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Interferon β and Anti-PD-1/PD-L1 Checkpoint Blockade Cooperate in NK Cell-Mediated Killing of Nasopharyngeal Carcinoma Cells

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Abstract

Nasopharyngeal carcinoma (NPC) is a highly malignant epithelial cancer linked to EBV infection. Addition of interferon-β (IFNβ) to chemo- and radiochemotherapy has led to survival rates >90% in children and adolescents. As NPC cells are sensitive to apoptosis via tumor necrosis factor-related apoptosis inducing ligand (TRAIL), we explored the role of TRAIL and IFNβ in the killing of NPC cells by natural killer (NK) cells. NPC cells, including cells of a patient-derived xenograft were exposed to NK cells in the presence or absence of IFNβ. NK cells killed NPC- but not nasoepithelial cells and killing was predominately mediated via TRAIL. Incubation of NK cells with IFNβ increased cytotoxicity against NPC cells. Concomitant incubation of NK- and NPC cells with IFNβ before coculture reduced cytotoxicity and could be overcome by blocking the PD-1/PD-L1 axis leading to the release of intracellular TRAIL from NK cells. In conclusion, combination of IFNβ and anti-PD-1, augmenting cytotoxicity of NK cells against NPC cells, could be a strategy to improve NPC-directed therapy and warrants further evaluation *in vivo*.

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Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor arising from the surface epithelium of the posterior nasopharynx. Infection by Epstein–Barr-Virus (EBV), ethnic background and environmental carcinogens play a major role in its pathogenesis [1,2]. Survival rates in patients with localized disease range between 80% and 90%, and are usually less than 20% in patients with metastatic disease or relapse [3]. State of the art treatment in adults consists of radiochemotherapy, usually with doses around 70 Gy to the primary tumor [4]. In children and adolescents radiochemotherapy is usually preceded by several blocks of cisplatin-containing chemotherapy allowing lower dosing of radiotherapy [5–8]. In the studies NPC-93 and -2001 of the German Society of Pediatric Oncology and Hematology (GPOH) overall and event-free survival rates >90% have been achieved, applying radiation doses less than 60 Gy [6,7]. Both protocols are unique by the fact that neoadjuvant chemotherapy and radiochemotherapy were followed by a 6 months lasting maintenance period with interferon beta (IFNβ). The introduction of IFNβ in these protocols was based on a previous response rate of 26% in patients refractory to conventional therapy [9,10]. Type 1 interferons, such as IFNα and β have been reported to display direct and indirect anti-tumor activities [11,12]. Recently, we were able to demonstrate that IFNβ, at concentrations achievable in patients, induces apoptosis in NPC cells, including cells of a patient-derived xenograft [13]. IFNβ induced apoptosis by expressing the death ligand TRAIL on the surface of NPC cells and the subsequent induction of apoptosis via an intact TRAIL-receptor signaling pathway. TRAIL is also expressed on lymphocytic effector cells such as T lymphocytes and NK cells, and NPC tumors have been shown to be sensitive to cytotoxicity of EBV-specific T lymphocytes [14,15]. In addition, IFNβ has been shown to

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increase the expression of TRAIL on lymphocytic effector cells [16,17]. On the other hand, it was recently demonstrated, that IFNβ may upregulate expression of the negative checkpoint regulator PD-L1 on tumor cells, thereby possibly downregulating an anti-tumor response [11,12]. Inhibition of the PD-1/PD-L1 interaction has been demonstrated to be of clinical benefit in the treatment of various tumors, such as Hodgkin lymphoma and malignant melanoma [18,19]. Both tumors like NPC are characterized by marked lymphocytic infiltration and expression of PD-L1. In this paper, we have analyzed the killing of NPC cells by NK cells and its possible modulation by IFNβ and PD-1/PD-L1 checkpoint-inhibition.

Materials and Methods

Cell Lines and Culture

Two NPC cell lines and one nasopharyngeal epithelial cell line as a control were used in this study. The EBV-negative cell line CNE-2 was kindly provided by Prof. Pierre Busson (Gustave Roussy Institute, Paris, France) [20]. The EBV-positive cell line C666–1 was a gift from Prof. Fei-Fei Liu, University of Toronto, Canada [21]. The SV40T-antigen immortalized nasopharyngeal epithelial cell line NP69 [22] was obtained from Prof. George Tsao (The Chinese University of Hong Kong, Hong Kong, China). All cell lines were cultured as described before [13]. The authenticity of NPC cell lines (C666–1, CNE-2) and the immortalized nasopharyngeal epithelial cell line (NP69) was investigated by DNA fingerprinting analysis using the AmpF/STR Identifiler PCR Amplification Kit (Applied Biosystem, Foster City, CA, USA) and detected on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. Data were analyzed and allele(s) of each locus were determined by GeneScan and Gene-Mapper TM ID Software (Applied Biosystems). STR profiles of NPC cell lines and the nasopharyngeal epithelial cell line NP69 are as previously described [13].

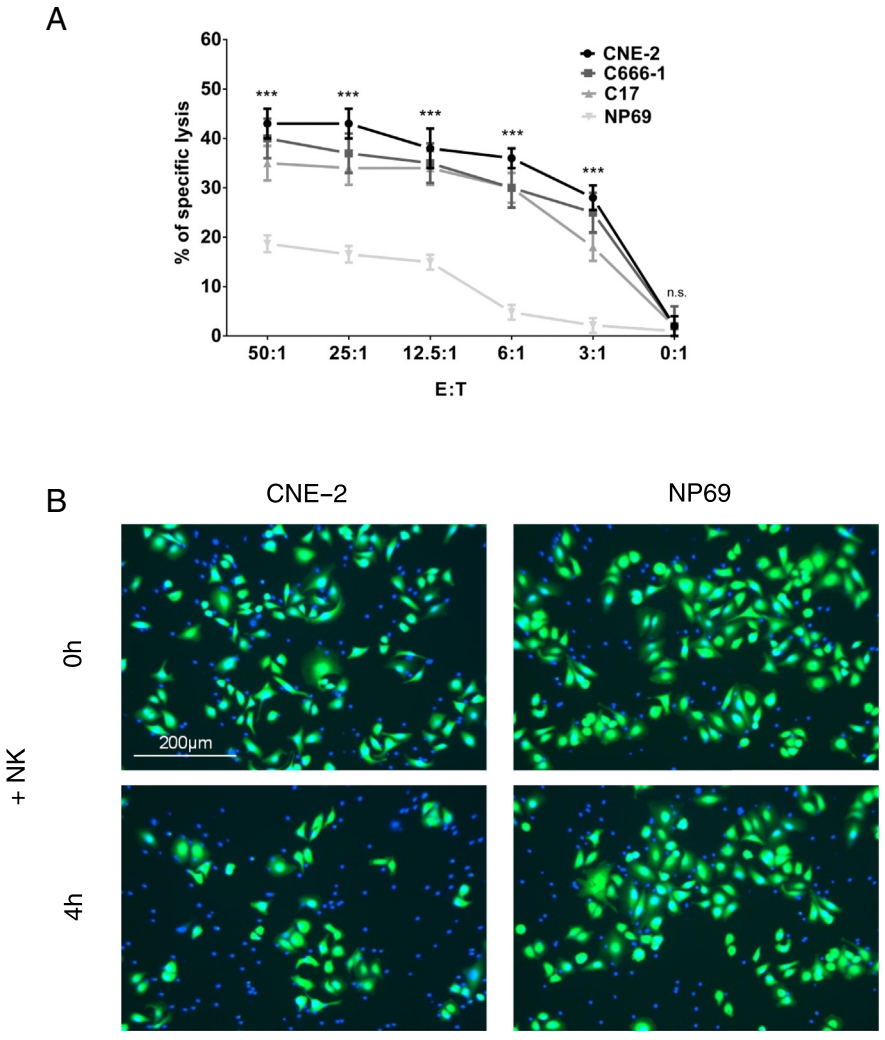
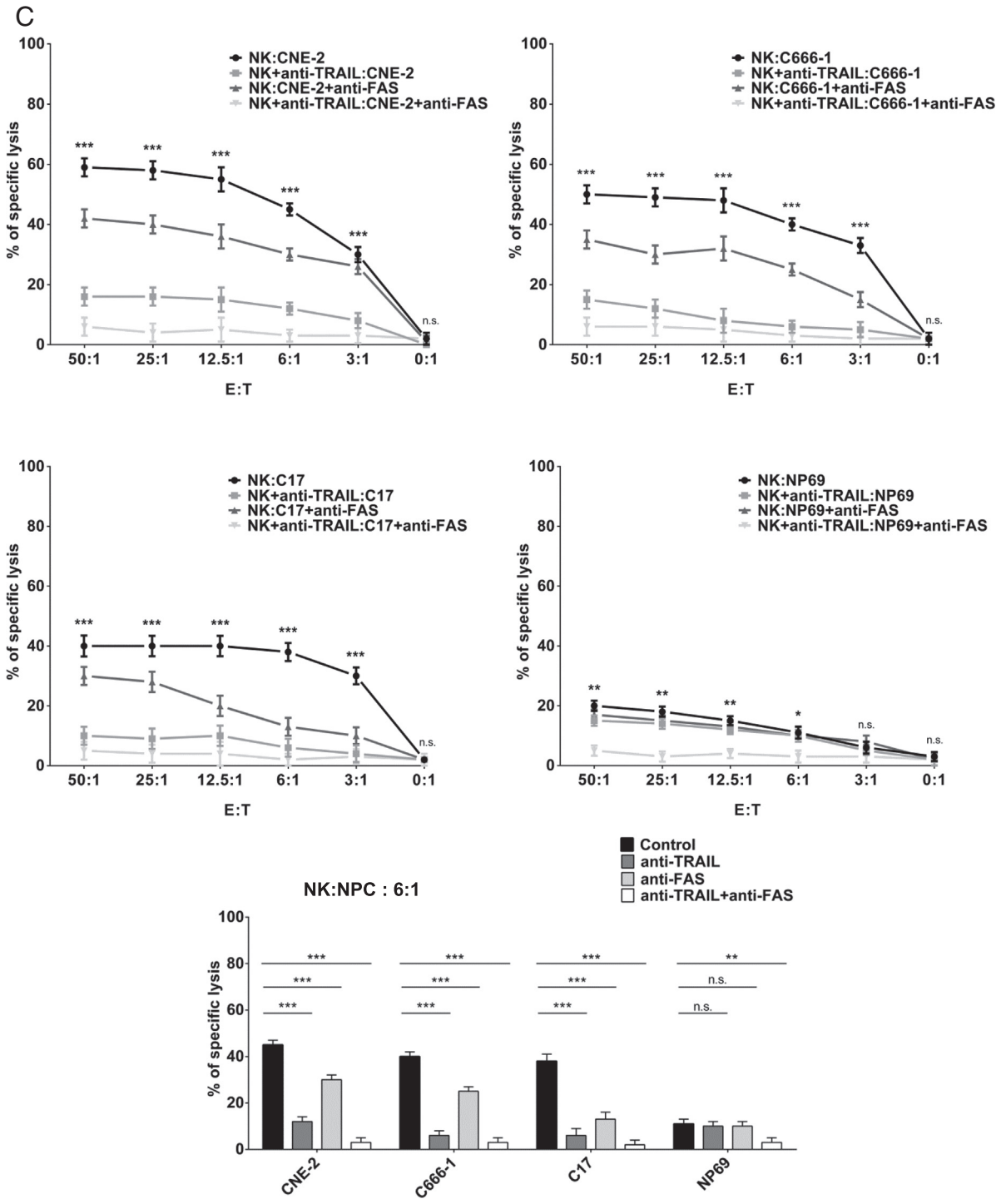


Figure 1. Killing of NPC cells by NK cells. (A) NK cells kill NPC cells starting at an effector:target (E:T) ratio of 3:1. Minor killing of nasoepithelial cells (NP69) at low E:T ratios. Cytotoxicity assays were performed in quintuplicates coculturing NK cells isolated from healthy donors and target cells labeled with calcein for 4 h. Lysis of target cells was determined by measurement of calcein in collected supernatants by an ELISA reader. (B) Bright-field and fluorescence overlay images of NPC cells cocultured with NK cells. NPC cells were stained with Calcein AM, NK cells with Hoechst 33258. (C) Preincubation of NK cells with 100 ng/ml anti-TRAIL antibody or preincubation of NPC cells with anti-FAS antibody, clone ZB4, inhibits killing of NPC cells by NK cells to 71,42% and 54,29%, respectively. Blocking of both pathways with the respective antibodies reduces killing of NPC cells by NK cells up to 90%. Data are presented as means ± S.E.M. Asterisks indicate statistically significant differences between all cell lines in one ratio-group (two-way ANOVA; $*P < .05$; $**P < .01$; $***P < .001$).



