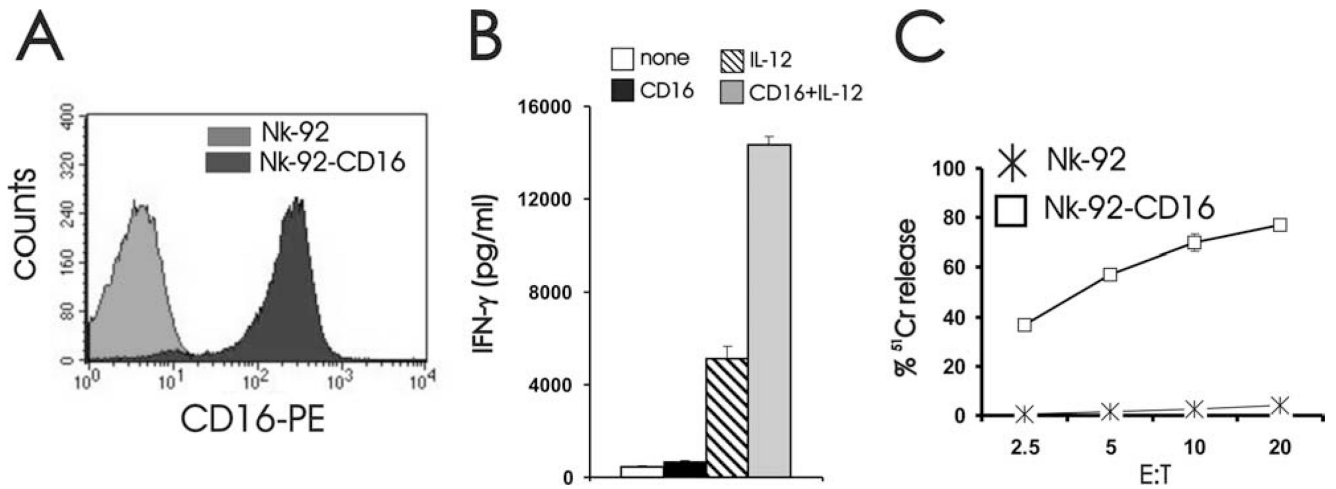
TGF- $\beta$  inhibits NK cell IFN- $\gamma$  and TNF- $\alpha$  production following CD16 activation. Primary human (CD56<sup>+</sup>) NK cells were simultaneously untreated or treated with TGF- $\beta$  (10 ng/ml) and stimulated with immobilized hIgG and/or IL-12 (10 ng/ml) or IL-2 (15 ng/ml) overnight, after which cell pellets were collected and analyzed for (A) IFN- $\gamma$  transcript by real-time RT-PCR (\*, \*\*, and \*\*\*,  $p < 0.001$  for all conditions), for (B) IFN- $\gamma$  protein by ELISA (\*, \*\*, and \*\*\*,  $p < 0.001$  for all conditions), and for (C) TNF- $\alpha$  protein by ELISA (\*, \*\*, and \*\*\*,  $p \leq 0.02$  for all conditions). Results are representative of no less than four such experiments with similar results.



