TNF α Augments Cytokine-Induced NK Cell IFN γ Production through TNFR2

Wagdi Almishri Tania Santodomingo-Garzon Tyson Le Danuta Stack
Christopher H. Mody Mark G. Swain

Immunology Research Group, Snyder Institute, Liver Unit, Division of Gastroenterology and Hepatology,
Cumming School of Medicine, University of Calgary, Calgary, Alta., Canada

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Abstract

NK cells play a central role in innate immunity, acting directly through cell-mediated cytotoxicity and by secreting cytokines. TNF α activation of TNFR2 enhances NK cell cytotoxicity, but its effects on the other essential function of NK cells – cytokine production, for which IFN γ is paramount – are poorly defined. We identify the expression of both TNF α receptors on human peripheral blood NK cells (TNFR2 > TNFR1) and show that TNF α significantly augments IFN γ production from IL-2-/IL-12-treated NK cells in vitro, an effect mimicked by a TNFR2 agonistic antibody. TNF α also enhanced murine NK cell IFN γ production via TNFR2 in vitro. In a mouse model characterized by the hepatic recruitment and activation of NK cells, TNFR2 also regulated NK cell IFN γ production in vivo. Specifically, in this model, after activation of an innate immune response, hepatic numbers of TNFR2-expressing and IFN γ -producing NK cells were both significantly increased; however, the frequency of IFN γ -producing hepatic NK cells was significantly reduced in TNFR2-deficient mice. We delineate an important role for TNF α , acting

through TNFR2, in augmenting cytokine-induced NK cell IFN γ production in vivo and in vitro, an effect with significant potential implications for the regulation of innate and adaptive immune responses.

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Introduction

The immune system is classically divided into innate and adaptive arms. NK cells are an important component of the innate immune system, and as such NK cells are critically involved in host immune responses to infectious agents (e.g. viruses and fungi) and to malignantly transformed cells [1–3]. In response to tissue injury, NK cells are rapidly recruited from the blood into affected tissues, where they are subsequently activated and directly and/or indirectly impact immune responses [3]. Activation of NK cells during pathological responses can occur as a result of direct cell-cell interactions or through indirect mechanisms including the release of cytokines from other innate immune cells within an inflamed tissue which

W.A. and T.S.-G. contributed equally to this work.

subsequently activate NK cells [3–6]. The cytokines most commonly implicated in this process of NK cell activation are IL-2, IL-12, IL-15, and IL-18 [5–7]. Subsequent immune effector roles of NK cells within tissues can be mediated through both cytotoxic and cytokine-producing capacities [5, 8]. IFN γ is a major cytokine produced by activated NK cells, and release of IFN γ within tissues has profound immunomodulatory effects [9]. Specifically, IFN γ release can lead to downstream activation of other innate and adaptive immune cells, enhanced recruitment of immune cells into affected tissues, a shift in cytokine responses to a more Th1-biased response, and direct antiviral effects [9, 10]. However, the roles of other cytokines, including TNF α , in enhancing NK cell production of IFN γ are not well understood.

TNF α is a pleiotropic cytokine that can be produced by numerous immune cells, including T cells, macrophages, NK cells, and NKT cells [11]. Enhanced TNF α production is an important regulator of innate immunity in a variety of diseases, in both patients and animal models [11, 12]. To exert its biological effects, TNF α interacts with two cell surface receptors, TNFR1 and TNFR2 [12, 13]. TNFR1 is ubiquitously expressed on all cells, whereas expression of TNFR2 is more restricted, being found mainly on certain T cell subtypes (i.e. CD4 and CD8), endothelial cells, and cells within the brain [11–13]. In general, the immunoregulatory effects of TNF α have been mainly attributed to interactions of TNF α with TNFR1, which in turn involves intracellular signaling pathways containing a death domain [11]. However, although less well characterized, TNFR2 (which is not linked to a death domain) has been increasingly implicated in the effects of TNF α in immune regulation [14]. It has been appreciated for many years that TNF α can augment the effects of IL-2 to induce NK cell differentiation and activation, and enhance NK cell-driven cytotoxicity towards target cells in vitro [15, 16]. This effect of TNF α on NK cell-mediated cytotoxicity has been attributed, at least in part, to TNF α stimulation of TNFR2 expressed on both human and murine NK cells [17, 18]. However, an effect of TNF α upon NK cell production of IFN γ has not been directly examined, but has clear potential immunological implications. In a recent study, the potential involvement of TNF α in enhancing NK cell IFN γ release was noted in an in vitro NK cell coculture system with macrophages; however, the TNFR subtype involved in this effect was not characterized [19].

In this series of experiments, we demonstrate a significant expression of TNFR2 on the surface of NK cells and delineate a central role for TNF α interacting with

TNFR2 in NK cell activation and IFN γ production in both human and murine NK cells. These observations highlight the importance of TNF α -TNFR2 interactions in NK cell production of IFN γ . Moreover, our current findings may provide new insight into the clinical consequences and commonly reported adverse outcomes associated with TNF α -neutralizing/-inhibiting therapeutic strategies in patients with infectious and autoimmune diseases [20], in which both IFN γ and NK cells are known to play important immunomodulatory roles [2, 7, 10, 21].