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Patient-Derived Xenograft

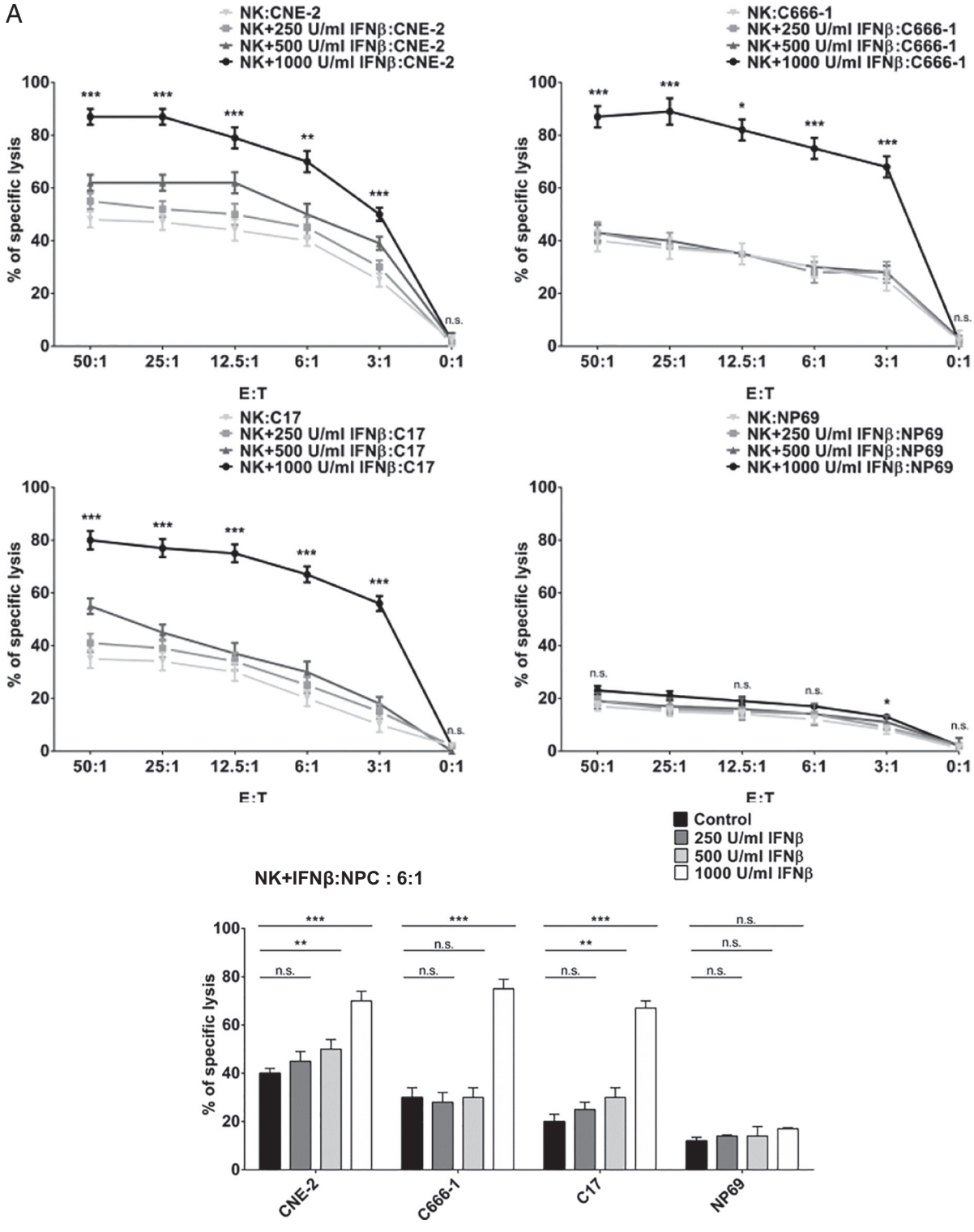
The xenograft C17 was established from a patient with an EBV-positive metastatic NPC by Prof. Pierre Busson, Paris in nude mice [23]. For the experiments described below, single cells suspensions were derived from freshly isolated C17 tumor fragments by collagenase cell dispersion. C17 cells were kept in culture using RPMI1640 Medium (Gibco) supplemented with 25 mM HEPES, 7,5% fetal bovine serum (Gibco) and 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco).

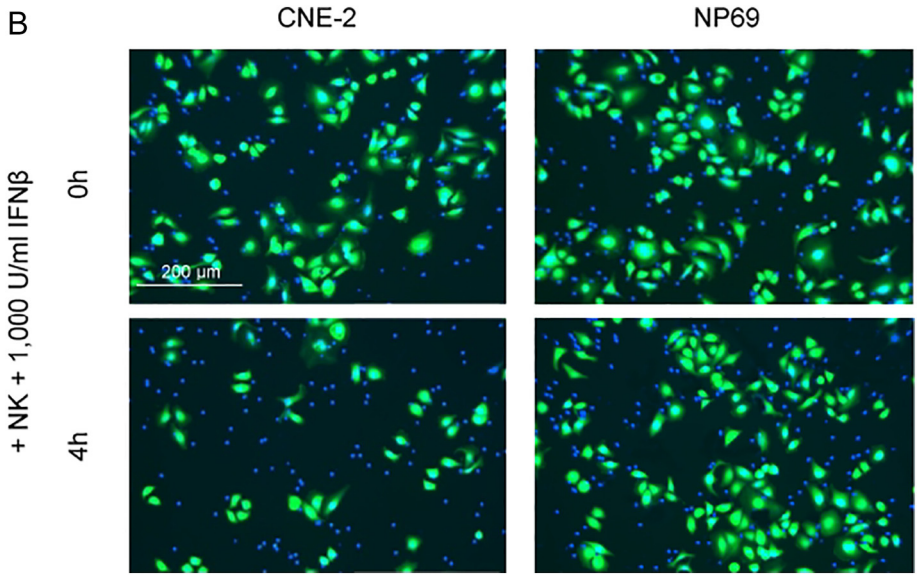
Isolation of Primary Human NK Cells

Human peripheral blood mononuclear cells (PBMC) were purified from buffy coats of 20 healthy donors using Ficoll–Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Informed consent was obtained from all donors for the use of buffy coat cells for research. Positive magnetic selection of $CD56^+$ cells from PBMCs was performed according to the manufacturer's instructions (Miltenyi; Bergisch Gladbach, Germany). Purified NK cells were cultivated in RPMI1640 medium supplemented with 10% FCS and 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco).

Reagents

Human recombinant interferon beta (IFNβ) was obtained from R&D System (NY, USA). The primary mouse monoclonal antibody against TRAIL, clone 2E5 was purchased from Enzo Life Science (Paris, France). The caspase inhibitors: Z-VAD-fmk, Z-IETD-fmk and Z-DEVD-fmk and primary mouse monoclonal antibody against PD-1, clone 913,429 were obtained from R&D System (Wiesbaden, Germany). The primary mouse monoclonal antibody against FAS Ligand, clone NOK-1, was purchased from Abcam (Cambridge, UK). Anti-FAS antibody, clone ZB4, was obtained from Millipore (California, USA), anti-human B7-H1/PD-L1 monoclonal antibody, clone 130,021, and anti-human B7-DC/PD-L2 antibody, clone 176,611, were obtained from R&D System (Minneapolis, USA). To assess TRAIL- and FAS Ligand-mediated cytotoxicity, we used freshly eluted GST-TRAIL fusion protein, produced as described before [24] and FAS Ligand obtained from Enzo Life Science (Paris, France).





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Interferon-β Treatment

To analyze the effect of IFNβ on the killing of NPC cells by NK cells, effector cells, target cells or both were incubated with IFNβ as indicated. NK cells, growing in suspension, were seeded at 1 x 10^6 cells/ml and treated with various concentration of IFNβ (0–1,000 U/ml) for 24 h. NPC cells were cultured to 70–80% confluence and treated in the presence or absence of 1,000 U/ml IFNβ for 24 h. Treated and untreated cells were evaluated for surface expression of NK cell ligands as well as PD-1, PD-L1 and PD-L2 by flow cytometry. Sensitivity of NPC cells to NK-cell mediated lysis was measured by calcein release assay.

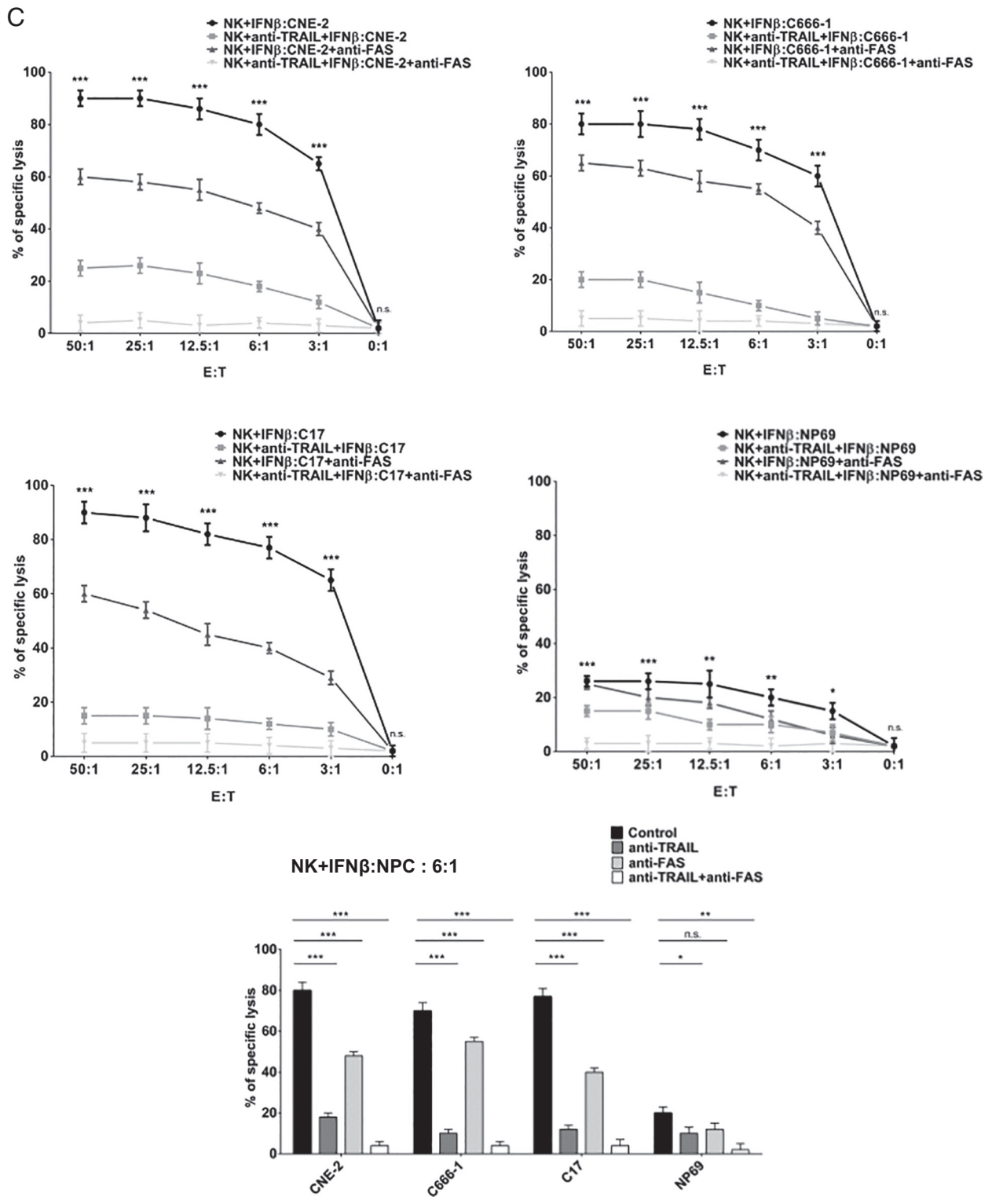
Calcein Release Assay

Calcein-acetoxymethyl (Calcein-AM) is proved to be specific and sensitive for the detection and tracking of apoptosis in living cells [25,26]. The acetoxymethyl ester of calcein is a lipid-soluble fluorogenic diester that passively crosses the cell membrane in an electrically neutral form, then being converted by intracellular esterases, which are active only in living cells, into the negatively charged, green fluorescent calcein. The fluorescence based calcein-AM release assay was used here to assess NK cell-induced cytotoxicity. NPC cells were washed and resuspended in 15 µM calcein-AM (Thermo Fisher; Eugene, USA) for 30 min at 37 °C, before co-incubation with NK cells at different effector to target (E:T) ratios (50:1, 25:1, 12,5:1, 6:1, 3:1, 0:1) for 4 h at 37 °C. 4% Triton (Merck; Darmstadt, Germany) was added to ensure maximum calcein release in controls. After centrifugation, cell free supernatant was transferred to a Cellcarrier Plate (Sarstedt; Nümbrecht, Germany) to measure relative fluorescence units (RFU) using the spectrophotometer (TECAN Infinite 200 Pro, Tecan, Männedorf, Switzerland). The percentage of specific lysis was calculated as follows: ((RFU value in respective treatment – RFU value in control (spontaneous release))/(RFU value Triton (maximum release) – RFU value in control (spontaneous release)) × 100).