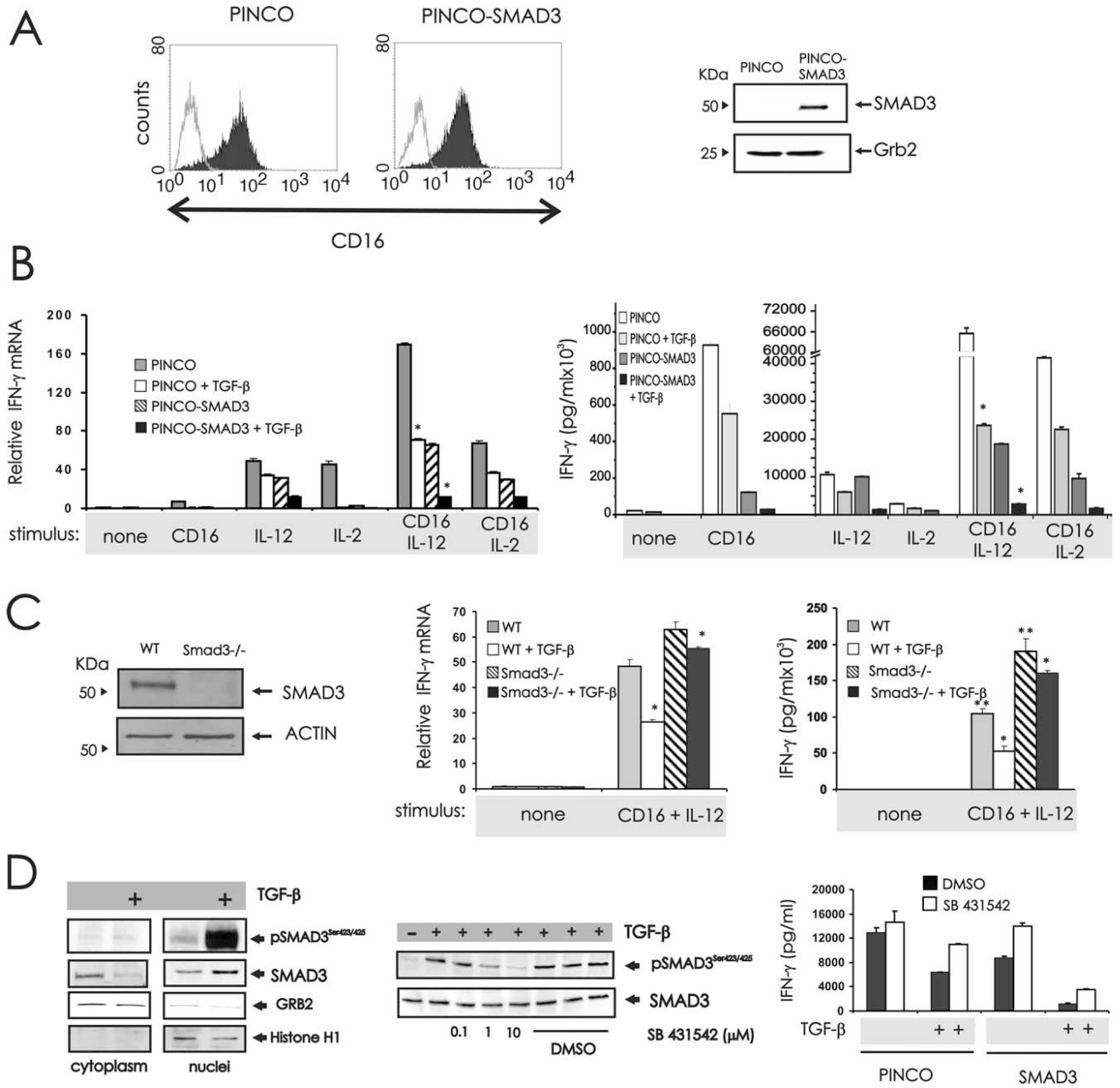
**FIGURE 2.**

Effect of TGF- $\beta$  on expression of CD16 and selective signaling intermediates in primary human NK cells. **A**, Primary human CD56<sup>+</sup> NK cells were either untreated or treated overnight with TGF- $\beta$  and then analyzed for CD16 surface expression by FACS analysis. The histogram shown is representative of seven separate experiments with virtually identical results. **B**, CD56<sup>+</sup> NK cells were untreated or treated with TGF- $\beta$  (10 ng/ml) and simultaneously stimulated with a mouse anti-human CD16 Ab (10  $\mu$ g/ml) plus an anti-mouse F(ab')<sub>2</sub> fragment of Ab (50  $\mu$ g/ml) and/or IL-12 (10 ng/ml) for 15 min. Western blotting analysis was performed on total lysates showing no appreciable quantitative change in phospho-STAT4<sup>Tyr693</sup>, phospho-ERK<sup>Thr202/Tyr204</sup>, or phospho-p38<sup>Thr180/Tyr182</sup> despite

the presence or absence of TGF- $\beta$ . The presence of the phospho-SMAD3<sup>Ser423/425</sup> band in lanes 2, 4, and 6 confirms activation of primary NK cells by TGF- $\beta$ . GRB2 was assessed as a housekeeping gene product to ensure equal loading. This experiment was performed three times with virtually identical results. Similar experiments were performed substituting IL-2 (15 ng/ml) for IL-12 and measuring STAT5, ERK, and p38, with similar results as noted in the text.

