**Cord Blood Natural Killer Cells Expressing a Dominant Negative TGF- β Receptor:
Implications for Adoptive Immunotherapy for Glioblastoma**

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Cord

ABSTRACT

Cord blood (CB) natural killer (NK) cells are promising effector cells for tumor immunotherapy, but are currently limited by immune suppressive cytokines in the tumor microenvironment like Transforming Growth Factor β (TGF- β). We observed that TGF- β inhibits expression of activating receptors like NKG2D and DNAM1, and decreases killing activity against glioblastoma tumor cells through inhibition of perforin secretion. To overcome the detrimental effects of TGF- β , we engrafted a dominant negative TGF- β receptor II (DNRII) on CB-derived NK cells by retroviral transduction and evaluated their ability to kill glioblastoma cells in the presence of TGF- β . Following manufacture using GMP compliant methodologies and transduction with DNRII, CB-derived DNRII-transduced NK cells expanded to clinically relevant numbers and retained both their killing ability and their secretion of IFN- γ upon activation. More importantly, these cells maintained both perforin expression and NKG2D/DNMA1 expression in the presence of TGF- β , allowing for recognition and killing of glioblastoma tumor cells. Hence, NK cells expressing a DNRII should have a functional advantage over unmodified NK cells in the presence of TGF-beta-secreting tumors and may be an important therapeutic approach for patients with cancer.

INTRODUCTION

Glioblastoma multiform (GBM) is the most aggressive diffuse glioma of astrocytic lineage [1] and also the most common brain and CNS malignancy, accounting for 45.2% of malignant primary brain and CNS tumors, and 54% of all gliomas [2]. Despite recent advances, the prognosis remains poor with a median survival of 15 months [3] and a relative survival of only 13.7% at two years [2] with less than 5% of patients surviving at 5 years [4]. Therefore, the development of novel targeted immune based therapies could be a critical advance for improving outcomes in patients with chemotherapy-resistant recurrent/refractory GBM.

NK cells (CD3-CD56+ lymphocytes) can rapidly kill tumor cells without prior immunization or MHC restriction [5]. Their cytotoxic activity is triggered by multiple factors: (a) lack of appropriate expression of the self-major histocompatibility complex (MHC) on target cells, leading to the absence of signals from killer immunoglobulin-like receptors (KIR) that normally hold NK cells in check; (b) recognition of upregulated ligands on target cells (stress-induced ligands MICA/MICB and UL16-binding proteins); (c) robust recognition of antibody-coated target cells, which activates the type IIIA Fc receptor (FcRgIIIA or CD16) and triggers NK cell-mediated ADCC; and (d) cytokines including IFN- γ and interleukins (IL)-12, IL-18, IL-2, IL-21 and IL-15 that enhance the activation or promote the maturation of NK cells [6-8]. Although evidence supports the role of NK cells in tumor immune-surveillance at an early stage of disease [9], the upregulation of MHC Class I molecules on tumor cells at advanced disease stages together with the presence of inhibitory cytokines such as TGF- β , IL-6 and IL-10 in the tumor microenvironment and the shedding of soluble ligands as decoys, tends to favor tumor growth over the activation of NK cells [10].

It has become clear that a substantial proportion of patients with GBM do not receive a benefit from the missing ligand response of NK cells. Reasons for this include evidence that NK cells from these patients are dysfunctional and negatively affected by the TGF- β -rich tumor microenvironment. Tumors may also upregulate class I upon encounter with NK to evade killing [11]. Indeed, TGF- β is a potent immune suppressive strategy employed by most human cancers with devastating effects on NK cell function [10, 12]. IFN- γ production by NK cells is considered essential for stimulation of the Th1 response, and TGF- β attenuates this activity as well as the cytolytic activity of these cells [13, 14]. Indeed, targeted killing by NK cells, which depends upon productive engagement of the NK receptors NKp46, NKp30, NKp44, and NKG2D [15], is significantly decreased by exogenous TGF- β [16]. Furthermore, studies using mice harboring immune cells resistant to TGF- β signaling have enhanced tumor rejection compared with controls [17]. Finally, down modulation of NKG2D has been associated with elevated TGF- β levels in cancer patients [18]. These observations support an immunosuppressive effect of TGF- β on NK cells under physiologic conditions in cancer patients and also provide a compelling rationale for blunting the inhibitory effect of TGF- β on NK cells when exploring this as an anti-cancer therapy.