Materials and Methods

Antibodies and Reagents for Human NK Cell Studies

The following reagents, antibodies, and appropriate isotype controls were obtained from the indicated sources. RPMI 1640 medium, HEPES, fetal bovine serum (FBS), 2-mercaptoethanol, nonessential amino acids, sodium pyruvate, penicillin-streptomycin, phosphate-buffered saline (PBS) were all obtained from Invitrogen, Life Technologies, Carlsbad, Calif., USA. FITC anti-human CD56 (clone B159) and APC Annexin V were obtained from BD Biosciences, Mississauga, Ont., Canada. Brefeldin A solution, monensin solution, cell stimulation cocktail, IC Fixation Buffer, permeabilization buffer, fixable viability dyes, human Fc receptor-binding inhibitor, and anti-human IFN γ eFluor 450 (clone 4S.B3) were all obtained from eBioscience Inc., San Diego, Calif., USA. Anti-human TNFR1/TNFRSF1A-APC (clone 16803), anti-human TNFR2/TNFRSF1B-APC (clone 22235), and anti-human TNFR1/TNFRSF1B polyclonal were all obtained from R&D Systems Inc. Minneapolis, Minn., USA. Specific TNFR2 agonist antibody (clone TY010) was kindly provided by Dr. Denise Faustman (Immunology Laboratories, Massachusetts General Hospital East, Charlestown, Mass., USA) [22].

Human Peripheral Blood NK Cell Isolation

For isolation of primary human NK cells, peripheral blood was obtained by venipuncture from healthy volunteers (in compliance with the University of Calgary Conjoint Health Research Ethics Board of the University of Calgary, Protocol No. 23363) and anticoagulated with heparin (10 U/ml blood). Peripheral blood mononuclear cells (PBMCs) were purified as previously described [23]. Briefly, blood was centrifuged on a Ficoll-Hypaque (GE Healthcare, Mississauga, Ont., Canada) density gradient and washed 3 times in Hanks' balanced salt solution (Invitrogen, Carlsbad, Calif., USA). NK cells were magnetically separated through LS columns using a MACS NK cell isolation kit (Miltenyi Biotec, Auburn, Calif., USA) as per the manufacturer's instructions. NK cells collected in the negative fraction were labeled with anti-human CD56 to assess the purity (which was routinely measured and consistently found to be between 95 and 97%) and to define CD56^{hi} and CD56^{lo} subsets.

Human NK Cell Flow Cytometry Analysis and Gating Strategy

Freshly isolated NK cells were subjected to direct immunofluorescence analyses using multicolor flow cytometry staining. Data from the samples were acquired either using a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif., USA) or an

Attune Acoustic Focusing flow cytometer (Applied Biosystems, Burlington, Ont., Canada). The data were analyzed using FlowJo software (Tree Star, Ashland, Oreg., USA) or Attune Cytometric Software v2.1 (Applied Biosystems). Gating proceeded as follows: gating of live cells, excluding duplet cells, followed by gating on forward scatter and side scatter areas to identify regions appropriate to define lymphocytes. CD56⁺ cells were identified in the lymphocyte gate. Cells expressing TNFR1 and TNFR2 were identified in the CD56⁺ gate. For IFN γ staining, cells were first stained for extracellular receptors, fixed, permeabilized, and then stained for IFN γ . Fluorescence-minus-one controls were used for the accurate designation of cells with fluorescence above background levels [24]. Appropriate isotype controls were used to determine the specificity of all antibodies used.

In vitro Human NK Cell Studies

To assess NK cell production of IFN γ , purified NK cells were allowed to rest overnight and were resuspended in 200 μ l of fresh RPMI 1640 complete medium supplemented with 10% (v/v) FBS, 2.0 mM L-glutamine, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin at a density of 1×10^6 cells/ml (250,000 cells/200 μ l) in flat-bottomed 96-well multiwell plates (Sarstedt Inc., Newton, Mass., USA). To stimulate submaximal IFN γ production from NK cells, cells were treated with IL-2 and IL-12 (each 20 ng/ml) for 24 h (concentrations were determined in pilot experiments and are consistent with those in previous reports) [25, 26]. For FACS (fluorescence-activated cell sorting) analyses, medium was supplemented with protein transport inhibitors (brefeldin A and monensin at 3.0 μ g/ml and 2 mM, respectively, for the last 5 h of incubation). To test for an effect of TNF α or TNFR2 agonist antibody, TNF α or TNFR2 agonist antibody was added to NK cell-containing wells (concentrations of 100 ng and 2.5 μ g/ml, respectively) [22] just prior to adding the IL-2 and IL-12. Addition of TNF α alone to the incubation medium did not increase NK cell Annexin V expression [stained with APC Annexin V (BD Pharmingen, San Jose, Calif., USA) and Fixable Viability Dye eFluor[®] 780 (eBioscience)] compared to NK cells incubated in buffer alone (online suppl. fig. 5; for all online suppl. material, see www.karger.com/doi/10.1159/000448077). For measurement of NK cell secretion of IFN γ into culture media, cell culture supernatants were harvested 24 h after stimulation. Samples were centrifuged (at 3,000 g) prior to aliquoting to remove debris. The cell culture supernatants were then stored at -20°C until assayed. IFN γ was measured in cell culture supernatant samples using a Luminex[®] assay [Luminex 100 system (Millipore, USA) assay performed by Eve Technologies Corporation, Calgary, Alta., Canada]. The results are expressed as pg/ml supernatant. To assess effects of cytokines (IL-2, IL-12, and TNF α) on NK cell TNFR2 expression, purified human NK cells were treated with IL-2 + IL-12 (each 20 ng/ml), TNF α alone (100 ng/ml), or a combination of all three cytokines for 24 h. At the end of the experiment, cells were harvested and cytokine-induced changes in NK cell TNFR2 expression evaluated by flow cytometry. TNFR2 expression was expressed both in terms of changes in frequencies of TNFR2-positive cells and mean fluorescence intensity (MFI).