**FIGURE 3.**

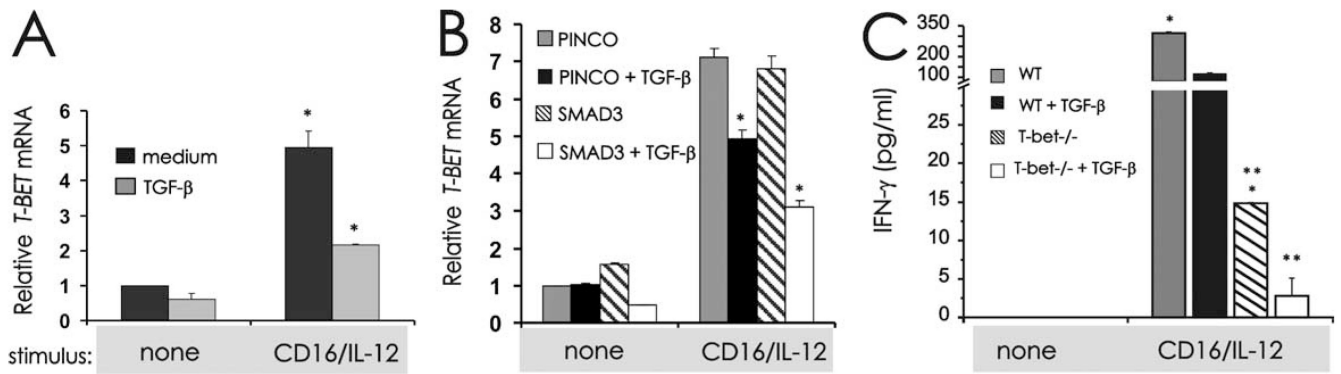
Generation and characterization of a CD16<sup>+</sup> NK-92 cell line. NK-92 cells were infected with PINCO-GFP-CD16A  $\alpha$ -chain virus and were sorted for coexpression of GFP and surface expression of CD16. Sorted NK-92-CD16 cells were (A) analyzed for the expression of CD16 by flow cytometry and were functionally assessed for their ability to (B) produce IFN- $\gamma$  and (C) mediate ADCC.

