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IFN-γ Stimulated Human Umbilical-Tissue-Derived Cells Potently Suppress NK Activation and Resist NK-Mediated Cytotoxicity In Vitro

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Umbilical cord tissue represents a unique source of cells with potential for cell therapy applications for multiple diseases. Human umbilical tissue-derived cells (hUTC) are a developmentally early stage, homogenous population of cells that are HLA-ABC dim, HLA-DR negative, and lack expression of co-stimulatory molecules in the unactivated state. The lack of HLA-DR and co-stimulatory molecule expression on unactivated hUTC may account for their reduced immunogenicity, facilitating their use in allogeneic settings. However, such approaches could be confounded by host innate cells such as natural killer (NK) cells. Here, we evaluate in vitro NK cell interactions with hUTC and compare them with human mesenchymal stem cells (MSC). Our investigations show that hUTC suppress NK activation, through prostaglandin-E2 secretion in a contact-independent manner. Prestimulation of hUTC or human MSC with interferon gamma (IFN-γ) induced expression of the tryptophan degrading enzyme indoleamine 2, 3 dioxygenase, facilitating enhanced suppression. However, resting NK cells of different killer immunoglobulin-like receptor haplotypes did not kill hUTC or MSC; only activated NK cells had the ability to kill nonstimulated hUTC and, to a lesser extent, MSC. The cell killing process involved signaling through the NKG2D receptor and the perforin/granzyme pathway; this was supported by CD54 (ICAM-1) expression by hUTC. IFN-γ-stimulated hUTC or hMSC were less susceptible to NK killing; in this case, protection was associated with elevated HLA-ABC expression. These data delineate the different mechanisms in a two-way interaction between NK cells and two distinct cell therapies, hUTC or hMSC, and how these interactions may influence their clinical applications.

Introduction

Human umbilical tissue-derived cells (hUTC) have been reported as having potential for cellular repair [1–4]. Immunogenicity studies revealed that a single injection of porcine unactivated umbilical cord tissue-derived cells (UTC) across a full major histocompatibility complex (MHC) barrier does not elicit a detectable adaptive immune response [5]. Animals injected once either systemically or subcutaneously with unactivated UTC had no detectable alloantibody production and a normal rejection pattern following in vivo skin graft challenge [5]. Mesenchymal stem cells (MSC) derived from bone marrow have been inferred nonimmunogenic based on their well-described immunomodulatory properties [6–8]. These properties have led to their development as cell therapeutics for inflammatory, transplantation, and autoimmune disorders [8–10]. These immunomodulatory properties allow allogeneic MSC to be deployed to limit graft rejection after hematopoietic stem cell transplantation [11] and the use of allogeneic MSC for the treatment of graft-versus-host disease (GvHD) [11,12]. Thus, these cells can be developed for allogeneic cell therapy in a manner that is characterized, standardized, and scaled for regulatory approval. Despite recent mechanistic advances [13], the comprehensive understanding of immune modulatory capabilities of hUTC or MSC remain to be tested. In particular, there is an incomplete understanding of how allogeneic cell therapy products interact with the cells of the recipient's innate and adaptive immune systems. An understanding of the interaction between potential allogeneic cell therapy products and recipient immune cells will be crucial for the rational development of effective and commercially attractive allogeneic cell-based therapies.

Natural killer (NK) cells represent a major lymphoid effector cell population of the innate immune arsenal [14].

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They play key roles in the recognition and destruction of virus-infected or aberrant host cells [14,15]. These cells shape subsequent adaptive immunity through secretion of cytokines, particularly interferon gamma (IFN-γ) [16]. Earlier, we and others have shown that adult human bone marrow-derived MSC are immunosuppressive, and counter-intuitively, that suppression can be enhanced or "licensed" by inflammatory mediators such as IFN-γ [7,17]. Thus, IFN-γ does not break, but enhances the immunomodulatory capacity of MSC. Given the time frame of development for adaptive immunity, if licensing of cell therapy occurs in vivo, then NK cells are a likely source of the licensing signal (IFN-γ) for MSC in vivo.