Mouse Studies

For all experiments, male wild-type C57BL/6 mice and TNFR2-deficient mice (on a C57BL/6 background) were used (B6.129S2-Tnfrsf1b^{tm1Mwm/J}, 8–10 weeks of age; The Jackson Laboratory,

Bar Harbor, Maine, USA). CD1d-deficient mice (C57BL/6 genetic background) were obtained as previously described [27]. All procedures in this study were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines established by the Canadian Council on Animal Care. To induce hepatic NK cell recruitment and activation, the mice were treated with a single intravenous (i.v.) injection of α -galactosylceramide (α GalCer; Alexis Biochemicals, San Diego, Calif., USA; 2 μ g in 100 μ l vehicle: 0.04% Tween 20 in sterile PBS) [28, 29]. Controls received 100 μ l of vehicle. α GalCer treatment rapidly activates hepatic NKT cells, which in turn secrete large amounts of numerous cytokines, including TNF α [30]. TNF α produced by activated hepatic NKT cells in this manner is a critical driver of the subsequent robust recruitment of NK cells into the liver, and NK cells recruited in this fashion are activated and produce IFN γ [28, 31, 32].

Murine Hepatic Lymphocyte Isolation and Flow Cytometry Analyses

Eight, 16, and 48 h after α GalCer or vehicle administration, hepatic lymphocytes were isolated using methods as previously described [27, 29]. Briefly, livers were perfused with ice-cold normal saline and were then treated with digestion buffer [0.05% collagenase 2 (Cedarlane Laboratories, Burlington, Ont., Canada) and 0.02% DNase I (Roche Diagnostics, Laval, Que., Canada) in Hanks' balanced salt solution with Ca^{2+} and Mg^{2+}] for cell isolation. Hepatic lymphocytes were isolated by discontinuous Percoll[®] gradient (GE Healthcare Bio-Sciences, Baie-d'Urfé, Que., Canada) as previously described [27, 29]. Cell viability was assessed by Trypan Blue dye exclusion. Single-cell suspensions ($0.5\text{--}1.0 \times 10^6$ cells/sample) were prepared in binding buffer (1% FBS in PBS) for flow cytometry staining using CellQuest software (Becton Dickinson). For cell surface staining, $0.5\text{--}1 \times 10^6$ cells were incubated with various antibodies for 30 min at 4°C . Anti-mouse antibodies included: PerCP anti-mouse CD3e (145-2C11; BD Pharmingen), FITC or PE anti-mouse NK1.1 (PK136; BD Pharmingen), PE anti-mouse CD120b receptor (TNFR2, TR75-8; BD Pharmingen), and FITC anti-mouse TNF α (MP6-XT22; BD Pharmingen). Hepatic NKT cells were identified as being CD3⁺ PBS-57 loaded CD1d tetramer positive (provided by the NIH Tetramer Facility, Emory University Vaccine Center, Atlanta, Ga., USA). Hepatic NK cells were defined as being NK1.1⁺CD3⁻ cells. Intracellular expression of IFN γ was assessed on permeabilized cells as previously described using a Cytofix/Cytoperm Plus Kit (BD Pharmingen) with PE anti-mouse IFN γ (XMG 1.2; BD Pharmingen).

In vitro Stimulation of Murine Splenic NK Cells

Single-cell suspensions were prepared from the spleens of naive wild-type and naive TNFR2-deficient mice using RPMI 1640 medium (supplemented with 10% fetal calf serum, nonessential amino acids, L-glutamine, β -mercaptoethanol, and penicillin-streptomycin; all reagents from Invitrogen, Canada). Briefly, spleens from naive wild-type and TNFR2-deficient mice were gently squeezed between sterile frosted slides, passed through a 70- μ m cell strainer (BD Pharmingen), and then placed in ammonium chloride lysis buffer to remove red blood cells. Next, freshly isolated splenocytes were enriched for NK cells using a negative selection NK cell isolation kit (130-090-864; Miltenyi Biotec). Enriched NK cells (1×10^6 cells/well) in supplemented RPMI 1640 medium were treated in vitro with the following murine recombinant cytokines: unstimu-

lated, murine IL-2 (10 ng/ml; eBioscience) alone; murine IL-12 (5 ng/ml; Peprotech, London, UK) alone; murine TNF α (10 ng/ml; Peprotech) alone, or either the combination of rmIL-2 + rmIL-12 or rmIL-2 + rmIL-12 + rmTNF α for 16 h at 37°C and 5% CO₂. Following in vitro stimulation, supernatants were collected for quantification of IFN γ levels by Luminex[®] assay (Eve Technologies Corporation). Additionally, NK cells were removed and stained extracellularly with FITC anti-mouse NK1.1 mAb (BD Pharmingen) and then intracellularly for PE anti-mouse IFN γ (BD Pharmingen) as described above.

Murine Peripheral Blood Isolation and Flow Cytometry Analyses

For evaluation of CD27 and TNFR2 expression on murine NK cells, blood from naive mice was collected into BD Vacutainers containing EDTA and PBMCs isolated by discontinuous Percoll[®] gradient (GE Healthcare Bio-Sciences) as previously described [27, 29]. The PBMCs were then stained with anti-mouse CD3 Alexa Fluor[®] 488 (17A2; BioLegend, San Diego, Calif., USA), anti-mouse PerCP-Cyanine5.5 NK1.1 (PK136; eBioscience), anti-mouse APC/Cy7 CD27 antibody (LG.3A10; BioLegend), anti-mouse PE TNFR2 antibody (TR75-89; BioLegend). NK cells were first identified as CD3⁺NK1.1⁺ cells, and were then further divided into CD27^{lo} and CD27^{hi} subsets. Differential expression of TNFR2 on the CD27^{lo} and CD27^{hi} subsets was then determined.

Statistics

All data are shown as means $\pm$ SEM. Statistical significance was assessed using an unpaired Student *t* test for comparisons between two groups, or with an ANOVA followed by the Student-Newman-Keuls post hoc test for comparisons between more than two groups, using GraphPad InStat 3 software (GraphPad Software Inc., La Jolla, Calif., USA). Differences between means were considered significant when *p* < 0.05.