Analysis of NK Cell Cytotoxicity

NPC cells were incubated with the following caspase inhibitors for 1 h prior to adding NK cells: Pan-caspase-inhibitor Z-VAD-fmk (10 µM), caspase-8-inhibitor Z-IETD-fmk (10 µM) and caspase-3-inhibitor Z-DEVD-fmk (10 µM). To analyze the contribution of death ligands in NK cell-mediated killing, NK cells were incubated with the blocking anti-TRAIL mAb, clone 2E5 (100 ng/ml) and NPC cells were incubated with the anti-FAS antibody, clone ZB4 mAb (100 ng/ml); cells were pretreated for 1 h with the respective

Figure 2. IFNβ augments killing of NPC cells by NK cells predominately via TRAIL. (A) Killing of NPC cells by NK cells starts at an E:T ratio of 3:1 and is markedly enhanced by preincubation of NK cells with IFNβ (1,000 U/ml) for 24 h. Minor killing of nasoepithelial cells (NP69) at low E:T ratios. Target cells were labeled with calcein, plated in a 96-well plate and incubated with NK cells for 4 h at the indicated E:T ratios. Lysis of target cells was determined by measurement of calcein in collected supernatants by an ELISA reader. (B) Bright-field and fluorescence overlay images of NPC cells cocultured with NK cells treated with or without 1,000 U/ml IFNβ for 24 h. NPC cells were stained with Calcein AM, NK cells with Hoechst 33258. (C) Increase in the cytotoxicity of NK cells after treatment with IFNβ against NPC cells is predominately mediated by the TRAIL signaling pathway. NK cells pretreated with IFNβ were incubated with a blocking anti-TRAIL antibody before coculture with NPC cells, and/or NPC cells and nasopepithelial cells NP69 were treated with the FAS-blocking mAb for 1 h before coculture. NPC lysis was then measured as before via calcein release assay. Data are presented as means ± S.E.M. Asterisks indicate statistically significant differences between all cell lines in one ratio-group (two-way ANOVA; $*P < .05$; $**P < .01$; $***P < .001$). D) IFNβ increases surface expression of TRAIL on NK cells. Only minor upregulation of FASL was observed. TRAIL and FASL surface expression were measured by flow cytometry 24 h after incubation of NK cells with 1000 U/ml IFNβ. (E) Addition of recombinant TRAIL to cocultures of unstimulated NK cells and NPC cells augments killing of NPC cells to a similar extent as by NK cells pretreated with IFNβ. Cytotoxicity assays were performed in quintuplicates using the calcein release assay. (F) Transfection of NPC cells with TRAIL-receptor 1 and− 2 siRNA blocks killing of NPC cells by IFNβ-stimulated NK cells. Data are presented as means ± S.E.M. Asterisks indicate statistically significant differences between all cell lines in one ratio-group (two-way ANOVA; $*P < .05$; $**P < .01$; $***P < .001$).



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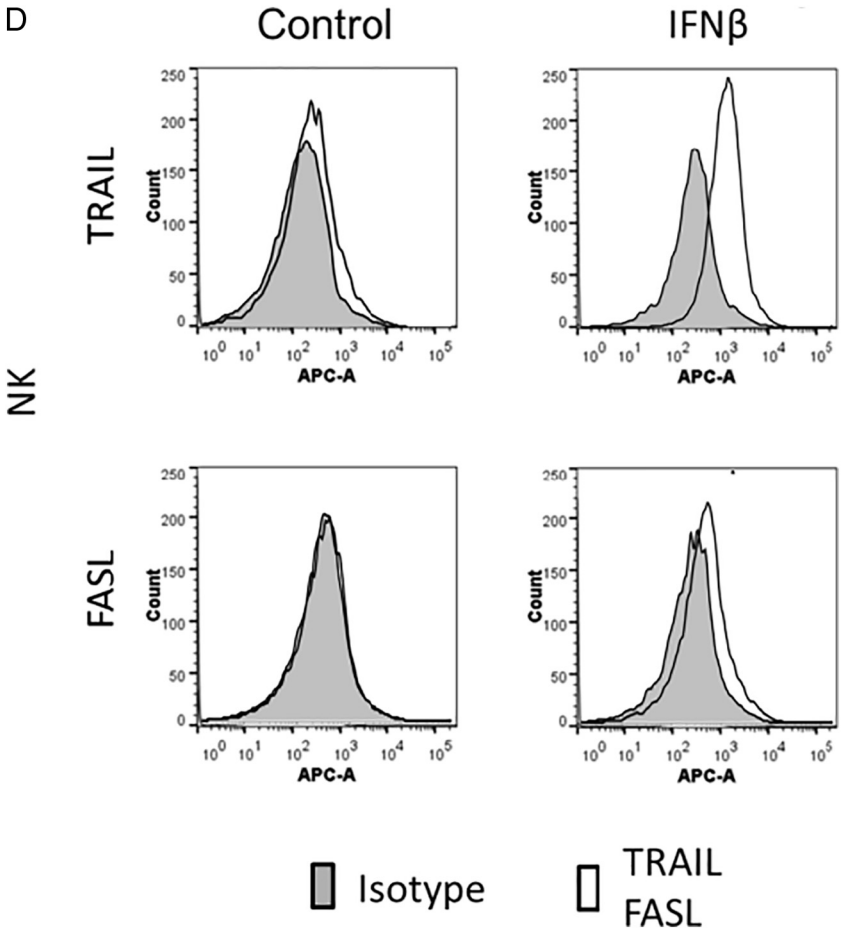
antibodies before coculture. In some experiments, NK cells were pretreated with 2.5 μg/ml conconavalin A (ConA; Sigma, St. Louis/USA) for 2 h to inactivate the perforin/granzyme B pathway. For inhibition of the PD-1/PD-L1/2 checkpoint NK cells were incubated with the PD-1 inhibitor nivolumab (Bristol-Myers; Anagni, Italien) for 1 h prior to coculture with NPC cells. Cytotoxicity was determined via calcein release assay as described above.

Flow Cytometry

NPC and NK cells were suspended at a density of 1 x 10^6 cells in 500 μl of medium. Where indicated cells had been pretreated with IFNβ at 1,000 U/ml for 24 h. For analysis of surface expression of death effectors NPC cells were incubated with the following anti-human monoclonal antibodies: anti-TRAIL (5 μl), anti-TRAIL receptor 1 (5 μl), anti-Trail receptor 2 (5 μl), anti-FASL (5 μl). For



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analysis of surface expression of checkpoint modulators, NPC and NK cells were incubated with anti-PD-1 (10 µl), anti-PD-L1 (10 µl) and anti-PD-L2 (10 µl) antibody. Incubation with primary antibodies was 1 h on ice. All cells were also stained with their corresponding isotype-matched control monoclonal antibodies. After washing in PBS three times (5 min each), APC-conjugated goat-anti-mouse antibody (1:200) was added to the cell suspensions and incubated for 1 h on ice. Subsequent to rinsing in PBS, samples were analyzed by flow cytometry. Data were analyzed by the FlowJo software (FlowJo LLC, Ashland, USA). Three independent experiments were performed for each assay.

RNA Extraction

Total RNA was isolated from C666–1, CNE-2, C17, NP69 and NK cells treated with 1,000 U/ml IFNβ for 0 h (control), 6 h or 24 h using the Maxwell RSC Simply RNA Tissue kit (Promega, Mannheim, Germany) according to the manufacturer's instructions. RNA quality was evaluated using the Agilent 4200 Tape Station (RNA screen tape assay) (Agilent, Santa Clara, USA) and quantification was performed using the Quantus Fluorometer (Promega).

RNA Library Construction and Sequencing

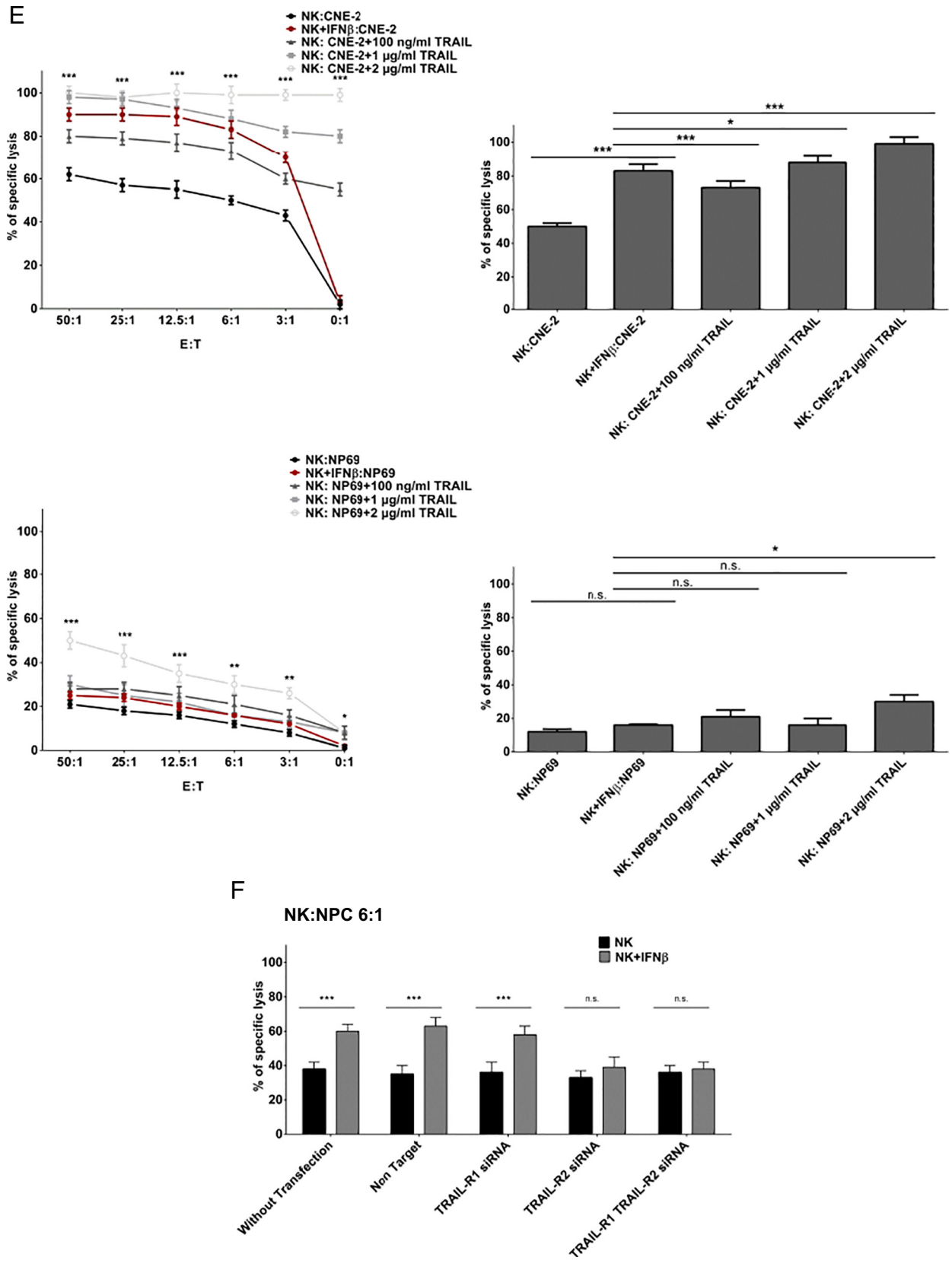
Libraries were generated from 1 µg of total RNA with the TrueSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, USA) and Ribo-Zero Gold Kit (Illumina) as described by the manufacturer. Quality and quantity of the RNA libraries were assessed using the 4200 Tape Station (D1000 screen tape assay) and the Quantus Fluorometer, respectively. The libraries were run on an Illumina NextSeq500 platform using the High Output 150 cycles Kit (2 x 76 cycles, paired-end reads, single index) (Illumina) resulting in 101.5 M reads per sample in average. Data were analyzed with an inhouse pipeline embedded in the workflow management system of the Quick NGS-environment [27].