Human NK cell function is regulated through the expression of a large repertoire of inhibitory and activating receptors [18], and activation is regulated by a balance of inhibitory and stimulatory signals. Cells lacking or with impaired expression of HLA-ABC are recognized and killed by NK cells [19]. In mice, NK1.1 and Ly49 receptors are important for murine NK cell function; however, these are not relevant for studies of human cells. The CD94 chaperone/NKG2 receptor family (A–D) is more important in the human context, and this complex recognizes the nonclassical HLA, HLA-E [16]. The other key signal group is the polymorphic killer immunoglobulin-like receptors (KIRs), which interact with HLA-ABC ligands in humans and deliver either inhibitory or stimulatory signals depending on the cytoplasmic KIR tail. Although polymorphic, KIR haplotypes can be classified into two broad types: type A and type B, both of which can suppress cytolysis. In contrast, NK-mediated killing of target cells depends on activatory signaling involving specific ligand–receptor interactions [18,20]. NK receptors involved in cytolysis are the natural cytotoxicity receptors (NCR) NKp30, NKp44, and NKp46, and the nonpolymorphic NK receptors NKG2D and DNAM-1. The ligands for these include the MHC Class I-related A and B molecules (MICA/B), UL16-binding proteins (ULBP), the poliovirus receptor (PVR), and Nectin-2 [20,21]. Therefore, NK cells possess the machinery to either activate or kill allogeneic cells, potentially enhancing or confounding cell therapies.

Rational and successful development of allogeneic cell therapies will require an understanding of the interaction between these cells and NK cells to determine longevity of therapeutic efficacy, cell dose, and frequency of administration. Recent reports have delivered apparently contradictory results. Mesenchymal cells derived from bone marrow or cells derived from umbilical cord seem to suppress NK cell proliferation and function in vitro [22–24]. However, IL-2- or IL-15-activated autologous and allogeneic NK cells were capable of killing MSC and fetal-derived stem cells [25,26]. Interestingly, IFN-γ-stimulated MSC and fetal stem cells were protected from NK cytolysis [25,26]. Thus, there is need for clarification of two questions: (1) to what extent and by what mechanisms do allogeneic hUTC or MSC cell therapy suppress activation of NK cells? and (2) to what extent and by what mechanisms do NK cells kill allogeneic hUTC and MSC?

In the current study, we sought to characterize the interaction between hUTC and MSC with NK cells in vitro. We show that hUTC or MSC suppress NK activation, and this is enhanced by IFN-γ prestimulation. This occurs through multiple mechanisms that depend on the activation state of the cell. Resting NK cells do not kill allogeneic hUTC, whereas activated NK cells can. hUTC can be partially protected from such killing by previous activation or licensing. The mechanisms for killing involved the exploitation of the NKG2D-perforin/granzyme pathway. Overall, this study clarifies the parameters and mechanisms by which hUTC and MSC interact with a critical confounding influence on cell therapy and suggest ways by which these can be overcome.

Materials and Methods

Isolation and culture of hUTC or MSC

The research was approved by the institutional review boards and biological ethics committee of the National University of Ireland, Maynooth. All human participants gave written informed consent. Human MSC from two different donors were isolated and expanded from aspirates of bone marrow by direct plating, as previously described [27,28]. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) and 1% penicillin/streptomycin (Sigma-Aldrich). When the cultures reached 80% confluence, cells were detached by treatment with 0.25% trypsin/EDTA (Bio Sciences Ltd.) and re-cultured at 1×10^6 cells/175 cm^2 tissue culture flask. The HLA haplotype of donor #030 was HLA*A01/33, B51/58, Cw06/07; donor #061 was HLA*A03/03, B07/35, Cw04/07. All MSC used fulfilled the ISCT criteria and displayed typical surface markers and tri-lineage differentiation capacity as previously described. MSC were rigorously monitored for contaminating cell populations and were used in experiments at passage 7. hUTC were isolated and cultured as described [1] and used at passage 11. In some experiments, hUTC or MSC were cultured with 50 ng/mL rhIFN-γ (Peprotech) for 48 h, to generate IFN-γ-stimulated hUTC or MSC.

Isolation and culture of NK cells

NK cells were isolated from the peripheral blood from a panel of healthy donors using an NK cell isolation kit (Miltenyi Biotec). Haplotype A and B donor NK cells were identified by KIR genotyping (below), and NK cells from representative donors were used in subsequent experiments. Purified NK cells were either used directly or cultured with 100 U/mL rhIL-2 and 10 ng/mL rhIL-15 (Peprotech) for 48 h in complete RPMI to obtain activated NK cells for cytotoxicity assays.