Clinical trials utilizing allogeneic NK cell therapy have used peripheral blood (PB) or cord blood (CB) (NCT01729091) as a cell source [19, 20]. Allogeneic cells have the advantage of circumventing the evasion caused by MHC expression, as they can be tailored to have mismatches against the tumor HLA. The use of cord blood as a source of allogeneic NK cells is advantageous because of their immediate availability in established cord blood banks allowing greater flexibility for the identification of HLA-compatible and KIR-mismatched lines. With more than 600,000 banked cord blood units worldwide [21], the global inventory of these cells represents a unique opportunity as a readily available donor source.

Here, we sought to determine whether cord blood-derived NK cells can be genetically engineered to resist the immune-dampening effects of TGF- β . In this report, we demonstrated that cord blood NK cells can be efficiently engineered to express a dominant negative receptor II for TGF- β (DNRII) and maintain their cytolytic activity against glioma tumor cells even after exposure to TGF- β . Our results pave the way for translation to the clinic for the treatment of patients with glioblastomas and other TGF- β secreting cancers using a NK-cell based immunotherapy approach.

RESULTS

Non-transduced and DNRII-transduced Cord Blood-derived NK cells are Phenotypically Similar

NK cells derived from four cord blood units were transduced with the retroviral vector encoding for DNRII. Six days after transduction, the expression of DNRII was analyzed by flow cytometry using the TGF- β RII antibody identifying a mean of 75.8% NK cells were DNRII positive (range, 60.6 to 88.1%) (**Figure 1A**). The antibody used in our study to evaluate the transduction efficiency and expression of the DNRII transgene did not readily detect wildtype TGF- β RII on non-transduced cord blood NK cells at day -12 of the culture (**Figure 1A**). To determine whether the transduction of NK cells using the retrovirus DNRII construct affect their phenotypic characteristics, analysis of expanded NT and DNRII-expressing NK cells was performed on day 12. As shown in **Figure 1B**, there were no differences in the expression of the activating receptors NKG2D, DNAM1 and the NCRs Nkp30, 44 and 46 between the NT and DNRII transduced NK cells. In addition, the contamination of T cells (CD3+CD56- lymphocytes) in the day 12 culture was consistently less than 1% in the NT and DNRII-expressing NK-cell products, validating our ex vivo expansion culture system where CD56+ cells are selected first, transduced second and then expanded using the K562-C9 cells (**Figure 1B**).

DNRII-expressing Cord Blood NK cells Expand to Clinically Relevant Numbers

To determine if the genetic modification of cord blood-derived NK cells hampered their proliferation, we expanded NK cells for 12 days and recorded the total nucleated cell counts. In our experiments, we observed a markedly lower fold cell expansion in the DNRII-expressing NK-cells compared to the NT NK cell products with a mean 769 (+/- 308) vs mean 1,376 (+/- 189) fold expansion respectively at day 12 of the cultures (**Figure 2A**). However, the reduction

in cell expansion was not due to the DNR11 transgene per se but rather the genetic modification since NK cells transduced with a control retroviral vector (encoding a GD2 Chimeric Antigen Receptor (CAR) [22] displayed an identical cell expansion profile as the DNR11 transduced NK cells (**Supplementary Figure 1**). Nevertheless, despite the reduced expansion of the transduced NK cells, based on our current experience using CB-derived NK cells clinically (NCT01729091) these expansion rates are sufficient to achieve the NK cell numbers required for clinical use. However, because uncontrolled growth of NK cells is a potential concern after transformation with the DNR11 transgene, we evaluated the ability of NK cells to expand in culture in the absence of supporting growth factors. On day 12, non-transduced and DNR11-transduced NK cells were cultured for an additional 7 days with or without IL-15 and cell counts performed every 2-3 days. Transduced and non-transduced cord blood NK cells were able to expand when cultured in the presence of IL-15. However, as shown in **Figure 2B**, we did not observe autonomous growth of the transduced and non-transduced cord blood NK cells grown in the absence of IL-15.