Confocal Microscopy

NK cells were allowed to settle onto poly-L-lysine (Sigma) -coated coverslips for 15 min. Cells were then fixed with 4% paraformaldehyde (Sigma) and incubated with monoclonal antibody recognizing TRAIL (Alexis Biochemicals, San Diego, CA, USA; 1:200) for 60 min in PBS containing 0.1% Tween 20 and 5 mg/ml BSA (PBST/BSA) followed by 30 min incubation with Alexa Fluor™ 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, USA; 1:200 in PBST/BSA). The nuclear marker Hoechst 33258 was added for 10 min. Samples were mounted between slide and coverslip in gel mounting medium (Sigma) and analyzed on a confocal microscope LSM 510 laser scanning confocal/Confocor 2 microscope (Zeiss, Jena, Germany) using a 40 x DIC oil immersion objective and LSM 510 software; acquired images were imported into ImageJ (National Institute of Health; http://rsbweb.nih.gov/ij/).

Determination of sTRAIL Serum Levels

Supernatants from NK cells after stimulation with 1,000 U/ml IFNβ for 24 h, incubated with or without the anti-PD-1 inhibitor



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nivolumab at 10 μg/ml for 1 h, and then cocultured or not for 4 h with C666–1 cells either pretreated or not with 1,000 U/ml IFNβ for 24 h, were measured using a commercial ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Briefly, 100 μl of each sample and standards were tested in triplicates. After 1 h incubation and plate washing, 100 μl of HRP substrate was

added to each well. After further incubation for 1 h and washing the plate, 100 µl TMP substrate solution was added to each well followed by stop solution. Absorbance was read in a microplate reader at 450 nm. Samples were diluted, as necessary, for the absorbance (450 nm) to fall within the range of the standard curve. Final results were given as pg/ml.

Transfection of siRNA

Cells were seeded at 10^5 cells/well in 24 well plates. When the cells reached about 75%–85% confluency, culture medium was aspirated, cells washed with PBS, followed by transfection with Lipofectamine (Invitrogen, Carlsbad, CA, USA) of siRNA against TRAIL, TRAIL-receptor-1, TRAIL-receptor-2 or scrambled siRNA. After 16 h the transfection mix was replaced with normal growth medium and the cells were incubated with NK cells, 1,000 U/ml IFNβ, or 100 ng/ml recombinant TRAIL for the indicated time periods. Transfection efficiency was monitored by measuring surface expression of TRAIL or TRAIL-receptor 1 and – 2 by flow cytometry. Three independent experiments were performed for each assay.

Cell Cycle Analysis

Propidium-iodide staining of nuclei was used for cell cycle analysis as described before [13].

Statistical Analysis

Data are represented as a mean ± S.E. Each set of data represents the mean from at least three independent experiments conducted in quintuplicates for calcein release assays and triplicates for flow cytometric analyses. Differences between groups were examined for significant differences by two-way ANOVA. The level of statistical significances was set at $P < .05$.

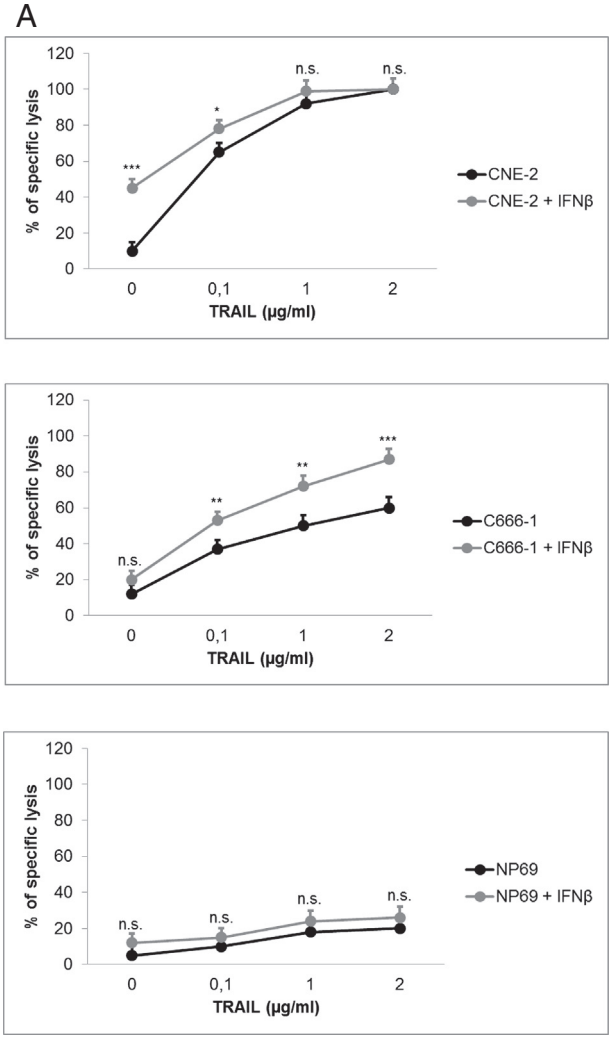

Figure 3. Preincubation of NPC cells with IFNβ decreases the cytotoxicity of IFNβ-pretreated NK cells. (A) Preincubation of NPC cells with IFNβ increases their sensitivity to cytotoxicity by recombinant TRAIL. NPC cells were incubated with 1,000 U/ml IFNβ for 24 h and then treated with recombinant TRAIL at 0, 0.1, 1 or 2 µg/ml for 4 h. Cytotoxicity was measured by the calcein release assay as described before. (B) Pretreatment of NPC cells with IFNβ reduced their sensitivity against lysis by NK cells activated with IFNβ. NK and NPC cells were incubated in the presence or absence of IFNβ (1,000 U/ml) for 24 h. Cells were then washed and cocultured at the indicated E:T ratios for 4 h. Cytotoxicity assays were performed in quintuplicates using the calcein release assay. Data are presented as means ± S.E.M. Asterisks indicate statistically significant differences between all cell lines in one ratio-group (two-way ANOVA; *$P < .05$; **$P < .01$; ***$P < .001$).

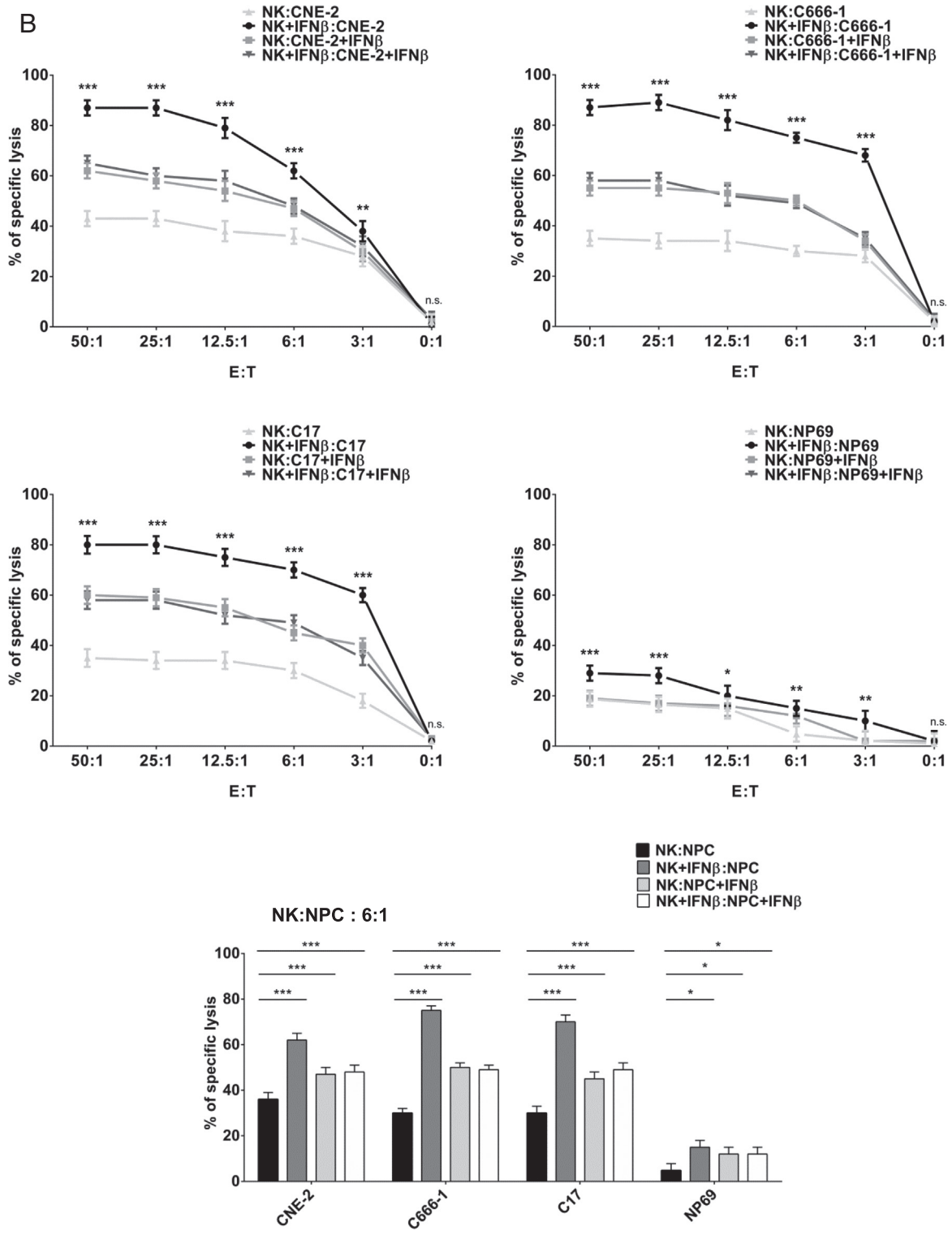

Figure 3. (*continued.*)

Results

NK Cells Kill NPC Cells

In a first experiment, we tested whether NK cells showed cytotoxic activity against NPC cells. NPC cells, labeled with calcein, were incubated with NK cells at increasing effector/target (E:T) ratios (0:1–50:1) for 4 h. The concentration of calcein in the supernatant was then measured as a marker for NPC cytotoxicity. NK cells induced high level of calcein release in an E:T ratio-dependent manner in both NPC cell lines and PDX cells C17. In contrast, only low calcein release was observed in the nasopharyngeal epithelial cell line NP69. Even at a low effector/target ratio of 6:1, 35.78 ± 2.13%

Table 1. IFNβ upregulates PD-L1 mRNA expression in NPC cell lines and PDX cells

Molecule	C666-1			CNE-2			C17			NP69		
	Control	6h IFNβ	24h IFNβ	Control	6h IFNβ	24h IFNβ	Control	6h IFNβ	24h IFNβ	Control	6h IFNβ	24h IFNβ
PD-1	0	0	0.010	0	0.019	0	0	0	0	0.031	0	0
PD-L1	0.040	0.220	0.240	0.060	0.630	0.450	0.160	O.267	0.102	1.250	3.620	0.700
PD-L2	0.030	0.080	0.030	0.110	0.210	0.330	0.308	0.420	0.210	0.450	1.050	0.740
CTLA-4	0	0	0	0	0	0	0.097	0.064	0.073	0	0	0
CD80	3.900	3.730	3.040	0.040	0.037	0.030	0.238	0.269	0.217	0.050	0.080	0.180
CD86	0.007	0.011	0.013	0.006	0.005	0.003	0.098	0.096	0.152	0.001	0.001	0.002

Values are given as TPM (transcripts per kilobase million). mRNA expression was analyzed by RNAseq before, 6 h and 24 h after incubation with 1,000 U/ml IFNβ.

of cells of NPC cell line CNE-2, 30.21 ± 3.80% of cell line C666–1 and 30.69 ± 2.95% of PDX cells C17 were lysed by NK cells, whereas cytotoxicity in nasoepithelial cells NP69 was 5.33 ± 3.01% (Figure 1, *A* and *B*). The same pattern of cytotoxicity was observed using NK cells from different healthy donors (n = 20). In summary, these results suggest that human primary NK cells can effectively kill NPC cells.