Monoclonal antibodies and cytofluorometric analysis

Antibodies used for flow cytometric labeling included FITC-conjugated anti-human CD29, CD44, CD90, HLA-ABC, CD31, CD94, and CD56; PE-conjugated anti-CD34, CD45, CD106, CD117, HLA-DR, CD54, CD11b, HLA-E, CD155, MICA/B, NKG2D, NKG2C, and NKp30; and APC-conjugated anti-CD105, HLA-G, CD112, CD69, NKG2A, and NKp44 (eBioscience; or R&D systems). Isotype controls included FITC, PE, or APC-conjugated mouse IgG1, IgG2a, or PE-conjugated mouse IgG2b (eBioscience). For cell surface labeling, cells were incubated with saturating amounts of monoclonal antibodies or appropriate isotype controls for 30 min at 4°C. Cells were then washed five times and examined by flow cytometry (FACScalibur; Becton

Dickinson). Data were analyzed using Cellquest software (Becton Dickinson).

Cytotoxicity assays

To test the lytic potential of NK cells against hUTC or MSC, a commercial total cytotoxicity and apoptosis detection kit was used. Briefly, NK cells (effector cells) were cultured with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) labeled hUTC or MSC (target cells) at different effector:target ratios for 4 h. Cells were then stained with SR-FLICA™ poly-caspase apoptosis and 7-AAD reagent (Immunochemistry Technologies LLC), which enabled the detection of both early and late apoptotic cells, respectively. The percentage of total cell cytotoxicity was then determined. In some experiments, hUTC or MSC were preincubated with anti-human CD54 (ICAM-1), or HLA-ABC blocking antibodies (10 μg/mL) or mouse IgG1 control antibody (10 μg/mL) for 40 min before addition of NK cells. In other experiments, rhIL-2 (100 U/mL)/rhIL-15 (10 ng/mL)-activated NK cells were cultured with anti-human NK receptor blocking antibodies NKp30, NKp44, or NKG2D (10–20 μg/mL) or mouse IgG1 control antibody (10–20 μg/mL) for 30 min or with a granzyme B inhibitor (Z-AAD-CMK; Enzo Life Sciences) at 50 μM or 100 μM for 45 min, before addition to hUTC or MSC. Concentrations of blocking antibodies had been previously optimized.

Cytokine production

To evaluate NK activation in vitro, resting NK cells (4×10^5) were cultured in a 96-well plate in complete RPMI containing 100 U/mL rhIL-2 and 10 ng/mL rhIL-15 (Peprotech), with or without hUTC or MSC at a 5:1 ratio for 48 h. In some experiments, 0.4 μm transwell inserts (Cruinn Diagnostics Ltd.) were used to separate NK cells from hUTC or MSC, and the prostaglandin inhibitor indomethacin (20 μM; Sigma-Aldrich), IDO inhibitor 1-MLT (20 μM; Sigma-Aldrich), anti-human TGFβ1 (5 μg/mL; R&D Systems), or control IgG (5 μg/mL; R&D Systems) were added to NK-hUTC or MSC co-cultures. After incubation, supernatants were harvested, and IFN-γ (R&D Systems) and PGE2 (Cayman Chemical Company) production were assessed by ELISA. TGFβ1 was quantified by flow cytometry using the FlowCytomix simplex kit (eBioscience).

KIR genotyping

Genomic DNA from NK cells was isolated using a QIAamp DNA mini kit (Qiagen). The presence or absence of specific KIR alleles was determined from 50–75 ng/μl DNA by polymerase chain reaction (PCR)-sequence specific priming (SSP) using a KIR Genotyping SSP kit (Bio Sciences Ltd.), according to the manufacturer's instructions. Type A donors were discriminated from Type B by lack of expression of KIR2DL2, KIR2DS1,2,3, KIR2DS5, and KIR3DS1 and expression of KIR2DS4 among others.

Real-time/reverse transcriptase PCR

Total RNA was extracted from cells using a Ribopure kit (Ambion), and RNA was reverse transcribed to cDNA using SUPERSCRIPT™ II reverse transcriptase (Invitrogen). cDNA (1 μg) was amplified in the presence of SYBR® Green PCR master mix (Invitrogen). Primers used are described in Table 1.