Results

Differential Expression of TNFR Subtypes on NK Cells

Conflicting reports have previously been published with regard to the relative differential expression of TNFR1 and TNFR2 on human NK cells, with the reported NK cell expression of TNFR1 ranging from <3 to 16% and the expression of TNFR2 ranging from 9 to 68% of NK cells [17, 33]. We found that TNFR2 expression was relatively enriched relative to TNFR1 within the NK cell population as a whole [expression as percent of total NK cells: TNFR1 29.0 $\pm$ 3.4% (*n* = 5 donors) and TNFR2 90.5 $\pm$ 2.8% (*n* = 6 donors)] (fig. 1). The NK cell population has typically been divided into two main groups based on CD56 expression. CD56^{hi} NK cells have classically been positioned as major cytokine producers, and CD56^{lo} NK cells as being mainly cytotoxic, although this separation by functional properties has recently been questioned [34, 35]. In our current study, we have

extended the phenotypic characterization of NK cell subpopulations for expression of TNF receptors. Flow cytometry analysis revealed a differential expression of TNFR2 within the CD56^{hi} and CD56^{lo} NK cell populations. Specifically, NK cell surface TNFR2 expression was greater in the CD56^{lo} subpopulation of NK cells (both as percent positive cells and as MFI) (fig. 1E, F; online suppl. fig. 4). Similarly, the proportion of CD56^{hi} NK cells expressing TNFR1 was significantly smaller than the CD56^{lo} NK cell population (online suppl. fig. 7). A differential expression of TNFR2 within these two NK cell populations is interesting and has not been previously reported.

Murine NK cells do not express CD56; however, CD27 expression on murine NK cells has been suggested to be equivalent to CD56 expression on human NK cells [36, 37]. Therefore, we determined differential TNFR2 expression on CD27^{hi} and CD27^{lo} murine peripheral blood NK cells by flow cytometry (fig. 2), and demonstrate findings similar to those obtained using CD56 in human NK cells (fig. 1).

Role of TNFR2 in Human NK Cell Production of IFN γ

TNFR2 has been implicated in the enhancement of NK cell cytotoxicity [17]; however, the role of TNFR2 in altering the other major property of NK cells, cytokine production, remains unclear. To address this issue, we examined NK cell production of IFN γ using an in vitro assay. We found that treatment of NK cells in vitro with TNF α alone, or with a specific TNFR2 agonistic antibody alone, did not induce NK cell IFN γ production (fig. 3A–C). In contrast, as reported previously, administration of a combination of IL-2 + IL-12 enhanced IFN γ production in NK cells (fig. 3A–C) [25, 26]. Surprisingly, coadministration of TNF α , or a TNFR2 agonistic antibody, to NK cells that were also treated with IL-2 + IL-12 resulted in a synergistic augmentation of IFN γ production (fig. 3A–C). We have shown that TNFR2 is differentially expressed on NK cell subpopulations (see above). Therefore, we sought to delineate the capacity of CD56^{hi} and CD56^{lo} NK cell subpopulations to produce IFN γ after TNF α costimulation. We found that IFN γ production was similar in CD56^{hi} and CD56^{lo} NK cell subpopulations in response to IL-2 + IL-12 treatment (as determined by MFI, a measure of IFN γ production per cell) (fig. 3D, E). However, addition of TNF α or the TNFR2 agonistic antibody to IL-2 + IL-12-treated NK cells synergistically increased overall IFN γ production, and induced more robust IFN γ production in CD56^{hi} NK cells compared to CD56^{lo} NK cells (fig. 3F–I).

Fig. 1. Flow cytometry analysis of TNFR1 and TNFR2 expression on human peripheral blood CD56⁺ NK cells. Purified peripheral blood NK cells from healthy donors were stained with CD56, TNFR1, or TNFR2 and analyzed by flow cytometry. **A** Representative flow cytometry dot plot showing the NK cell gating strategy (**a**) and the gating strategy used to determine CD56^{lo} (small rectangular gate) and CD56^{hi} (small circular gate) NK cells (**b**). SSC-A = Side scatter area. **B** Representative flow cytometry dot plot of

CD56⁺ NK cells vs. TNFR1 expression (mean 29.0 ± 3.4%, n = 5). **C** Representative flow cytometry dot plot of CD56⁺ NK cells vs. TNFR2 expression (mean 90.5 ± 2.8%, n = 6). **D** Total number of TNFR2⁺ NK cells in CD56^{lo} vs. CD56^{hi} NK cell subsets. *** p < 0.0001 (n = 3/group). **E** Frequency of CD56^{lo} vs. CD56^{hi} NK cells expressing TNFR2. *** p < 0.0001 (n = 3/group). **F** TNFR2 expression as MFI on CD56^{lo} vs. CD56^{hi} NK cells. *** p < 0.0001 (n = 3/group).

NK Cell Recruitment and Activation in the Liver of Mice after α GalCer Administration

The glycolipid α GalCer specifically activates hepatic NKT cells via their invariant T cell receptor, leading to the rapid and robust recruitment and activation of NK cells within the liver [27, 28, 32]. Therefore, this model was used to determine the *in vivo* role of TNFR2 in tissue-recruited NK cell activation and IFN γ production. Consistent with previous reports, administration of α GalCer to mice resulted in a significant increase in the overall numbers of hepatic NK1.1⁺CD3⁻ (NK) cells compared to vehicle-treated controls [27, 32]. Moreover, elevated hepatic NK cell numbers persisted for at least 48 h after treatment (fig. 4A; online suppl. fig. 1A). Administration of α GalCer also led to enhanced numbers of IFN γ -producing NK cells within the liver compared to vehicle-treated controls (fig. 4B; online suppl. fig. 1B). α GalCer treatment of mice rapidly (within 2 h) activates hepatic NKT cells which sub-