NK cells use two different pathways to eliminate target cells, a granule-dependent (perforin/granzyme-mediated) and a granule-independent (death ligand-mediated) mechanism [28]. The latter is executed by the death ligands TRAIL and FAS Ligand (FASL) expressed on the surface of NK cells which upon binding to their respective receptors on target cells can induce apoptosis [29]. As we have previously shown that NPC cells bear receptors for TRAIL and exogenous TRAIL is able to induce apoptosis in NPC cells [13], we asked whether and to what extent the induction of cytotoxicity in NPC cells by NK cells was mediated by TRAIL. Therefore, NK cells were incubated with an inhibiting anti-TRAIL antibody for 1 h prior to coculture with NPC cells; similarly, to study involvement of the FASL/FAS pathway in the cytotoxicity of NK cells, NPC cells were incubated with a FAS blocking antibody 1 h prior to coincubation with NK cells. As shown in Figure 1*C*, killing of NPC cells by NK cells was inhibited by 57.5% to 71.42% with anti-TRAIL- and 42.2% to 54.29% with anti-FAS antibody. Blocking of both pathways with the respective antibodies reduced killing of NPC cells by NK cells up to 90%. Killing of NPC cells by granule-dependent cytotoxicity therefore appears to occur only to a minor extent which was supported by the observation that pretreatment of NK cells with the granzyme B inhibitor ConA only weakly inhibited NK cytotoxicity against NPC cells (Supplemental Figure 1). In addition, incubation of NPC cells with different caspase-inhibitors prior to coincubation with NK cells shows that blockade of caspase-8 which plays as major role in the induction of apoptosis after activation of death receptors but not granzyme B, reduces calcein-release of NPC cells (Supplemental Figure 2). The effect was observed in all NPC cell lines as well as PDX cells but not in nasoepithelial cells. This data suggests that NK cell-induced cytotoxicity against NPC cells is predominately mediated by death ligands TRAIL and FASL, and only to a small extent by the perforin/granzyme system.

Treatment of NK Cells With IFNβ Augments Killing of NPC Cells Predominately via TRAIL

As type-I interferons are known to upregulate expression of TRAIL on lymphocytes [16], we wondered whether preincubation of NK cells with IFNβ would increase their killing activity against NPC cells. Therefore, NK cells were treated with various concentrations of IFNβ (0–1,000 U/ml) for 24 h and then coincubated with NPC cells at different E:T ratios. The upper concentration of 1,000 U/ml was chosen, as this was the highest concentration measured in patients treated with IFNβ for multiple sclerosis [30,31]. The experiment shows that preincubation of NK cells with IFNβ significantly increased NK killing of all NPC cells in a dose-dependent manner. At an IFNβ concentration of 1,000 U/ml, calcein release increased from about 40% to approximately 90% and from 40% to 80% in the two NPC cell lines and PDX cells, respectively, whereas no significant change was observed in nasoepithelial cells NP69 (Figure 2, *A* and *B*).

We then investigated whether the augmentation of NK cell cytotoxicity depended on the death ligand pathways (TRAIL and/or FASL) or the perforin/granzyme B system. NK cells pretreated with IFNβ were incubated with a blocking anti-TRAIL antibody before coculture with NPC cells, and/or NPC cells and nasoepithelial cells NP69 were treated with the FAS-blocking mAb for 1 h before coculture. Additionally, NK cells pretreated with IFNβ were either incubated for 1 h with ConA or fixed with paraformaldehyde to block granzyme B activity. NPC lysis was then measured via the calcein release assay as before. The results demonstrate that the increase in the cytotoxicity of NK cells after treatment with IFNβ against NPC cells is predominately mediated by the TRAIL signaling pathway and to a smaller extent by the FASL/FAS signaling pathway (Figure 2*C*). IFNβ did not augment killing via granzyme B/perforin. (Supplemental Figure 3, *A* and *B*).

We next asked the question whether the increased killing of NPC cells by NK cells pretreated with IFNβ is due to an increased expression of TRAIL and FASL by IFNβ. As shown in Figure 2*D* and Supplemental Figure 4, IFNβ upregulated expression of TRAIL and to a minor extent of FASL on NK cells after an incubation of 24 h. Addition of recombinant TRAIL to cocultures of unstimulated NK cells and NPC cells increased cytotoxicity against NPC cells to a similar extent as pretreatment of NK cells with IFNβ indicating that on engagement of unstimulated NK cells with NPC cells only a part of TRAIL receptors on NPC cells were saturated and that adding TRAIL either externally as recombinant molecule or by IFNβ-stimulated NK cells leads to a dose-dependent increase in NPC killing (Figure 2*E*). Recombinant FASL added to cocultures of unstimulated NK cells and NPC cells increased cytotoxicity of NK cells against NPC cells only to a small amount (Supplemental Figure 5).

In order to confirm that TRAIL signaling plays a key role in the cytotoxicity of NK cells toward NPC cells, especially in the setting of IFNβ-pretreated NK cells, TRAIL-receptor 1 and– 2 expression in target cells was silenced by specific siRNA before coincubation with NK cells. The efficiency of siRNA-knock down was monitored by measuring apoptosis after treatment with recombinant TRAIL via

flow cytometry (Supplementary Figure 6). As shown in Figure 2*F*, transfection of NPC cells with TRAIL-receptor siRNA against TRAIL-receptors but not scr-RNA (nontarget) reduced killing by IFNβ-stimulated NK cells. These results indicate that the cytotoxicity of IFNβ-stimulated NK cells against NPC cells predominately depends on the activation of the TRAIL-signaling pathway.

Pretreatment of NPC Cells with IFNβDecreases Their Sensitivity to Lysis by IFNβ-Activated NK Cells

We next asked the question whether preincubation of NPC cells with IFNβ would further increase the cytotoxicity of IFNβ-pretreated NK cells. In a first experiment NPC cells were incubated IFNβ (1,000 U/ml) for 24 h and then exposed to different concentrations of recombinant TRAIL. As shown in Figure 3*A* pretreatment of NPC but not nasoepithelial cells with IFNβ increased their sensitivity to TRAIL-mediated killing. Also, NPC cells pretreated with IFNβ were more sensitive to killing by non-stimulated NK cells than untreated cells (Figure 3*B*). In contrast, pretreatment of NPC cells with IFNβ reduced their sensitivity against lysis by NK cells activated with IFNβ (Figure 3*B*).

IFNβ Leads to Activation of Inhibitory Checkpoints

As pretreatment with IFNβ of either NK cells or NPC cells increased the sensitivity of NPC cells to NK cell cytotoxicity, but incubation of both cell populations with IFNβ had a smaller effect on NPC lysis than exclusive pretreatment of NK cells with IFNβ, we asked whether IFNβ would impair killing by upregulation of inhibitory checkpoint molecules on both NK and NPC cells. As the PD-1/PD-L1/2 and CTLA-4/B7 checkpoints are amenable to pharmaceutical intervention [32], we focused on the expression of these molecules on NK and NPC cells and their possible modulation by IFNβ. Table 1 shows RNA expression by RNAseq of the checkpoint members above in NPC cell lines before, 6 h and 24 h after incubation with IFNβ. Whereas IFNβ did not modulate mRNA expression of the CTLA-4 members, it upregulated mRNA expression of PD-L1, and to a smaller extent of PD-L2 in all NPC cell lines and PDX cells. Increased expression of PD-L1 but not PD-L2 after incubation of NPC cells with IFNβ was also seen on the cell surface using flow cytometry (Figure 4*A*). No surface expression of PD-1 was observed in NPC cells, either untreated or treated with IFNβ. In addition, unstimulated NK cells weakly expressed PD-1

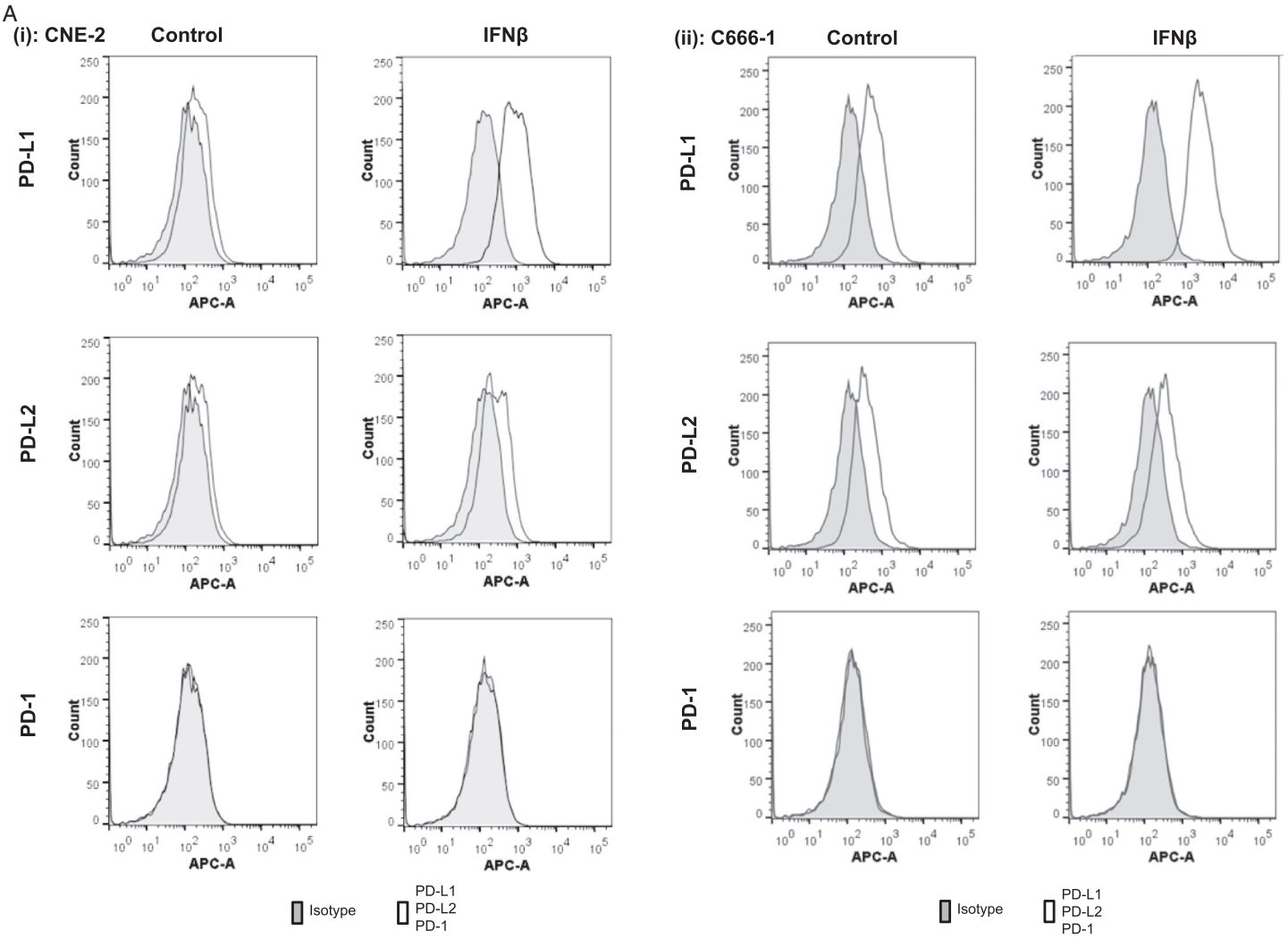

Figure 4. IFNβ activates the PD-L1/PD-1 checkpoint. (A) IFNβ induces surface expression of PD-L1 but not PD-L2 and PD-1 in NPC cells. PD-L1, PD-L2 and PD-1-expression was measured 24 h after incubation with 1,000 U/ml IFNβ. (B) IFNβ upregulates surface expression of PD-1 on NK cells. Cells were incubated with 1,000 U/ml IFNβ for 24 h and PD-1, PD-L1, PD-L2 expression was analyzed by flow cytometry.