Samples were run in duplicate, and accumulation of gene-specific PCR products was measured continuously by means of fluorescence detection over 36 cycles. Gene expression was calculated relative to the endogenous control, and analysis was performed using the $2^{-\Delta\Delta CT}$ method. cDNA (1 μg) was also analyzed for the expression of human IDO mRNA by semi-quantitative reverse transcriptase (RT)-PCR. PCR products were resolved by 1.3% (w/v) agarose gel electrophoresis.

Statistical analysis

Statistical significance was assessed using Prism5 software (GraphPad Software, Inc.). For group comparisons, data were analyzed using one-way ANOVA, with Tukey's multiple comparison test to measure significance. For other comparisons, data were analyzed using the two-tailed Student's *t*-test. *P*-values of $^*P\le0.05$, $^{**}P\le0.01$, and $^{***}P\le0.001$ were considered statistically significant.

Results

hUTC or MSC suppress NK activation, which is enhanced by IFN-γ stimulation

The capacity of hUTC or MSC to suppress NK activation was explored in vitro. Differences in KIR haplotypes can affect the outcome of hematopoietic stem cell transplantation [29]; for that reason, it was speculated that mesenchymal-like cells might differentially influence activation of NK cells from either haplotype A or B donors. Therefore, NK cells from healthy donors were typed for KIR alleles, and representative donors representing the A or B haplotype were used. In order to determine whether IFN-γ-stimulated hUTC or MSC suppressed NK activation in vitro, NK cells (haplotype A or B) were activated by a cocktail of IL-2/IL-15 and co-cultured with hUTC or MSC. NK activation was assessed by cytokine production. Human MSC potently suppressed NK activation, significantly inhibiting IFN-γ secretion from NK cells at all NK to MSC ratios (Fig. 1). No difference was observed between NK cells from different haplotype groups. hUTC also significantly suppressed NK activation but only at a high hUTC concentration (5:1 and 1:1), (Fig. 1C, F). Thus, unlike MSC, nonactivated hUTC only suppress NK activation at high ratios.

Table 1. Primers Used for Reverse Transcriptase–Polymerase Chain Reaction

Target	*Forward primer/reverse primer pairs*
ULBP-1	5′-AAGGCCTGGTGGATGAAAGGCC-3′
	5′-AGGGTGAGGGGCTCAATGGGTAT-3′
ULBP-2	5′-GCCGCTACCAAGATCCTTCTGTGC-3′
	5′-TCTGTGCTTTCCAGGCCGTTGTG-3′
ULBP-3	5′-GGCAACAGTGGTGTGAGGTCCA-3′
	5′-CAGCCAGTTCCAGTCTGAGCCT-3′
IDO	5′-CGCTGTTGGAAATAGCTTC-3′
	5′-CAGGACGTCAAAGCACTGAA-3′
GAPDH	5′-GGTGAAGGTCGGAGTCAACG-3′
	5′-CAAAGTTGTCATGGATGACC-3′

ULBP, UL16-binding proteins.