SMAD2 Phosphorylation by TGF- β is inhibited in DNR11-Expressing Cord Blood NK cells

To demonstrate the protective effect of the DNR11 receptor, we looked at downstream TGF- β signaling in cord blood NK cells. TGF- β was added to NT and DNR11-transduced NK cells for 45 minutes after which the presence of the phosphorylated (Ser465/467) form of SMAD2 (pSMAD2) was analyzed by western blot. As shown in Figure 3, pSMAD2 is detected in NT NK cells and control cells (Jurkat cells) when exposed to TGF- β . In contrast, pSMAD2 is absent in the DNR11-transduced NK cells exposed to TGF- β (**Figure 3**). Hence, these results confirmed that in DNR11-transduced NK cells, signal transduction of TGF- β was efficiently blocked by the DNR11 receptor transgene. The non-phosphorylated form of SMAD2 was detected in all the NK cells cultures irrespective of the presence or absence of TGF- β .

NKG2D and DNAM1 expression is not modulated by TGF- β in DNR11-expressing Cord Blood NK cells.

The activating receptors NKG2D and DNAM1 play an important role in the recognition and killing of malignant glioma tumor cells including cancer stem cells [23, 24]. However, the expression of these two NK-cell receptors is strongly down regulated by TGF- β [24-26]. Therefore, we determined the effect of TGF- β on the expression of NK-cell activating receptor after 5 days of exposure to TGF- β at a range of doses (2.5, 5, 10 and 20 ng/mL). As expected, cord blood NK cells exposed to TGF- β displayed a marked loss of NKG2D and DNAM1 receptor expression (**Figure 4**). An identical pattern of expression loss was also observed for CD16, NKp30 and NKp44 when non transduced NK cells were exposed to TGF- β and only NKp46 did not appear to be affected by exposure to TGF- β irrespective of the dose tested (**Supplementary Figure 2**). In contrast, DNR11 transduced NK cells maintained expression of the NKG2D and DNAM1 receptors on their cell surface even after exposure to TGF- β (**Figure 4**).

DNR11-expressing cord blood NK cells maintain their cytolytic activity after exposure to TGF- β .

On day 12 of ex vivo expansion, NT and DNR11-expressing NK cells were cultured an additional 5 days in the presence or absence of TGF- β (5ng/mL) and cytotoxic activity was evaluated in a standard 4-hour ^{51}Cr release assays. After exposure to TGF- β , the ability of NT NK cells to kill the U87 cell line was significantly reduced by a mean of 65% (range 60 to 96% inhibition; $p = 0.0057$) (**Figure 5A & 5C**). In contrast, the DNR11-transduced NK cells cultured in the presence of TGF- β maintained their cytolytic capability (**Figure 5A & 5C**). The same pattern of inhibition was observed when NK cells were tested against the cell line K562 (**Figure 5B & 5D**) and the

neuroblastoma cell line LAN-1 (**Supplementary Figure 3**) showing that the protective effect of DNR11 is applicable against other tumors known to secrete TGF- β [27]. The difference in killing levels between the K562 and U87 cell lines by the cord blood-derived NK cells can be attributed to a difference in the target cell phenotypes with the glioma cell line U87 expressing lower levels of the NK-cell activating ligands, higher expression of the inhibitory MHC class I molecules (**Supplementary Figure 4**) and probably expression of anti-apoptotic molecules from the Bcl-2 subfamily [28] compared to the K562 cells.

DNR11-expressing and Non-Transduced cord blood NK cells retain their ability to secrete IFN- γ after exposure to TGF- β .