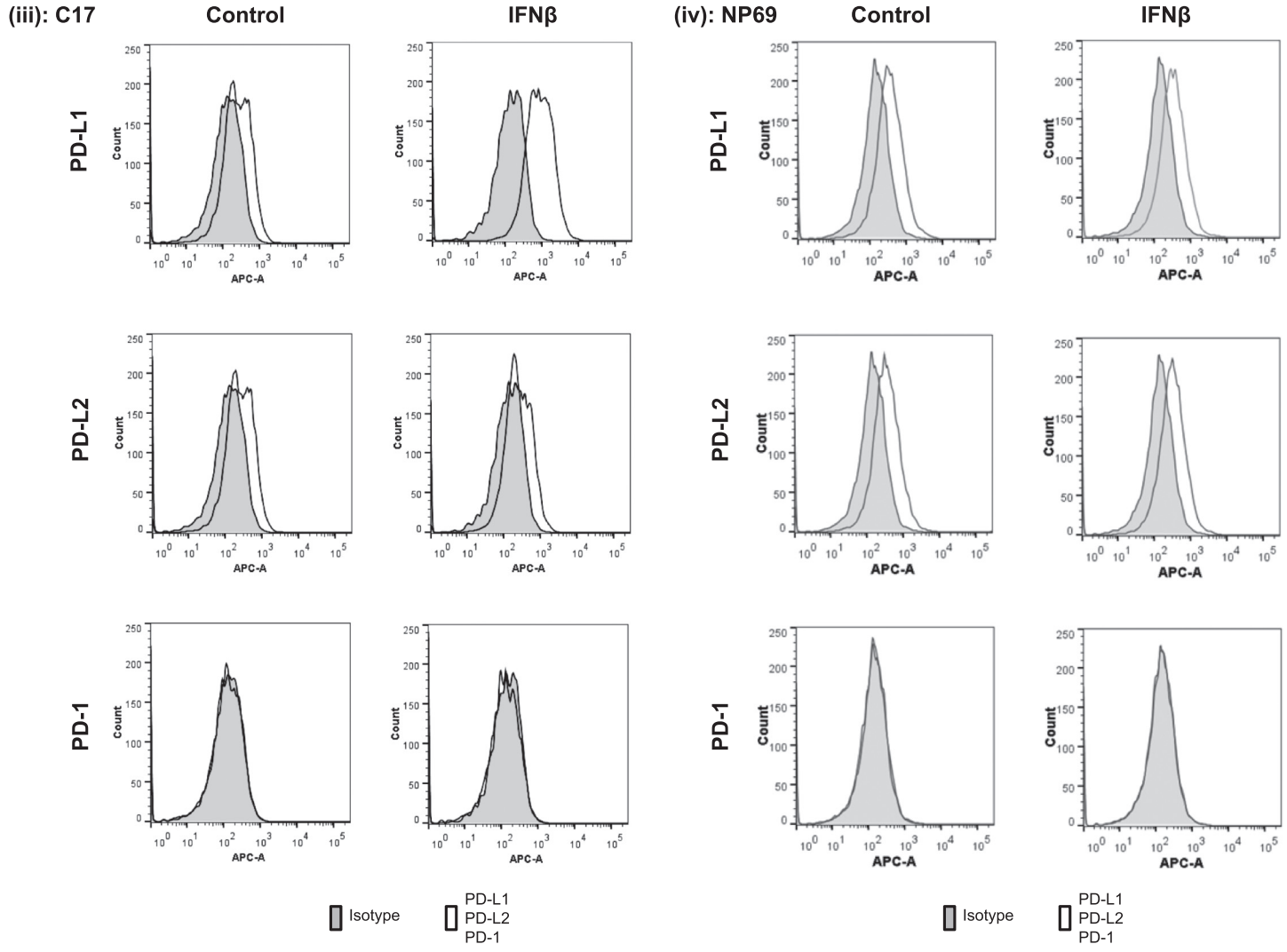

Figure 4. (*continued.*)

which in contrast to CTLA-4 increased after incubation of cells with IFNβ as shown by RNAseq and flow cytometry (Table 2, Figure 4*B*).

Incubation of IFNβ-Pretreated NK Cells With an Anti-PD1 Antibody Increases Their Toxicity Against NPC Cells Pretreated With IFNβ

Having shown that IFNβ increases expression of PD-1 on NK cells and PD-L1 on NPC cells, we asked the question whether activation of the PD-1/PD-L1 checkpoint by IFNβ could explain that pretreatment of NPC cells with IFNβ decreased their sensitivity against IFNβ-pretreated NK cells and whether blockade of this checkpoint with an anti-PD-1 antibody could increase killing. To study this, IFNβ-pretreated NK cells were incubated with the anti-PD-1 antibody nivolumab for 1 h before coculturing them with IFNβ-pretreated NPC cells. Figure 5*A* demonstrates that addition of nivolumab markedly increased sensitivity of IFNβ-pretreated NPC cells to NK cell lysis, making them more sensitive to lysis by IFNβ-pretreated NK cells than NPC cells without IFNβ pretreatment. Nivolumab only slightly increased killing of IFNβ-pretreated NK cells against untreated NPC cells, of unstimulated NK cells against IFNβ-pretreated NPC cells and of unstimulated NK cells against unstimulated NPC cells indicating that marked checkpoint activation only occurred when both effector and targets were stimulated with IFNβ (Figure 5*A* and Supplemental Figure 7). When the experiment above was repeated in the presence of an anti-TRAIL-, or anti-Fas-antibody, or in the presence of concanvalin A to block the respective NK effector pathways, it could be shown that the increased killing after PD-1 blockade was predominately mediated through TRAIL (Figure 5*B*).

PD-1 Blockade Increases the Cytotoxicity of IFNβ-Activated NK Cells Against IFNβ-Treated NPC Cells by Releasing Intracellular TRAIL From NK Cells

We then wondered whether the increase of cytotoxicity of IFNβ-activated NK cells by anti-PD-1 blockade was due to upregulation of TRAIL. Therefore, TRAIL-surface expression was measured by flow cytometry in IFNβ-activated NK cells treated with or without nivolumab. TRAIL surface expression was slightly increased after nivolumab exposure (Figure 6*A*). Interestingly, when IFNβ-pretreated NK cells got cocultured with IFNβ-treated NPC cells, TRAIL expression was lost in NK cells treated with nivolumab but not in cells without nivolumab. Similarly, TRAIL expression was markedly reduced in IFNβ-treated NK cells when cocultured with untreated NPC cells, suggesting that killing of NPC cells by NK cells

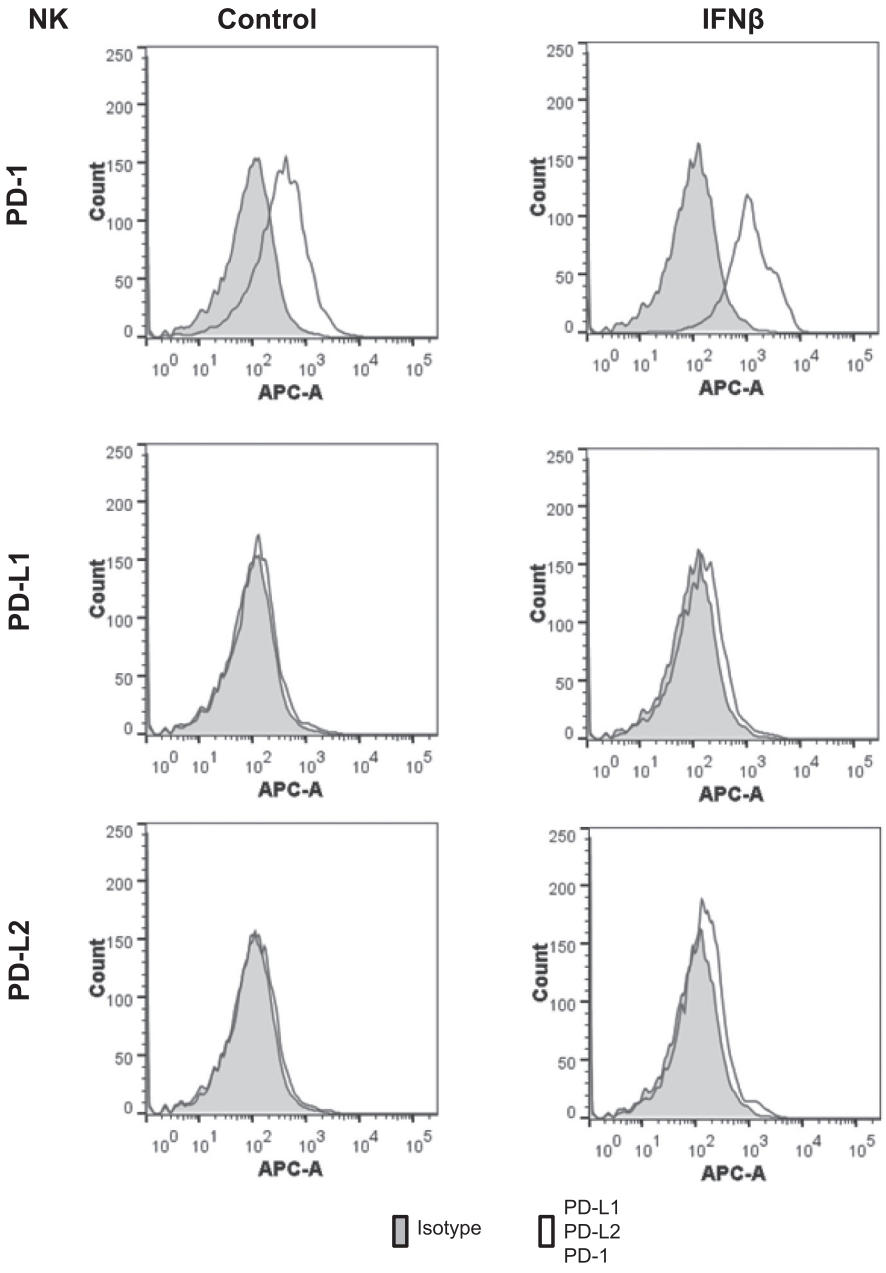

Figure 4. (*continued.*)

was associated with decreased surface expression of TRAIL on NK cells. This was confirmed by confocal microcopy depicting that TRAIL expression of IFNβ-activated NK cells cocultured with IFNβ-activated NPC cells got lost when cells were incubated with nivolumab (Figure 6*B*). As staining of IFNβ-activated NK cells for TRAIL was predominately intracytoplasmic, we examined the concentration of soluble TRAIL (sTRAIL) in the supernatant of cocultures. As shown in Figure 6*C*, treatment of IFNβ-activated NK cells with the anti-PD-1 antibody nivolumab led to a marked increase of sTRAIL after coculture with IFNβ-activated NPC cells compared to cells treated without nivolumab. To investigate whether the increase in sTRAIL was from NK cells or NPC cells, the experiment was repeated with NPC cells treated or not with siRNA against TRAIL. No difference was observed in the amount of sTRAIL measured in the supernatant of cocultures indicating that sTRAIL in cocultures stemmed from NK cells (Supplemental Figure 8). Therefore, it can be assumed that the PD-1/PD-L1 interaction between IFNβ-treated NK- and NPC cells inhibits the release of

Table 2. IFNβ upregulates PD-1 and PD-L1 mRNA expression in NK cells

Molecule	NK		
	Control	6h IFNβ	24h IFNβ
PD-1	0.049	0.244	0.260
PD-L1	0.202	0.963	1.173
PD-L2	0.197	0.220	0.246
CTLA-4	0.093	0.129	0.133
CD80	0.211	0.697	0.591
CD86	0.331	0.207	0.797

Values are given as TPM (transcripts per kilobase million). mRNA expression was analyzed by RNAseq before, 6 h and 24 h after incubation with 1,000 U/ml IFNβ.

cytoplasmic TRAIL from NK cells thereby limiting their cytotoxicity. Upon checkpoint-blockade with anti-PD1 antibody, intracellularly stored TRAIL gets released from IFNβ-activated NK cells increasing their cytotoxicity.