**FIGURE 4.**

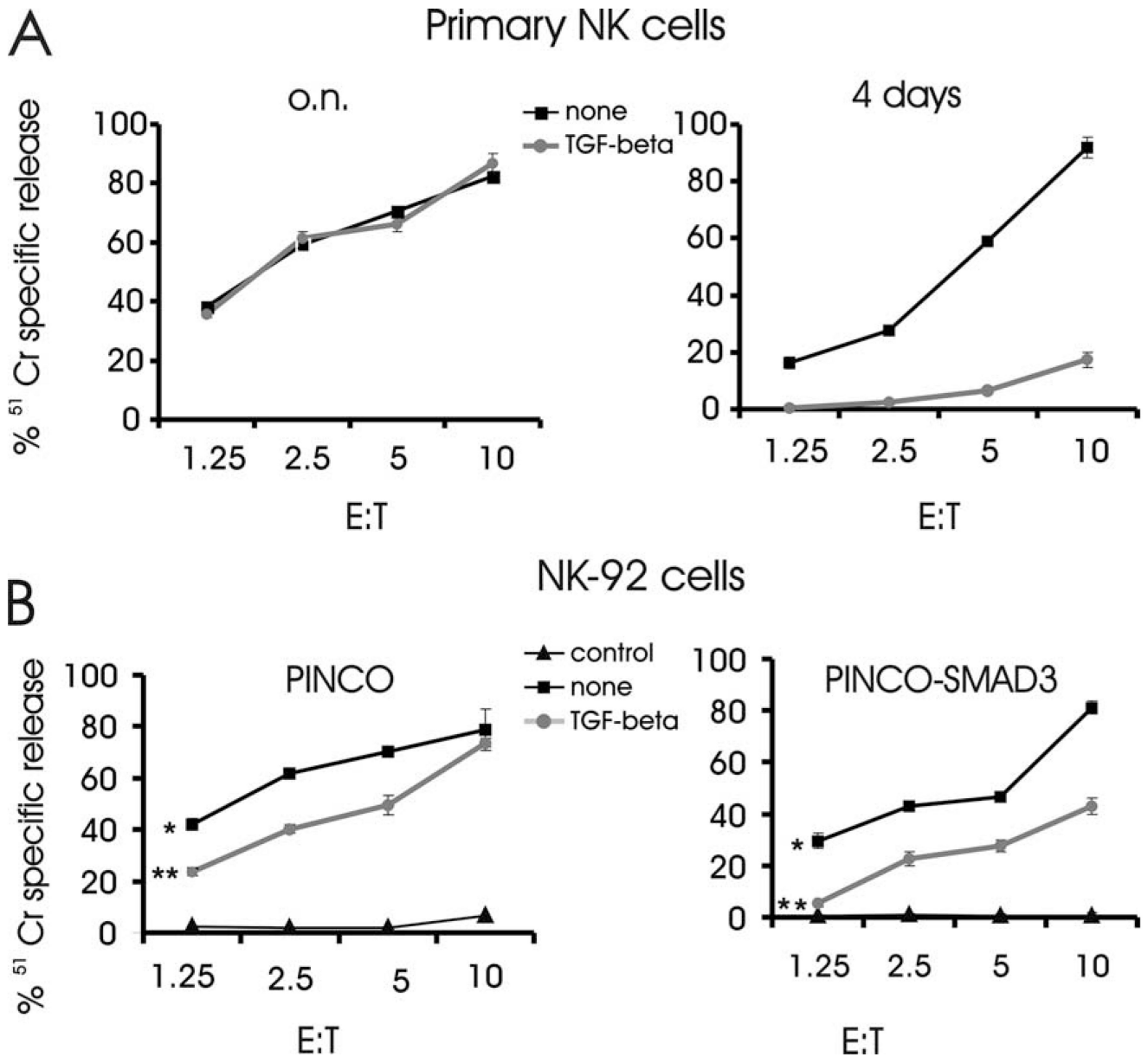
CD16-mediated NK cell IFN- $\gamma$  production is inhibited by a TGF- $\beta$  pathway that involves SMAD3. **A**, NK-92 cells retrovirally infected with PINCO (NK-92 PINCO) or PINCO-SMAD3 (NK-92 PINCO-SMAD3) were infected with a PINCO-CD16A  $\alpha$ -chain virus and then sorted for expression of CD16. **B**, CD16<sup>+</sup> NK-92 PINCO and CD16<sup>+</sup> NK-92 PINCO-SMAD3 cells were next untreated or treated with TGF- $\beta$  (10 ng/ml) and simultaneously stimulated with immobilized anti-CD16 Ab and/or IL-12 (10 ng/ml) or IL-2 (15 ng/ml) overnight. **B, left**, Cell pellets were then analyzed by real-time RT-PCR for IFN- $\gamma$  transcript (\*,  $p < 0.03$ ,  $n = 3$ ). **B, right**, Cell supernatants were analyzed for the absolute amount of IFN- $\gamma$  protein by ELISA (\*,  $p < 0.001$ ,  $n = 3$ ). **C, left**, NK cells from wt and smad3<sup>-/-</sup> mice were analyzed for SMAD3 expression by Western blot. Cells were then costimulated in vitro for