To further address the impact of TGF- β on cord blood derived NK cells, IFN- γ production was assayed after co-culture with U87 tumor cells as well as control target cells (K562). As expected, the NT- and DNR11-NK cells produced IFN- γ when co-cultured with K562 and U87 (**Figure 6**). However, exposure of the NK cells to TGF- β (5ng/mL) for 5 days appeared to result in an increase – albeit moderate – of IFN- γ production by either the expanded NT- or DNR11-transduced NK cells (**Figure 6**).

Perforin level is Reduced in Non-Transduced but Preserved in DNR11-transduced Cord Blood NK cells in the Presence of TGF β .

In previous reports, a loss of cytolytic activity despite an increase in IFN- γ release has been associated with defective perforin levels in T cells and NK cells [29-31]. To determine whether the same mechanism is at play in ex vivo expanded cord blood NK cells exposed to TGF- β , intra-cellular levels of perforin and granzyme B were measured by flow cytometry. As shown in **Figure 7**, after 5 days of exposure to TGF- β , non-transduced cord blood NK cells had a significant reduction in perforin levels as determined by intracellular flow cytometry ($p = 0.0021$).

In contrast, perforin expression was largely preserved in DNR11-transduced cord blood NK cells exposed to TGF- β ($p = 0.0496$) (**Figure 7**). No changes in Granzyme B expression was observed in DNR11-transduced and non-transduced expanded cord blood NK cells when exposed to TGF- β for 5 days (**Figure 7**).

DISCUSSION

In the present study, we demonstrate that expression of a TGF- β dominant negative receptor (DNR11) effectively protects cord blood-derived NK cells against the detrimental effects of this powerful immune-suppressive cytokine. Although a previous study showed that the NK-cell line NK92 can be rendered resistant to TGF- β [32], we believe that cord blood-derived NK cells are a more appropriate source of effector cells as they can be expanded from banked cryopreserved cord blood units as an “off the shelf therapeutic”. [33] Moreover, NK-cell products derived from banked cord blood units can be selected according to KIR expression and do not need to be irradiated unlike NK-derived cell lines such as NK-92.

Our group has developed an efficient method for the transduction of cord blood NK cells with a mean transduction efficiency of 75.8% (range 60.6% to 88.1%) [34]. Although the transduction procedure decreases the expansion of DNR11-transduced cord blood NK (and vector control transduced NK cells), clinically relevant numbers of DNR11-expressing cord blood NK cells were still easily generated using our approach. Because autonomous growth of primary effector cells like T cells or NK cells expressing the DNR11 transgene is a potential concern, we evaluated the expansion of DNR11-transduced cord blood NK cells in the absence of growth factors (i.e. IL-15).

We did not observe any autonomous growth similar to the observations reported using T cells expressing the DNR11 transgene [35].

Although glioblastoma tumor cells can efficiently be killed by ex vivo expanded cord blood-derived NK cells, it is evident that exposure of expanded cord blood NK cells to a TGF- β rich microenvironment has a detrimental effect on their cytolytic activity. U87 cells secrete TGF- β , emphasizing its use by these malignant cells as an immune suppressive strategy (**Supplementary Figure 5**). Indeed, TGF- β -mediated downregulation of the activating receptor NKG2D allows glioblastoma tumor cells to avoid their recognition by NK cells and thereby resist elimination [24]. In our study we showed marked downregulation of the activating receptors NKG2D and DNAM1 on expanded cord blood NK cells after exposure to TGF- β . The decrease in expression of these two activating receptors was associated with a dramatic decrease in the killing capability of the glioblastoma tumor cell U87 by the expanded cord blood NK cells. However, DNR11-transduced cord blood NK cells maintained their NKG2D and DNMA1 expression levels and were able to kill the U87 tumor cells even after 5-day exposure to TGF- β hence validating our hypothesis that cord blood NK cells can be manipulated to resist the effects of TGF- β .