Discussion

In this paper we have demonstrated that (**1**) NK cells effectively kill NPC cells and that killing occurs predominately via the TRAIL signaling pathway, that (**2**) IFNβ increases the cytotoxic activity of

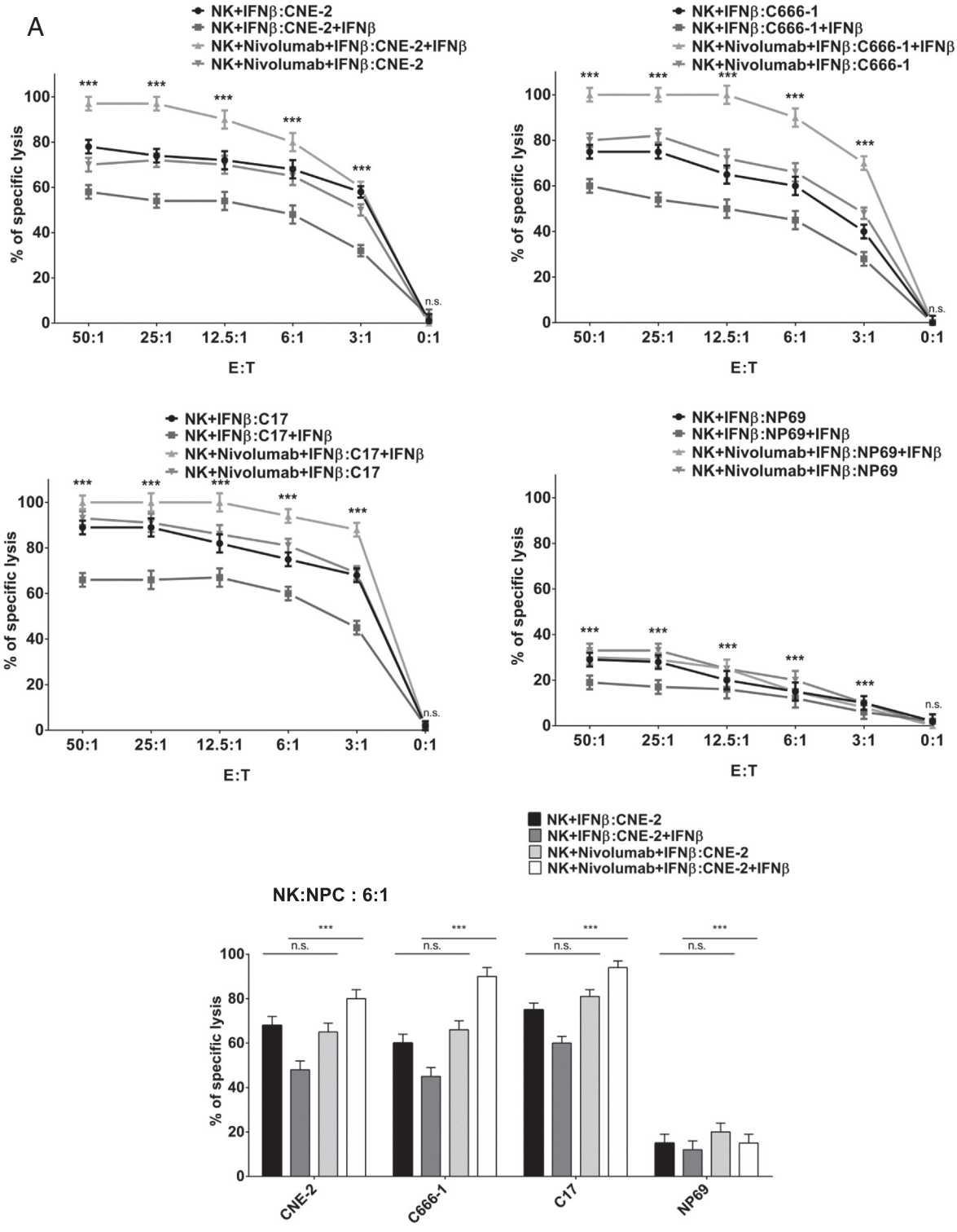

Figure 5. Inhibition of PD-1 increases killing of NPC cells by NK cells in the presence of IFNβ. (A) Pretreatment of NK cells with the PD-1 inhibitor nivolumab leads to a marked increase in killing of NPC cells when effector and targets are both pretreated with IFNβ (1,000 U/ml), as compared when only NK cells are exposed to IFNβ (B) Preincubation of IFNβ-activated and anti-PD1-treated NK cells with anti-TRAIL antibody (100 ng/ml) inhibits the killing of IFNβ-pretreated NPC cells by 80%. Treatment of IFNβ-activated and anti-PD1-treated NK cells with Con A or treatment of IFNβ-pretreated NPC cells with an anti-FAS-antibody reduces killing by 15% and 20%, respectively. Cytotoxicity assays were performed in quintuplicates using the calcein release assay. Data are presented as means ± S.E.M.Asterisks indicate statistically significant differences between all cell lines in one ratio-group (two-way ANOVA; **P* < .05; ***P* < .01; ****P* < .001).

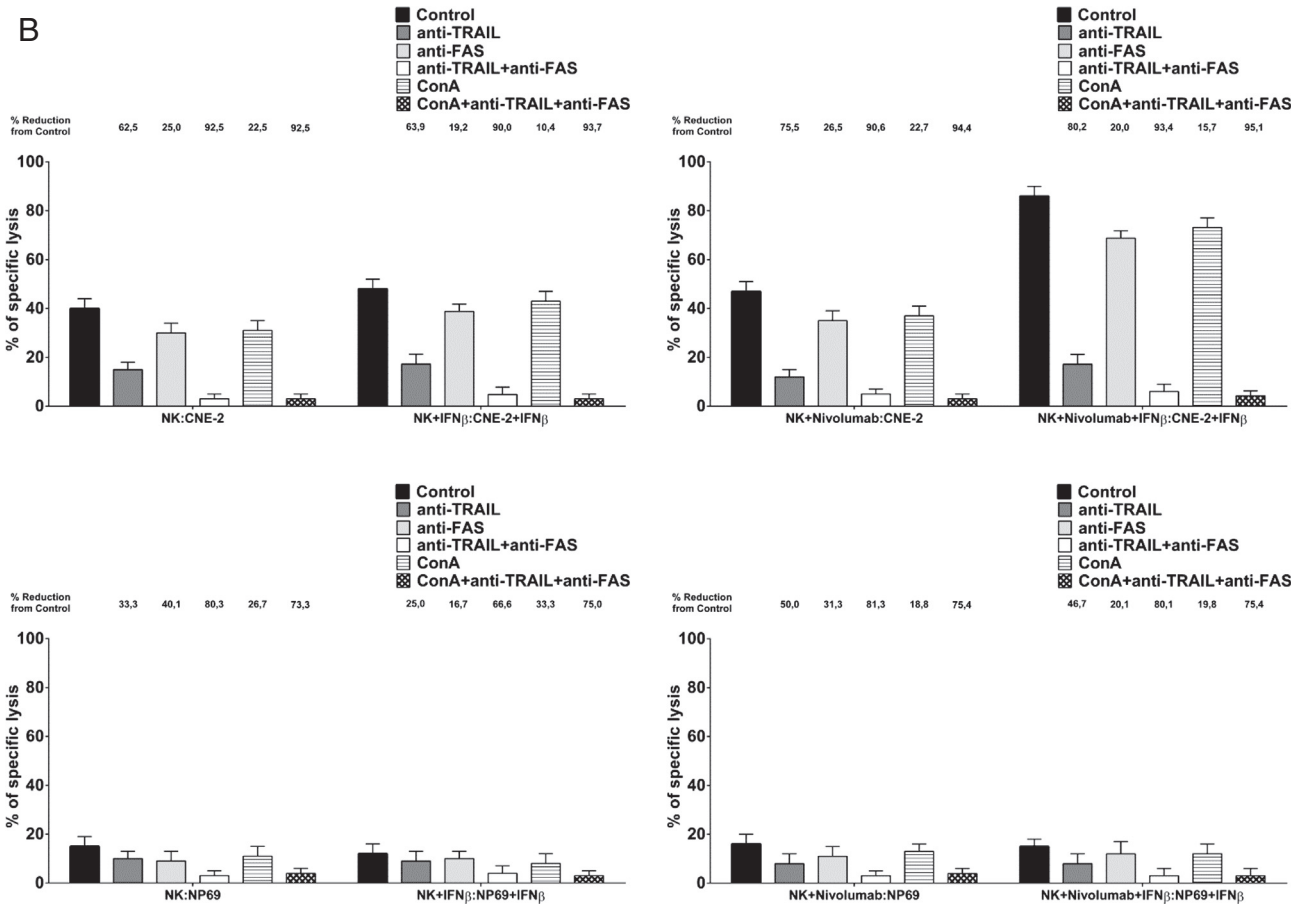

Figure 5. (*continued.*)

NK cells against NPC cells, that (**3**) blocking of the PD-1/PD-L1 checkpoint further increases the killing activity of NK cells against NPC cells in the presence of IFNβ and that **(4)** the increased killing after PD-1/PD-L1 checkpoint blockade is linked to the release of soluble TRAIL from IFNβ-activated NK cells (Figure 7). Our results point to a potential clinical benefit of a combination therapy of IFNβ and PD-1/PD-L1 checkpoint blockade in patients with NPC.

NPC is an immunogenic tumor as the majority of NPC tumors are characterized by a marked lymphomononuclear infiltrate and immune effector mechanisms such as the transfer of EBV-specific T lymphocytes, cytokine-induced killer cells or blockade of the anti-PD-1/PD-L1 checkpoint can lead to tumor control in patients [33–36]. We have previously shown that NPC cells in contrast to nasoepithelial cells have an intact TRAIL signaling pathway and that exposure of NPC cells to IFNβ induced expression of TRAIL in the majority of NPC cell lines studied, leading to subsequent activation of the TRAIL-receptor signaling pathway and apoptosis [13]. TRAIL is also used by lymphocytic effector cells such as T lymphocytes and NK cells to induce apoptosis in susceptible target cells [16,37,38]. Using NK cells from healthy donors, we were able to show that these cells induce cell death in different NPC cells, including cells of a patient-derived xenograft. Cell death was predominately induced by TRAIL, whereas the granzyme B/perforin system played only a minor role in cytotoxicity.