sequently produce TNF α , and these activated NKT cells constitute the major TNF α -producing cell type in the liver after α GalCer treatment [31]. We have confirmed that α GalCer does indeed rapidly activate hepatic NKT cells to produce TNF α , as reflected in a significant increase in the percentage of hepatic NKT cells producing TNF α 2 h after α GalCer treatment compared to vehicle-treated mice (64.9 ± 2.6 vs. 4.1 ± 0.7%; n = 5 mice/group; p < 0.0001) (online suppl. fig. 6). We next determined whether NK cells isolated from livers of α GalCer-treated mice expressed TNFR2. Indeed, numbers of hepatic TNFR2⁺ NK cells were increased in α GalCer-treated compared to vehicle-treated mice (fig. 4C–E). Moreover, hepatic recruitment of TNFR2⁺ NK cells after α GalCer administration was dependent upon the presence of NKT cells, as NK cells were not recruited to the liver in CD1d knockout (KO) mice (which are NKT cell deficient) [27] treated with α GalCer (fig. 4C–E).

Fig. 2. Flow cytometry analysis of TNFR2 expression on murine peripheral blood CD27⁺ NK cells. Peripheral blood from naïve mice was analyzed by flow cytometry to determine NK cell CD27 and TNFR2 expression. **A** Frequency of CD27^{lo}- and CD27^{hi}-expressing NK cells within the total NK cell population. *** $p \leq 0.0001$ vs. CD27^{lo} group ($n = 5$ mice). **B, C** Frequency (**B**) and total number (**C**) of TNFR2⁺ cells in CD27^{hi} and CD27^{lo} subsets.

*** $p \leq 0.0001$ vs. CD27^{lo} ($n = 5$ mice). **D, E** Representative flow cytometry dot plots showing the NK cell gating strategy (**D**) and the gating strategy used to determine CD27^{lo} (**b**) vs. CD27^{hi} (**a**) NK cells (**E**). **F** Representative flow cytometry dot plot of TNFR2 expression on CD27^{hi} NK cells. **G** Representative flow cytometry dot plot of TNFR2 expression on CD27^{lo} NK cells.

TNFR2 Regulates Murine NK Cell IFN γ Production in vivo and in vitro

To assess the role of TNFR2 in IFN γ production by liver-recruited NK cells in vivo, we treated TNFR2-deficient and wild-type mice with α GalCer. Surprisingly, α GalCer-treated TNFR2 KO mice demonstrated significantly greater total NK cell recruitment to the liver than did similarly treated wild-type mice (fig. 5A). Although the overall total number of hepatic IFN γ ⁺ NK cells was not altered in TNFR2 KO versus wild-type mice treated with α GalCer (online suppl. fig. 2), the percentage of IFN γ ⁺ NK cells within the liver 16 h after α GalCer administration was significantly reduced in TNFR2-deficient mice compared to similarly treated wild-type controls (fig. 5B), and the fluorescent intensity of IFN γ labeling in NK cells was also lower (fig. 5C, D). To further establish the role of TNF α signaling via TNFR2 in NK cell IFN γ production, TNFR2 KO or wild-type splenic NK cells were purified by MACS and cultured with rmIL-2, rmIL-12, and rmTNF α for 16 h. Levels of IFN γ in culture su-

pernatants were then quantitated by Luminex[®] assay. We found a significant decrease in IFN γ levels in TNFR2 KO splenic NK cells treated with rmIL-2 + rmIL-12 + rmTNF α compared to similarly treated wild-type splenic NK cells (fig. 5E). A similar pattern of results was obtained when intracellular IFN γ expression in NK cells was determined by flow cytometry (fig. 5F). Importantly, NK cells isolated from TNFR2 KO mice are not defective in their overall ability to produce IFN γ , since splenic NK cells isolated from wild-type and TNFR2 KO mice stimulated with PMA (phorbol 12-myristate 13-acetate) and ionomycin in vitro demonstrate similar IFN γ expression (online suppl. fig. 3).

Effect of IL-2 + IL-12 and TNF α on Human NK Cell Expression of TNFR2

The effect of cytokine-mediated activation of NK cells upon the expression of TNFR2 is unknown, as is the effect of TNF α stimulation. Therefore, we assessed the impact of stimulation of NK cells with IL-2 + IL-12

Fig. 3. Effect of TNFα or TNFR2 agonistic antibody on IFNγ production by human CD56⁺ NK cells in vitro. Human peripheral blood NK cells were cultured for 24 h in the presence of buffer alone, TNFα alone, TNFR2 agonist antibody alone, IL-2 + IL-12, IL-2 + IL-12 + TNFα, or IL-2 + IL-12 + TNFR2 agonist antibody. **A** NK cell IFNγ expression as MFI. *** p ≤ 0.001 group F vs. groups A, B, C, and D; ### p ≤ 0.001 group E vs. groups A, B, C, and D; +++ p ≤ 0.001 group D vs. groups A, B, C, E, and F. **B** IFNγ production in NK cell culture supernatant determined by ELISA. *** p ≤ 0.001 group F vs. groups A, B, C, and D; ** p ≤ 0.01 group F vs. group E; ### p ≤ 0.001 group E vs. groups A, B, C, and D; +++ p ≤ 0.001 group D vs. groups A, B, C, E, and F. **D, F, H** CD56^{hi} and CD56^{lo} NK cell IFNγ production after 24 h of incubation with IL-2 + IL-12 (**D**; n.s., not significant), IL-2 + IL-12 + TNFR2 agonist antibody (**F**; * p ≤ 0.05), or IL-2 + IL-12 + TNFα (**H**; ** p ≤ 0.01). Data are shown as the mean ± SEM of 4 replicates from 1 donor. Similar results were obtained from 4 additional donors. **C** Representative flow cytometry histograms depicting IFNγ expression for the data presented in **A**. Buffer