To further analyze the effects of TGF- β on ex vivo expanded cord blood NK cells, non-transduced NK cells exposed to TGF- β for 5 days were co-cultured with K562 and U87 tumor cells and supernatant harvested at 24 hours to measure the release of IFN- γ , a hallmark of NK-cell activation.[36]. We observed a moderate increase in IFN- γ release by activated cord blood NK cells exposed to TGF- β , even though the killing activity was blunted. Previous reports in perforin knock out mice have shown that T cells (CD8+) as well as NK cells displayed the same type of behavior with failure to kill target cells associated with a hypersecretion of IL-2, TNF,

IFN- γ as well as chemokines [29]. The authors concluded that a delayed death of the target cells was responsible for an increase in synapse time hence the increase in cytokines release. We hypothesized that the same mechanisms were at play in our experimental model and therefore looked at perforin expression in cord blood NK cells after exposure to TGF- β . Decreased perforin expression after TGF- β treatment was detected in unmodified CB-derived NK cells but not in CB-derived NK cells expressing the DNRII transgene. In addition, we observed that specific blocking of perforin in cord blood NK cells by Concanamycin A (CMA) was responsible for the loss of the U87 tumor cells killing and was associated with an increase of IFN- γ release by cord blood NK upon activation (**Supplementary Figure 6**). These two critical observations indicated that effect of TGF- β on perforin expression in ex vivo expanded NK cells is a major tumor immune evasion strategy, which can be overcome by genetically modifying CB-derived NK cells with the DNRII. In addition to perforin, TGF- β also affects the expression of granzyme B, a protease found in the granules of cytotoxic lymphocytes and NK cells [25]. We did not observe reduced expression of granzyme B in ex vivo expanded cord blood NK cells after treatment with TGF- β , which may be related to the activated state of the NK cells prior to exposure to TGF- β . However, we did not look at the gene expression of CB-derived NK cells exposed to TGF- β and therefore cannot exclude that longer exposure to TGF- β would affect not only the expression of the activating receptors NKG2D and DNAM1 but also expression of granzyme B as well as perforin.

If NK-cell based therapy in glioblastoma is to be of clinical value, it will be necessary to generate NK cells that would not only kill tumor cells, but also resist the immune-suppressive effects of TGF- β . The negative impact of TGF- β on the cytolytic activity of NK-cells is devastating and must be abrogated to ensure the success of NK-cell-based adoptive cell therapy. The results reported here demonstrate that engrafting a dominant negative receptor (DNRII) protects cord

blood-derived NK cells against the detrimental effects of TGF- β . For future studies, it will be meaningful to define the contribution of DNR-II to the anti-tumor activity of NK cells in vivo and to compare the cytotoxic activity and function of DNRII-expressing NK cells, with or without the incorporation of a tumor specific chimeric antigen receptor.

In summary, we have shown that TGF- β negatively affects the function of expanded cord blood NK cells. Further, we demonstrate for the first time that these detrimental effects can be overcome by genetically transforming ex vivo expanded CB-derived NK cells to express a dominant negative receptor for TGF- β . We believe that administration of DNRII-expressing third party cord blood-derived NK cells will be a highly beneficial “off the shelf” therapeutic not only for patients with glioblastoma but also for the treatment of most solid tumors which utilize TGF- β secretion as a potent immune evasion strategy.