18 h using immobilized anti-mouse CD16 mAb and rmIL-12 (50 ng/ml) in presence or absence of TGF- $\beta$  (10 ng/ml) and analyzed for (*middle*) relative expression of IFN- $\gamma$  by real-time RT-PCR (\*,  $p < 0.05$ ,  $n = 3$ ) and for (*right*) absolute amount of IFN- $\gamma$  protein by ELISA (\*,  $p < 0.02$ ,  $n = 3$ ; \*\*,  $p < 0.05$ ,  $n = 3$ ). *D, left*, CD16<sup>+</sup> NK-92 PINCO-SMAD3 cells were untreated or treated with TGF- $\beta$  for 30 min. Western blotting analysis was performed on cytoplasmic and nuclear extracts using an anti-phospho-SMAD3<sup>Ser423/425</sup> and anti-SMAD3 Abs. Anti-GRB2 and anti-histone H1 Abs were used to assess for the purity of the cytoplasmic and nuclear extracts, respectively. *Middle*, CD16<sup>+</sup> NK-92 PINCO-SMAD3 cells were pretreated with the indicated dose of SB 431542 or vehicle DMSO for 40 min and then stimulated for 40 min with TGF- $\beta$ . Western blotting analysis was performed on total cellular extracts using an anti-phospho-SMAD3<sup>Ser423/425</sup> and anti-SMAD3 Abs. *Right*, CD16<sup>+</sup> NK-92 PINCO and PINCO-SMAD3 cells were pretreated with 1  $\mu$ M SB 431542 or DMSO for 40 min and then costimulated overnight with immobilized anti-CD16 Ab and IL-12 in presence or absence of TGF- $\beta$ . Cell supernatants were collected and analyzed for IFN- $\gamma$  by ELISA.