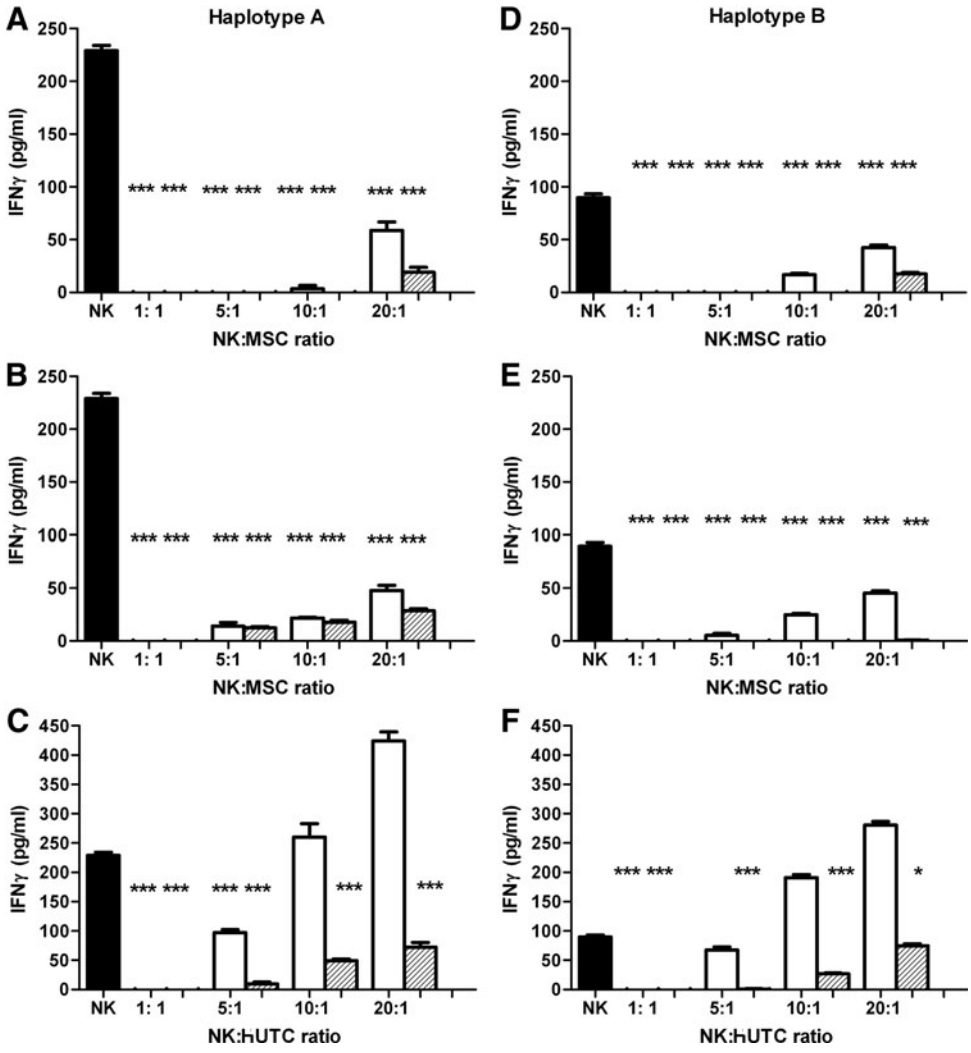

FIG. 1. IFN-γ-stimulated hUTC or MSC potently suppress NK activation compared with nonstimulated cells. Freshly isolated NK cells from either haplotype A **(A–C)** or haplotype B **(D–F)** donors were stimulated with IL-2 and IL-15 and co-cultured with nonstimulated hUTC or MSC or IFN-γ-stimulated γhUTC and γMSC for 48 h at a ratio of 1:1–20:1 (NK: MSC/HUTC). IFN-γ levels from supernatants of NK cells co-cultured with donor 030 MSC (*empty bar*)/γMSC (*hatched bar*) **(A, D)**, donor 061 MSC/γMSC **(B, E)** or hUTC and γhUTC **(C, F)** were determined by ELISA. Results are representative of three independent experiments and are shown as the means ± SE of triplicate samples. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$ (differences between activated NK cells and nonstimulated hUTC or MSC or IFN-γ-stimulated hUTC or MSC co-cultures). hUTC, human umbilical tissue-derived cells; NK, natural killer; MSC, mesenchymal stem cells; IFN-γ, interferon gamma.

IFN-γ prestimulation of MSC enhances other immune modulating capacities of MSC [7,17]. Interestingly, IFN-γ stimulation of both hUTC (γhUTC) and MSC (γMSC) suppressed NK activation more effectively, and at lower concentrations than cells not stimulated with IFN-γ (Fig. 1). Since cells from haplotype A and B donors behaved in a similar manner (Fig.1 and data not shown), all subsequent data are representative for both haplotypes.

hUTC or MSC suppress NK activation through prostaglandin secretion, and after IFN-γ stimulation, through additional mechanisms

In order to identify the factors involved in hUTC or MSC suppression of NK activation, known mediators of suppression were investigated. Prostaglandins are key mediators that are involved in other aspects of MSC-mediated immune modulation [7,30]. Co-cultures of NK cells with nonstimulated hUTC or MSC showed elevated quantities of PGE2 (Fig. 2A). Prostaglandin secretion by hUTC or MSC represented a strong candidate that might potentially mediate suppression of NK activation, and we have previously shown that MSC used in this study secrete PGE2 [7]. To test this hypothesis, NK cell co-culture experiments were performed with hUTC or MSC in the presence or absence of the prostaglandin antagonist, indomethacin. Blocking prostaglandins with indomethacin restored NK-derived IFN-γ levels in hUTC or MSC co-cultures compared with nontreated co-cultures (Fig. 2B). This effect is most profound in hUTC, as blocking prostaglandins with indomethacin leads to elevated IFN-γ (Fig. 2B). This suggests that in the absence of prostaglandins, hUTC behave similar to any other allogeneic cell and have an activatory effect. Further investigation revealed that suppression of NK activation through PGE2 was contact independent, as IFN-γ secretion from NK cells was unchanged when the cells were separated by transwells in the presence of indomethacin (Fig. 2C).