(far left, black dashed line), TNFR2 agonist (left, gray dotted line), TNFα (middle, black dotted line), IL-2 + IL-12 (right, gray solid line), IL-2 + IL-12 + TNFα (far right, black long-dashed line), IL-2 + IL-12 + TNFR2 agonist (far right, gray long-dashed line). **E** Representative flow cytometry histograms depicting IFNγ expression for the data presented in **D**. The solid line represents IFNγ expression by the CD56^{lo} subset and the dotted line represents IFNγ expression by the CD56^{hi} subset in the presence of IL-2 + IL-12. **G** Representative flow cytometry histograms depicting IFNγ expression for the data presented in **F**. The solid line represents IFNγ expression by the CD56^{lo} subset and the dotted line represents IFNγ expression by the CD56^{hi} subset in the presence of IL-2 + IL-12 + TNFR2 agonist. **I** Representative flow cytometry histograms depicting IFNγ expression for the data presented in **H**. The solid line represents IFNγ expression by the CD56^{lo} subset and the dotted line represents IFNγ expression by the CD56^{hi} subset in the presence of IL-2 + IL-12 + TNFα. **C, E, G, I** The shaded histograms represent isotype staining controls.

Fig. 4. TNFR2-expressing NK cells are recruited to the liver in mice after αGalCer treatment. Recruitment of NK cells (identified as NK1.1⁺CD3⁻ cells) to the liver after αGalCer administration was evaluated by flow cytometry. Mice were treated with either αGalCer (2 μg i.v./mouse) or vehicle; 8, 16, and 48 h later, hepatic lymphocytes were isolated. **A** Time course of NK cells recruited to the liver after αGalCer treatment. There was an increase in the number of NK1.1⁺CD3⁻ cells recruited to the liver in αGalCer-treated mice in comparison to vehicle-treated mice. * $p \leq 0.01$ (n = 6/group) vs. vehicle-treated group. **B** Time course of IFNγ-producing NK cells recruited to the liver after αGalCer treatment. There was an increase in the number of IFNγ⁺NK1.1⁺CD3⁻ cells recruited to the liver in αGalCer-treated mice in comparison to vehicle-treated mice. * $p \leq 0.01$ vs. vehicle-treated group (n = 6/group). **C, D** There

were increases in the frequencies (**C**) and numbers (**D**) of TNFR2⁺ NK cells recruited to the liver 16 h after αGalCer treatment in comparison to vehicle-treated mice. The frequencies and total numbers of TNFR2⁺ NK cells recruited to the liver after αGalCer treatment did not change in CD1d KO mice compared to vehicle-treated wild-type mice. **C** *** $p < 0.001$ vs. vehicle-treated group (n = 6/group). **D** * $p \leq 0.01$ vs. vehicle-treated group (n = 6/group). NK cell deficiency (i.e. CD1d KO mice) prevented the hepatic recruitment of TNFR2⁺ NK cells to the liver after αGalCer treatment. # $p \leq 0.01$ vs. wild-type αGalCer-treated mice. **E** Representative flow cytometry dot plots for the data presented in **C**. **a** Vehicle-treated group. **b** Wild-type mice 16 h after αGalCer treatment. **c** CD1d KO mice 16 h after αGalCer treatment.

in vitro, in the presence or absence of TNFα, on total NK cell – and CD56^{hi} and CD56^{lo} NK cell – expression of TNFR2. We now demonstrate that activation of NK cells with IL-2 + IL-12 increased the percentage of NK cells expressing TNFR2 (fig. 6A). In contrast, incubation of NK cells with TNFα alone decreased the percentage of NK cells expressing TNFR2 (fig. 6A). Moreover, this pattern of IL-2 + IL-12 and TNFα-stimulated alterations in the percentages of TNFR2-expressing total NK cells was paralleled in the CD56^{lo} NK cell subset (fig. 6B). However, CD56^{hi} NK cells demonstrated a more pronounced enhancement in the frequency of TNFR2-expressing cells after stimulation with IL-2 + IL-12 versus

the CD56^{lo} subset (fig. 6C). In contrast to our findings in CD56^{lo} NK cells, TNFα alone did not alter the TNFR2-expressing NK cell frequency amongst the CD56^{hi} NK cell subset (fig. 6C). Moreover, addition of TNFα to IL-2 + IL-12-stimulated NK cells did not significantly change the percentage of NK cells expressing TNFR2 (fig. 6A–C). However, when NK cell TNFR2 expression was examined by MFI, addition of TNFα significantly attenuated the upregulation of TNFR2 expression induced by IL-2 + IL-12 in the total NK cell population (fig. 6D) and in both the CD56^{lo} and CD56^{hi} NK cell subsets (fig. 6E, F).

Fig. 5. TNFR2 deficiency attenuates murine NK cell activation to produce IFN γ . **A** Hepatic IFN γ ⁺NK1.1⁺CD3⁻ cells were evaluated by flow cytometry 16 h after vehicle or α GalCer treatment. **Inset** Representative FACS profile demonstrating the hepatic NK cell gate. Bars represent means $\pm$ SEM of the data from 5 mice/group and indicate an increase in hepatic numbers of NK cells in α GalCer-treated TNFR2 KO vs. α GalCer-treated wild-type mice. ** $p \leq 0.01$ vs. wild-type α GalCer-treated mice; *** $p \leq 0.001$ vs. α GalCer-treated TNFR2 KO mice; * $p \leq 0.05$ vs. α GalCer-treated wild-type mice. **B** Frequency of IFN γ ⁺ NK cells in vehicle-treated mice and in wild-type and TNFR2 KO mice 16 h after α GalCer treatment. *** $p \leq 0.001$ vs. both α GalCer-treated groups; ^^^ $p \leq 0.001$ vs. α GalCer-treated TNFR2 KO mice ($n = 5$ mice/group). **C** NK cell production of IFN γ as measured by MFI in wild-type and TNFR2 KO mice treated 16 h previously with α GalCer. *** $p \leq 0.001$ vs. wild-type mice ($n = 5$ mice/group). **D** Representative flow cytometry histograms depicting the lower IFN γ expression in hepatic NK cells isolated from α GalCer-treated TNFR2 KO mice (solid line; 41%) vs. α GalCer-treated wild-type mice (dotted line; 55%). The shaded histogram represents the isotype. **E** TNFR2 KO or wild-type splenic NK cells were purified and cultured with rmIL-2 +