MATERIALS & METHODS

Cells and cell lines

NK cells were enriched from cord blood units obtained under IRB approved protocols at MD Anderson Cancer Center and Baylor College of Medicine (Houston, Texas) using CD56 microbeads (Miltenyi Biotech Inc; San Diego, CA) and either directly cultured or cryopreserved for further use. In this study, NK cells were grown in NK-media composed of SCGM-media (CellGenix GmbH; Freiburg, Germany) supplemented with 10% Fetal Bovine Serum (Hyclone™, GE Healthcare Life Sciences; Logan, UT), 2mM Glutamax (Gibco™, ThermoFisher Scientific; Waltham, MA) and Penicillin / Streptomycin (Gibco™). The Chronic Myelogenous Leukemia K562 cell line, Acute T Cell Leukemia Jurkat cell line and the glioblastoma U87 cell line were purchased from ATCC (Manassas, VA) and were maintained in culture in IMDM (Hyclone™) base medium containing 10% FBS (Hyclone™), Penicillin/Streptomycin (Gibco™) and 2mM Glutamax (Gibco™). The Neuroblastoma LAN-1 cell line was purchased from DSMZ (Leibniz, Germany) and was maintained in culture in RPMI (Hyclone™) base medium containing 10% FBS, Penicillin/Streptomycin and 2mM Glutamax. A genetically modified K562 cell line named K562-C9 [37] was used for the activation/expansion of non-transduced as well as transduced NK cells. These modified K562 express membrane bound IL21 and 41BBL. K562-C9 cells were grown in IMDM supplemented with 10% Fetal Bovine Serum, 2mM Glutamax and Penicillin / Streptomycin.

Plasmid construction and retrovirus production

The method used for the generation of retroviral vector encoding the truncated human type II TGF- β receptor (DNRII) has been previously described [38]. Briefly, a cassette encoding the DNRII transgene was cloned into a SFG retroviral vector and transient viral supernatant generated using the Phoenix-eco cell line. Retroviral supernatant was then used to transduce

(using multiple rounds of transduction) the packaging cell line PG13. In our work, the SFG plasmid construct used to generate the PG13 producer cell line did not contain the influenza virus hemagglutinin peptide epitope HA1 sequence. The GalV-pseudotyped replication-incompetent retrovirus supernatant encoding the DNR11 transgene was harvested and stored at -80°C until needed.

NK-cell transduction

To achieve high transgene expression in NK cells using a retroviral construct, we developed a GMP compliant procedure that required a single round of transduction [34]. Briefly, CD56+ selected NK cells derived from cord blood, either freshly isolated or cryopreserved, were pre-activated with 10ng/mL of hIL-15 (R&D Systems; Minneapolis, MN) in NK media for 24 hours [39]. NK cells were then collected, counted and activated with irradiated K562-C9 (100 Gy) at a ratio of one NK cell for two K562-C9 in presence of recombinant hIL-2 (200 IU/mL) (Proleukin; Chiron, USA). Activated NK cells were then transduced with the retroviral supernatants on day 4 in plates coated with recombinant fibronectin fragment CH-296 (Takara Shuzo, Japan). After transduction, the NK cells were further expanded for an additional 8 days using IL-2 (200 IU/mL) every 2-3 days and irradiated K562-C9 (100Gy and 1:2 ratio) for a second NK stimulation at day 7 of the culture. Phenotypic characteristics and functional activity of the NK cells were evaluated at day 12 of the culture.

Flow Cytometry of NK Cells and Targets

The following antibodies were used: CD3 (SK7), CD226 (DX11), NKp30 (p30-15), NKp44 (p44-8), NKp46 (9-E2), Perforin (delta G9), Granzyme B (GB11), CD112 (R2.525), HLA-ABC (G46-2.6) and HLA-DR (L243) from BD-Biosciences (San Jose, CA); NKG2D/CD314 (149810), MICA/B (159207), ULBP-1 (170818), ULBP-3 (166510), CD155 (300907) and TGF- β RII (Polyclonal Goat IgG) were purchase from R&D Systems; CD56 (MEM-188) and HLA-E (3D12)

were purchase from Biolegend (San Diego, CA). To detect the expression of a Chimeric Antigen Receptor (CAR) on transduced NK cells, we are using a secondary antibody to human Fc (DyLight™ 649-conjugated AffiniPure Mouse anti-human IgG, Fc γ fragment specific; Jackson ImmunoResearch Laboratories Inc; West Grove, PA). Cells were acquired with a FACSCalibur system (BD-Biosciences) and data analyzed using the Becton Dickinson CellQuest-Pro software.