Incubation of NK cells with IFNβ at concentrations achievable in patients increased cytotoxicity of NK cells and was mainly mediated by upregulation of TRAIL expression on NPC cells. Upregulation of TRAIL expression by type-I-interferons has been shown previously and the TRAIL promoter has been demonstrated to contain an interferon-responsive element [16,17,39]. Incubation of NPC cells with IFNβ also increased their susceptibility to NK cell-mediated cytotoxicity or exogenous TRAIL. However, preincubation with IFNβ decreased the sensitivity of NPC cells against IFNβ-activated NK cells which could be overcome by incubating NK cells with the anti-PD-1 inhibitor nivolumab. Here, we could demonstrate that IFNβ increased PD-L1 expression on NPC cells and induced PD-1 expression on NK cells. As in our NPC model, IFNβ has been shown to upregulate PD-L1 expression in different tissues such as human macrophages [40], hepatocytes [41], endothelial cells [42] and in monocytes and dendritic cells of patients with multiple sclerosis [43]. Similar to our observation, induction of PD-1 expression on NK cells has been described when cells were activated and expanded *in vitro* using anti-CD16 antibody and IL-2 [44]. When such activated NK cells were either cocultured *in vitro* with PD-L1 expressing myeloma cells or injected into mice with myeloma xenografts, their cytotoxicity was enhanced by blockade of the PD-1/PD-L1 checkpoint. Upregulation of PD-1 and PD-L1 by type I interferons has also been shown in a melanoma mouse model [45]. Here, targeted activation of the type I IFN system by poly (I:C) in the microenvironment of immune cell-poor melanoma in mice, resulted in cytotoxic immune cell recruitment, upregulation of PD-L1 expression in tumor tissue and increased expression of PD-1 on peripheral blood CD8 + T-cells. The addition of an anti-PD-1 antibody together with but not without poly (I:C), prolonged survival

of mice, indicating that activation of the type I IFN system as in our NPC system leads to subsequent functional activation of the PD-L1/PD-1 immune-inhibitory signaling axis. So far, there has been not much knowledge how blockade of PD-1 leads to increased cytotoxicity of NK cells. In T lymphocytes binding of PD-1 by its ligand PD-L1 or PD-L2 leads to phosphorylation of the PD-1

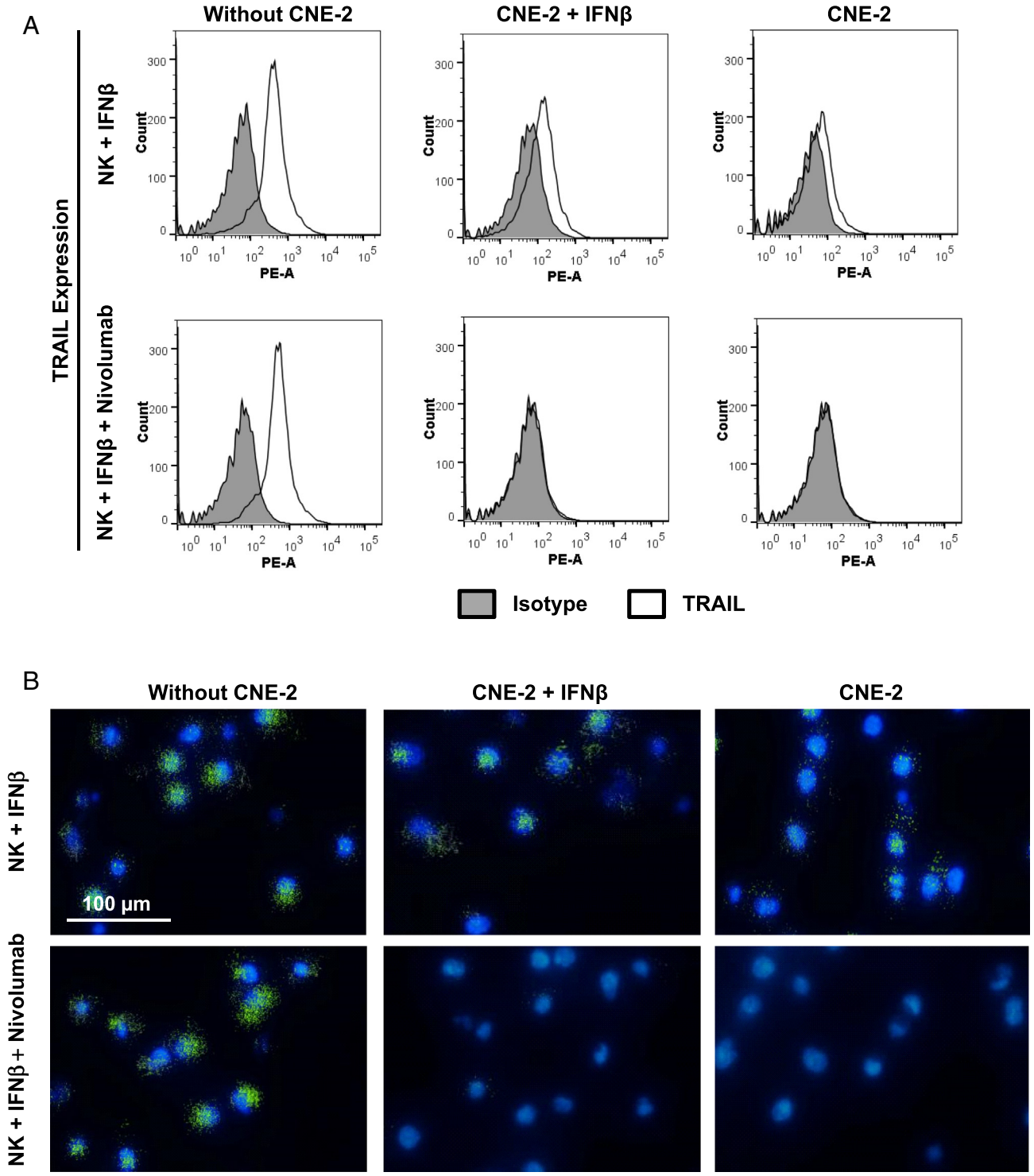

Figure 6. PD-1 blockade increases the cytotoxicity of IFNβ-activated NK cells against IFNβ-treated NPC cells by releasing intracellular TRAIL from NK cells. (A) Surface expression of TRAIL by FACS in IFNβ-activated NK cells treated or not with anti-PD-1 antibody, before or after 4 h coculture with IFNβ-pretreated or untreated NPC cells. Anti-PD-1 treatment leads to loss of TRAIL expression of IFNβ-activated NK cells when cocultured with IFNβ-pretreated NPC cells. (B) Confocal microscopy depicting predominately intracytoplasmic staining for TRAIL in IFNβ-activated NK cells. Loss of TRAIL in anti-PD-1-treated and IFNβ-activated NK cells after coculture with IFNβ-pretreated NPC cells. (C) Soluble TRAIL concentration measured by ELISA. Anti-PD-1 treatment leads to a marked increase in the concentration of sTRAIL in the supernatant of cocultures of IFNβ-activated NK cells with IFNβ-pretreated NPC cells. Data are presented as means ± S.E.M. Asterisks indicate statistically significant differences between all cell lines in one ratio-group (two-way ANOVA; $*P < .05$; $**P < .01$; $***P < .001$).

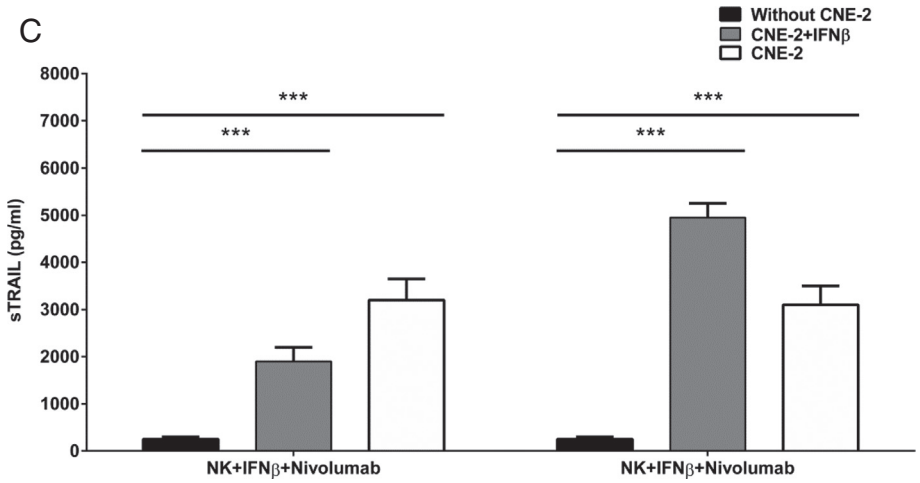

Figure 6. (*continued.*)

cytoplasmic domain and recruitment of the tyrosine phosphatase SHP-2, resulting in reduced phosphorylation of TCR signaling molecules leading to decreased T cell activation and cytokine production and cytokine release [46]. γδ-T lymphocytes have been shown to produce and release soluble TRAIL upon activation leading to apoptosis of lung cancer cells in one model [47]. Based on our experiments we can postulate that blockade of PD-1 in IFNβ-activated NK cells leads to the secretion of soluble TRAIL which then contributes to the killing of TRAIL-susceptible NPC cells.

Our results also suggest that the clinical application of NK cells, together with IFNβ and PD-1/PD-L1 checkpoint blockade could be of therapeutic potential in patients with NPC. Over the last years several clinical trials either with autologous or allogeneic NK cells have been performed in patients with cancer [48]. Infusion of large doses of NK cells have been generally shown to be safe [49]. In a clinical trial involving 10 patients with renal cell carcinoma, the infusion of autologous NK cells expanded and activated *ex vivo* with feeder cells and IL-2, led to a complete and partial remission in 4 and 2 patients, respectively [50]. Tumor responses after the transfer of autologous NK cells have also been observed in patients with glioma and breast cancer [51,52]. In the haploidentical setting the transfer of allogeneic NK cells has been shown to induce complete remissions in

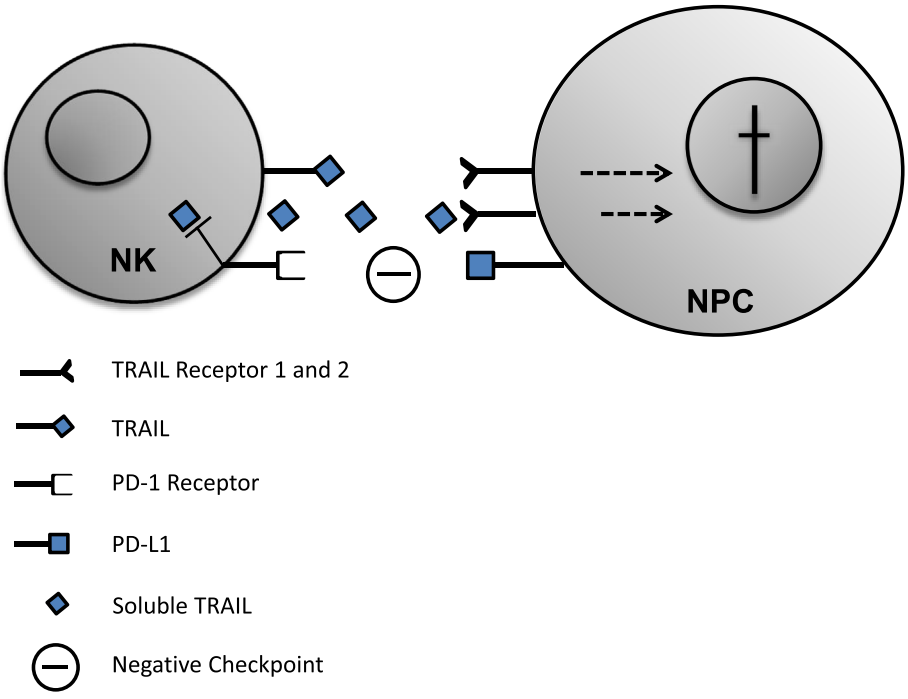

Figure 7. Mechanism of NK killing against NPC. Activation of NK cells by IFNβ induces cytotoxicity against NPC cells through upregulation of expression of surface and soluble TRAIL by NK cells and subsequent activation of the TRAIL-signaling pathway in NPC cells. This cytotoxicity is enhanced by blockade of the PD-1/PD-L1 checkpoint. Blockade of PD-1 in IFNβ-activated NK cells increases the secretion of soluble TRAIL contributing to the killing of TRAIL-susceptible NPC cells.

patients with acute lymphatic and myeloid leukemia, and neuroblastoma [53–55]. Transferred NK cells have been shown to persist and expand *in vivo* [56,57].

Conclusion

In conclusion, IFNβ augments NK-induced cytotoxicity against NPC cells through upregulation of TRAIL expression in NK cells and subsequent activation of the TRAIL-signaling pathway. PD-1 blockade further enhances cytotoxicity of natural killer cells in the presence of IFNβ toward NPC cells. The presented findings suggest that the combination of IFNβ with PD-L1/PD-1 blockade could be of clinical potential in NPC therapy and warrants further studies *in vivo*.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.04.017.

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