rmIL-12 + rmTNF α , and 16 h later IFN γ levels were quantitated in culture supernatant. * $p \leq 0.05$ comparing wild-type splenic NK cells cultured with rmIL-2 + rmIL-12 + rmTNF α with wild-type splenic NK cells cultured with rmIL-2 + rmIL-12 ($n = 4$ /group); # $p \leq 0.01$ comparing TNFR2 KO splenic NK cells cultured with rmIL-2 + rmIL-12 + rmTNF α with similarly cultured wild-type splenic NK cells ($n = 4$ /group). Baseline IFN γ release in vitro from TNFR2 KO and wild-type NK cells was similar (20.6 and 18.5 pg/ml, respectively; $n = 2$ /group). Stimulation of TNFR2 KO NK cells in vitro with IL-2 + IL-12 or IL-2 + IL-12 + TNF α resulted in a similar low-level release of IFN γ (IL-2 + IL-12 = 141.0 ± 9.8 pg/ml; IL-2 + IL-12 + TNF α = 145.3 ± 30.5 pg/ml; $n = 3$ /group; not significant). **F** Representative flow cytometry histograms ($n = 3$ replicates/group) showing the lower IFN γ expression in splenic NK cells isolated from TNFR2 KO mice (dashed line; 11%) 16 h after in vitro stimulation with rmIL-2 + rmIL-12 + rmTNF α vs. similarly activated wild-type NK cells (dotted line; 42%). IFN γ production from splenic NK cells isolated from wild-type mice and stimulated in vitro for 16 h with rmIL-2 and rmIL-12 is shown as the solid line (31%). The shaded histogram represents the isotype. Experiments were repeated at least twice, with similar results.

Fig. 6. Cytokine activation of human NK cells in vitro differentially regulates TNFR2 expression (the role of TNFα). Purified human peripheral blood NK cells were cultured for 24 h in the presence of buffer alone, TNFα alone, IL-2 + IL-12, or IL-2 + IL-12 + TNFα. **A** Changes in the frequency of TNFR2-expressing NK cells induced by activation with different cytokine mixtures. *** $p \leq 0.001$ buffer group vs. all other groups; ^^^ $p \leq 0.001$ TNFα group vs. all other groups ($n = 4/\text{group}$). **B** Changes in the frequency of CD56^{lo} NK cells expressing TNFR2. *** $p \leq 0.001$ buffer vs. TNFα group; ** $p < 0.01$ buffer group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups; ^^^ $p \leq 0.001$ TNFα group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups ($n = 4/\text{group}$). **C** Changes in the frequency of CD56^{hi} NK cells expressing TNFR2. *** $p \leq 0.001$ buffer group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups; ^^^ $p \leq 0.001$ TNFα group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups. **D–F** TNFR2 ex-

pression by NK cells as quantified in terms of MFI. **D** TNFR2 expression on all NK cells. *** $p \leq 0.001$ buffer group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups; ^^^ $p \leq 0.001$ TNFα group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups; ^^ $p \leq 0.01$ TNFα vs. buffer group; +++ $p \leq 0.001$ IL-2 + IL-12 vs. IL-2 + IL-12 + TNFα group ($n = 4/\text{group}$). **E** TNFR2 expression on CD56^{lo} NK cells. *** $p \leq 0.001$ buffer group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups; * $p \leq 0.05$ buffer vs. TNFα group; +++ $p \leq 0.001$ IL-2 + IL-12 vs. IL-2 + IL-12 + TNFα group; ^^^ $p \leq 0.001$ TNFα group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups. **F** TNFR2 expression on CD56^{hi} NK cells. *** $p \leq 0.001$ buffer group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups; +++ $p \leq 0.001$ IL-2 + IL-12 vs. IL-2 + IL-12 + TNFα group; ^^^ $p \leq 0.001$ TNFα group vs. IL-2 + IL-12 and IL-2 + IL-12 + TNFα groups. Similar results were obtained from 3 different donors.

Discussion

NK cells are critical players in innate immune responses and are important for antimicrobial defense and tumor immunosurveillance [5]. To carry out these tasks, NK cells are well armed for the cytotoxic destruction of transformed cells [1, 2, 5]. However, NK cells also mediate immunoregulatory effects through the release of numerous cytokines and chemokines, with IFNγ playing a key effector role [2, 5, 9]. IFNγ is a central regulator of both innate and adaptive immune responses by promoting viral clearance and suppressing viral replication, by recruiting other immune cells into inflamed tissues, through the polar-

ization of adaptive immunity towards a Th1 phenotype, and by promoting the maturation and activation of dendritic cells, macrophages, and T cells [38]. Importantly, both NK cells and IFNγ have also been linked to the development and/or exacerbation of autoimmunity [10, 21, 38, 39]. Production of IFNγ from NK cells can be induced by cell-cell contact, but also through cytokine stimulation [3, 4, 6]. The cytokines most closely associated with NK cell activation and IFNγ release include IL-2, IL-12, IL-18, and IL-15 [4, 9, 40]. TNFα can activate NK cells to enhance cytotoxicity [17]. However, an effect of TNFα upon NK cell production of IFNγ has not been defined, but is clearly of potential importance in better understanding

the role of TNF α in the NK cell-mediated regulation of innate immune responses and autoimmunity.