NK Cell function assessment after treatment with TGF- β

Recombinant human TGF- β 1 was purchased from PeproTech (Rocky Hill, NJ) and stock solution of TGF- β prepared at the concentration of 50ng/ μ L and stored at -80°C until needed. Prior using TGF- β in our NK-cell cultures, stock solution was diluted 1/10 in HCl (4mM) for a final concentration of 5ng/ μ L. After a 30-minute incubation time, HCl-diluted TGF- β was added to the NK-cell cultures at the indicated concentration.

To study the effect of TGF- β on NK cells [DNRII-expressing and non-transduced (NT)], the NK cell cultures were harvested on day 12 and 6x10⁶ NK cells from each condition were replated in 20 mL of NK-media in the presence or absence of TGF- β at a concentration of 5 ng/mL. NK-cell cultures were replenished with IL2 (200U/mL) and TGF- β on days 14. NK cells were then harvested on day 17 and the functional effect of TGF- β on expanded NK cells was studied.

Western Blot for Smad2 and pSmad2

Transcriptional activation of NK cells exposed to TGF- β was analyzed by Western blot. NK cells that were either non transduced or transduced with DNRII were treated with 5ng/mL human TGF- β (PeproTech) for 45 minutes at 37°C. Jurkat cells were similarly treated and used as control. Fifteen million cells were pelleted and re-suspended in RIPA lysis buffer (Pierce; Rockford, IL) containing protease inhibitors (Pierce) and incubated on ice for 10 minutes, then

centrifuged at 13,000 rpm for 10 minutes. Supernatants were harvested, mixed 1:2 with Laemmli buffer (Bio-Rad; Hercules, CA), boiled, and 100µg of proteins loaded on a precast 10 - 20% SDS-polyacrylamide gel (Bio-Rad). After transfer, nitrocellulose membranes (Invitrogen Life Technologies; Grand Island, NY) were probed with a rabbit anti-human antibody against Smad2 (86F7) (60kDa), and a rabbit antibody against Phospho-Smad2 (Ser465/467) (60kDa), at concentrations suggested by the manufacturer (both antibodies from Cell Signaling Technology; Danvers, MA). Membranes were then exposed to peroxidase conjugated secondary antibodies and protein expression was quantitated by chemi-luminescence detection (LumiGLO[®]; Cell Signaling Technology). Blots were stripped and re-probed with rabbit anti-β-Actin (45 kDa) (Cell Signaling Technology) to confirm equal protein loading.

Cytotoxicity Assays

Specific killing of targets cells has been determined by a standard ⁵¹Cr release assay as previously described [38]. Briefly, NK cells were co-incubated in triplicate for 4 hours with 5,000 ⁵¹Cr-labeled target cells (Amersham Pharmacia Biotech Inc.; Piscataway, NJ). The targets used were the cell lines K562 and U87 at various NK-Cell to Tumor-Cell ratios.

Measurement of cytokine production by ELISA

Half a million (0.5x10⁶) DNR-expressing versus non-transduced NK cells were cultured in a well of a 24-well plate in the presence of the glioblastoma tumor cell line U87 or the K562 cell line at an NK cell to tumor cell ratio of 5:1. Culture supernatant were collected after 24 hours and stored at -80°C for further analysis of IFN-γ content by Enzyme-Linked Immuno-Absorbent Assay (ELISA). Manufacturer instructions were followed (R&D systems).

Statistical analysis

To determine the statistical significance of the observed differences between experimental groups, the Student's *t-test* was used. $P < 0.05$ was considered significant using GraphPad Prism software.

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FIGURE LEGENDS

Figure 1: CB-NK cells are genetically modified to express the DNRII and maintain their expression of activating receptors. **A.** CB-NK cells were transduced with a retroviral vector encoding for DNRII and 6 days' post transduction were stained with PE-labeled anti-TGF- β RII or isotype control antibodies to evaluate the transduction efficiency. Left panel shows a representative flow analysis for the TGF- β RII expression on cord blood NK cells. The right panel shows the results for transduction efficiency of CB-NK cells (n=5). **B.** Representative flow analysis showing the NK-cell phenotype and CD3+CD56- T cells contamination of in cord blood NK cell products after 12 days' ex vivo expansion for non-transduced CB-NK cells and DNRII-expressing CB-NK cells.