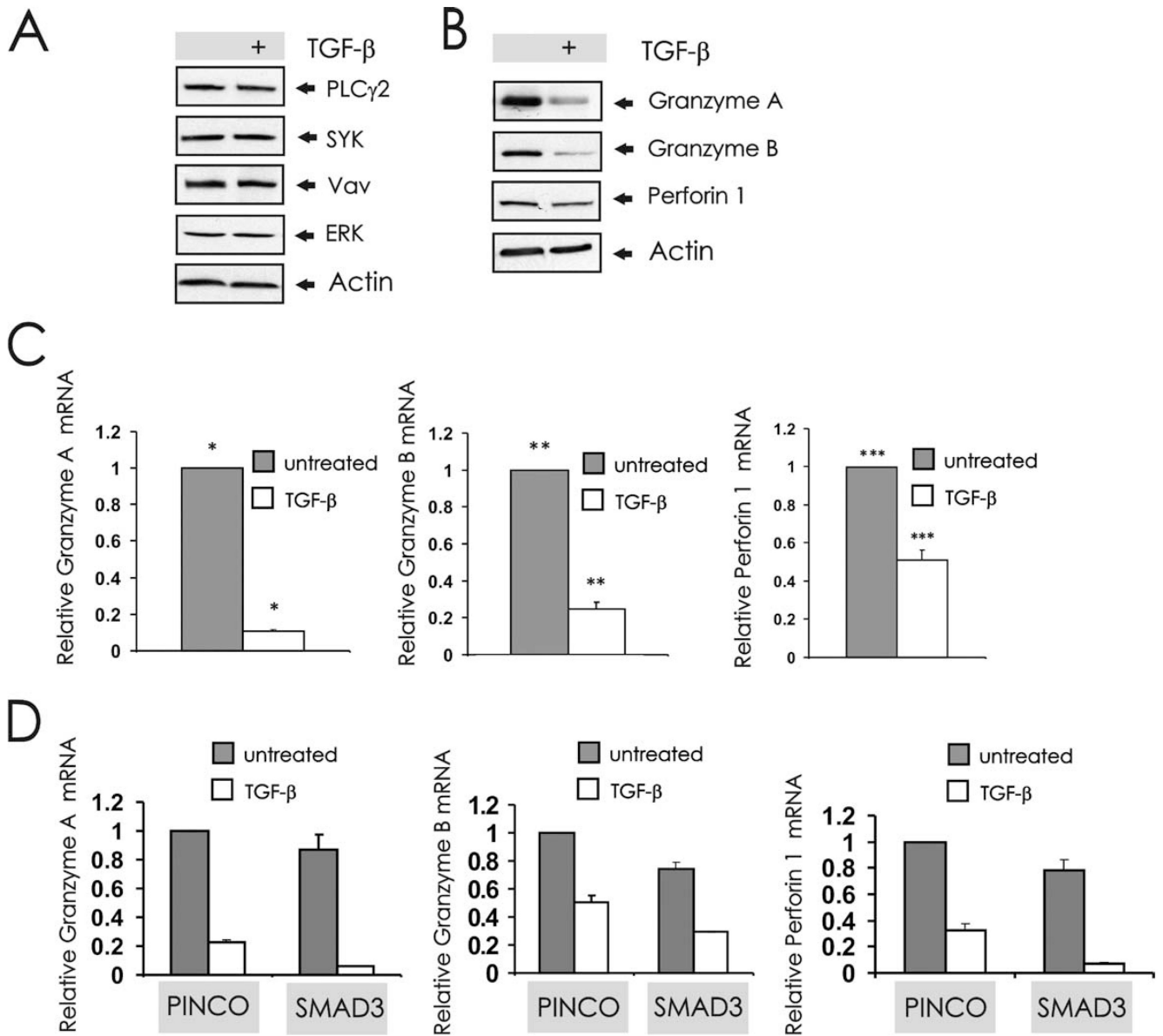


**FIGURE 5.**

TGF- $\beta$  targets *T-BET* for its suppression of IFN- $\gamma$  gene expression following NK costimulation by CD16 and IL-12. **A**, Primary human NK cells were activated with immobilized human IgG and IL-12 (10 ng/ml) overnight in the absence or presence of TGF- $\beta$  (10 ng/ml) and quantified for *T-BET* transcript (\*,  $p = 0.007$ ,  $n = 3$ ). **B**, CD16<sup>+</sup> NK-92 PINCO and CD16<sup>+</sup> NK-92 PINCO-SMAD3 cells were either untreated or treated with TGF- $\beta$  (10 ng/ml) and activated overnight with immobilized anti-CD16 Ab in presence of IL-12. Cell pellets were quantified for *T-BET* transcript (\*,  $p < 0.03$ ,  $n = 3$ ). **C**, NK cells from *tbet*<sup>-/-</sup> and wt mice were costimulated in vitro for 18 h using immobilized anti-mouse CD16 mAb and rmIL-12 (50 ng/ml). Supernatants were then quantified for IFN- $\gamma$  by ELISA (\*,  $p < 0.001$ ,  $n = 5$ ; \*\*,  $p < 0.002$ ,  $n = 3$ ).

**FIGURE 6.**

Effect of TGF- $\beta$  and SMAD3 on ADCC. **A**, Primary CD56<sup>+</sup> NK cells were incubated overnight (o.n.) or for 4 days with TGF- $\beta$  and then assayed for ADCC against <sup>51</sup>Cr-labeled P815 Abcoated target cells. **B**, NK-92 PINCO and PINCO-SMAD3 cells expressing CD16 were cultured in IL-2-free medium for 18 h and then left untreated or treated with TGF- $\beta$  for 24 h in absence of IL-2 and used as effectors for NK cell ADCC assays (\*,  $p < 0.01$ ,  $n = 5$ ; \*\*,  $p < 0.01$ ,  $n = 5$ ). Cytotoxicity against P815 Ab-coated target cells was tested in a 3-h <sup>51</sup>Cr-release assay. Each experiment is representative of at least three performed with similar results.

**FIGURE 7.**

Assessment of signaling and cytotoxic granules molecules involved in NK cell ADCC. Primary CD56<sup>+</sup> NK cells were untreated or treated for 4 days with TGF-β in presence of IL-2, after which cell pellets were collected and analyzed for (A) PLCγ2, SYK, Vav, and ERK proteins or (B) granzyme A, granzyme B, and perforin 1 proteins by Western blot. C, Granzyme A, granzyme B, and perforin 1 transcripts isolated from cells used in B were quantified by real-time RT-PCR (\*, \*\*, and \*\*\*,  $p < 0.001$  for all conditions,  $n = 4$ ). D, CD16<sup>+</sup> NK-92 PINCO and PINCO-SMAD3 cells were cultured in IL-2-free medium for 18 h and then left untreated or treated with TGF-β for 24 h and analyzed for granzyme A, granzyme B, and perforin 1 transcripts by real-time RT-PCR.