We speculated that TNF α may play an important role in regulating NK cell production of IFN γ , and furthermore we postulated that TNFR2 would be important for mediating this TNF α -related effect on NK cells. In keeping with this hypothesis, TNF α activation of TNFR2 has been previously shown to enhance NK cell cytotoxicity and cytokine production during NK cell and dendritic cell interactions in mice and humans [18, 41]. In our current study, we found that the majority of human peripheral blood NK cells express TNFR2, with >90% of CD56^{lo} and >50% of CD56^{hi} NK cells expressing TNFR2. Similar to our findings in human peripheral blood, differential TNFR2 expression was found on murine peripheral blood NK cells subdivided into CD27^{hi} versus CD27^{lo} subsets, which have been reported to parallel human NK subsets divided using CD56 expression [36, 37]. Moreover, we demonstrate that TNF α can activate both human and murine cytokine-stimulated NK cells via TNFR2 to augment IFN γ production. We also demonstrate with a mouse model of hepatic innate immune activation that TNFR2 expression is important for regulating the production of IFN γ by hepatic NK cells. Moreover, it has previously been shown in this model that TNF α is a critical mediator of liver injury [31]. We now show in this model that TNF α -TNFR2 interactions regulate the severity of liver injury, as TNFR2 KO mice are less susceptible to α GalCer-induced liver injury as reflected by reduced serum alanine aminotransferase levels in α GalCer-treated TNFR2 KO versus wild-type mice (levels as IU/l: vehicle = 24.0 ± 3.2 vs. wild-type + α GalCer = $246.2 \pm 34.4^*$ vs. TNFR2 KO + α GalCer = 69.8 ± 8.5 ; $n = 4-5/\text{group}$; $*p \leq 0.001$). These findings may have significant implications for the regulation of immune responses in general, and could potentially explain, at least in part, a number of observations associated with TNF α inhibition in the clinical setting.

TNF α plays a critical role in regulating the clinical expression of many immune-mediated diseases [42, 43]. As a result, inhibition of TNF α has become a widely used and effective therapy for many of these diseases, including inflammatory bowel disease, rheumatoid arthritis, and autoimmune liver disease [44, 45]. However, inhibition of TNF α in the clinical setting has been associated with the reactivation of a number of infectious diseases (e.g. hepatitis B and tuberculosis) and an increased risk for developing malignancy, indicating that TNF α is also important for the suppression of these diseases [46–48]. However, it remains unclear whether this is a direct or an indirect ef-

fect of TNF α [46]. Interestingly, TNF α inhibition has been shown to suppress IFN γ production from a mixed lymphocyte population in vitro, and to inhibit NK cell activation (as measured by cell surface CD69 expression); however, the mechanism underlying these effects was not defined [49, 50]. Recently, Serti et al. [19] demonstrated that monocytes isolated from patients with hepatitis C infection produce lower amounts of TNF α than those isolated from healthy donors. In addition, the monocytes isolated from these hepatitis C patients were less effective than monocytes isolated from healthy donors in stimulating IFN γ production from NK cells in an in vitro coculture system [19]. These findings suggest that TNF α -related antiviral effects may be mediated indirectly through the induction by TNF α of IFN γ production from NK cells, a pathway that is defective in hepatitis C-infected patients. Our current data are consistent with this suggested mechanism. In addition, we demonstrate that TNF α -induced NK cell production of IFN γ is augmented via stimulation of TNFR2. This paradigm also aligns with the observed reactivation of hepatitis B infection in patients treated with TNF α -neutralizing therapies, a disease in which IFN γ plays a key role in suppressing viral replication [46].

In response to infection or inflammation, NK cells are rapidly recruited to injured tissues, where they are in turn activated and establish immune-modulatory effector roles, including the production of IFN γ [4–6, 32, 40]. α GalCer treatment leads to the rapid activation of NKT cells within the liver and the subsequent production of numerous cytokines (including TNF α), which leads to the robust recruitment of NK cells to the liver [27, 32]. NK cells recruited in this fashion are activated and produce large amounts of IFN γ [31, 32]. Therefore, α GalCer treatment in the mouse provides a useful tool to examine the potential impact of TNFR2 deficiency in vivo upon the capacity of liver-recruited NK cells to produce IFN γ . We found that α GalCer treatment resulted in the rapid recruitment of TNFR2-expressing NK cells to the liver. Moreover, these liver-recruited NK cells produced IFN γ . Surprisingly, in the absence of TNFR2, greater numbers of NK cells overall were recruited to the liver, an observation that warrants future studies. In addition, a reduced frequency of IFN γ -producing liver-recruited NK cells was documented in TNFR2 KO versus wild-type mice treated with α GalCer. Moreover, IFN γ production per hepatic NK cell was decreased in TNFR2 KO mice versus wild-type controls treated with α GalCer, as measured by MFI. Consistent with these in vivo observations, we found by an in vitro murine NK cell culture system that TNF α

acting via TNFR2 plays a key role in enhancing cytokine-mediated IFN γ production. Similarly, we also showed that cytokine-induced IFN γ production from freshly isolated human peripheral blood NK cells was augmented by TNF α as well as by a specific TNFR2 agonistic antibody. These observations clearly highlight the importance of the TNF α -TNFR2 pathway in enhancing cytokine-driven NK cell IFN γ production.

Collectively, this work establishes an important role for TNF α , acting via TNFR2, in the regulation and augmentation of NK cell production of IFN γ . In addition, we report for the first time that cytokine stimulation, as well as TNF α activation, differentially regulates TNFR2 expression on human NK cells, a finding that likely has its importance in the regulation of NK cell responses within inflamed tissues. Moreover, we confirm that this TNF α -TNFR2 pathway is important for the establishment of the

presence of IFN γ -producing NK cells within the liver during an innate immune response. These novel findings have significant potential clinical implications for the role of TNF α and TNFR2 in the regulation of innate and adaptive immune responses.

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