Figure 2: Ex vivo expanded CB-NK cells expressing DNRII remain dependent on growth factors for survival and expansion in vitro. **A.** Mean fold expansion of CB-NK cell products derived from five separate experiments are shown. The non-transduced cultures demonstrated significantly greater CB-NK expansion than DNRII-expressing CB-NK cell products (p=0.0148). **B.** CB-NK cells harvested on day 12 were cultured for an additional 7 days in the presence or absence of the growth factor IL-15 (5ng/mL). Viable cells were counted by trypan blue exclusion every 2-3 days. Data in this panel represent the mean $\pm$ SD of 5 CB-NK cell lines.

Figure 3. Inhibition of SMAD2 Phosphorylation by TGF- β in Cord Blood NK cells expressing DNRII. After one hour of exposure to TGF- β (5ng/ml) as indicated, the presence or absence of phosphorylated Smad2 (pSmad2) was detected by Western Blotting. Presence of Smad2 was also performed by Western Blotting and Jurkat cells were used as positive control

for TGF- β induced phosphorylation of the Smad2 protein. Beta-actin was used in the experiment as protein loading control.

Figure 4: Expression of NKG2D and DNAM1 is maintained on CB-NK cells expressing DNR11. On day 12, non-transduced and DNR11-transduced CB-NK cells were cultured for an additional 5 days in the presence or absence of TGF- β (5ng/mL), and IL-2 (200IU/ml). Expression of NKG2D and DNAM1 was evaluated by flow cytometry. **A.** Representative surface expression of NKG2D and DNAM1 is shown. **B.** This panel shows the mean expression of the receptors NKG2D and DNAM1 in DNR11-transduced and non-transduced CB-NK cell products (n=5 in each group) after culture in the presence or absence of TGF- β (5ng/mL).

Figure 5: Exogenous TGF- β decreases the cytolytic activity of Non-Transduced Cord Blood-derived NK cells but not DNR11-transduced Cord Blood NK cells. On day 12 of culture, ex vivo expanded non-transduced and DNR11-transduced CB-NK cells were cultured for an additional 5 days in the presence or absence of TGF- β (5ng/mL), and IL-2 (200IU/ml). Specific lysis of the K562 and U87 targets was then evaluated in a standard ^{51}Cr release assay. **A & B.** Representative experiments show the percentage of specific lysis of the K562 and U87 targets respectively. **C & D.** The mean specific lysis of K562 and U87 respectively are shown (n = 4 in each group).

Figure 6: Cord Blood NK cells continue to produce IFN- γ in response to activation even after exposure to TGF- β . Non-transduced and DNR11-transduced NK cells were harvested on day 12 and cultured for an additional 5 days in the presence or absence of TGF- β (5ng/ml) and co-cultured with either K562 or U87 tumor cells at a 5:1 NK-cell to tumor-cell ratio. After 24 hours, supernatants were collected and analyzed for IFN- γ content by ELISA assay. **A & B.**

show the mean IFN- γ production at 24 hours after activation with K562 and U87 cells respectively (n=3).

Figure 7: In the presence of exogenous TGF- β , perforin expression is reduced in Non-Transduced NK cells but is preserved in DNRII-Transduced NK cells. On day 12 of ex vivo culture, the non-transduced and DNRII-transduced NK cells were cultured for an additional 5 days in the presence or absence of TGF- β (5ng/mL), and IL-2 (200IU/ml). Expression of perforin as well as granzyme B was then evaluated by flow cytometry. **A.** Representative expression level of perforin and Granzyme B in NT-NK versus DNRII-NK cells after exposure to TGF- β . **B.** Mean expression of perforin and Granzyme B in NT-NK and DNRII-NK cells after exposure to TGF- β (n=4).

A **B**

Figure 2

A

B

Figure 3

Figure 5

A

NK vs U87

B

NK vs K562

C

D

Figure 6

Figure 7