Stimulation of MSC with IFN-γ alters expression of immunosuppressive mediators; in particular, IDO and TGFβ1 have been implicated in this effect [7,31]. Therefore, the influence of these factors was examined in hUTC. Here, IFN-γ stimulation reduced PGE2 production from hUTC (Fig. 2A) but increased expression of TGFβ1 in prestimulated hUTC-NK co-cultures and this was greater than that seen in MSC co-cultures (Fig. 3A). IFN-γ stimulation also induced expression of the enzyme IDO in hUTC or MSC (Fig. 3B). Therefore, IFN-γ-stimulated hUTC or MSC were co-cultured with NK cells under activating conditions in the presence or

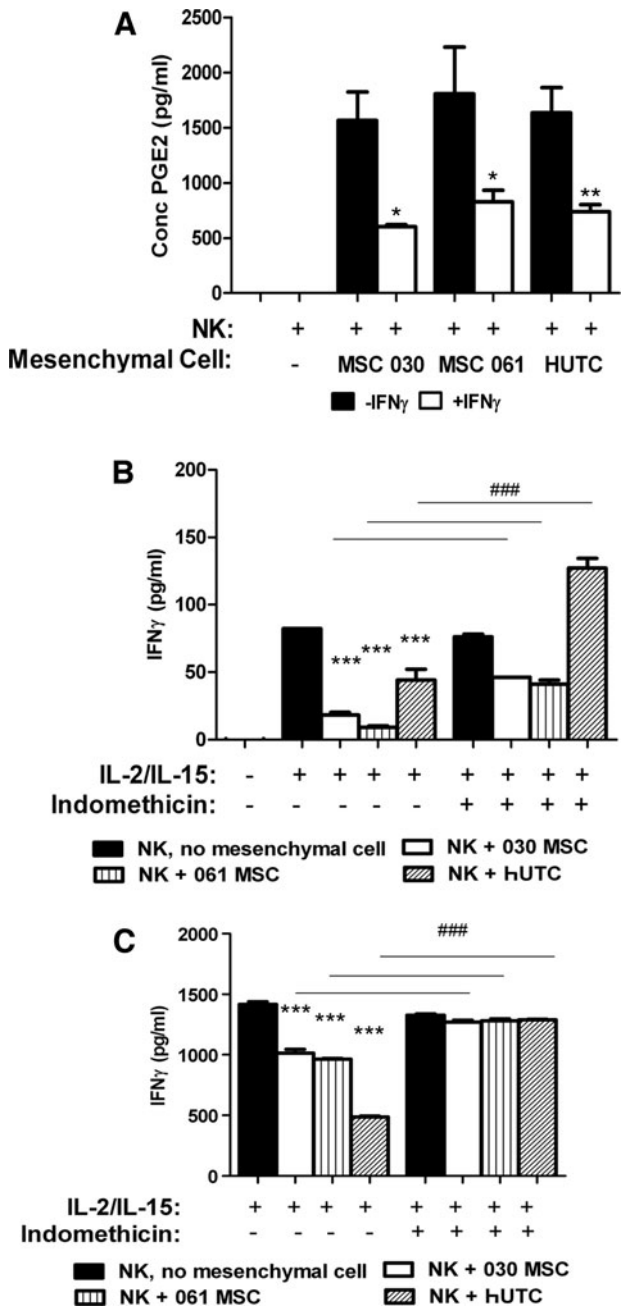

FIG. 2. Nonstimulated hUTC or MSC suppress NK activation through prostaglandin secretion. **(A)** Presence of PGE2 in co-cultures of activated (IL-2 and IL-15) NK with nonstimulated or IFN-γ–stimulated hUTC or MSC at 1:0.2 (NK:MSC) ratio after 48 h. Data represent the means ± SE from three combined experiments of duplicate samples. $^{*}P \le 0.05$, $^{**}P \le 0.01$ (nonstimulated hUTC or MSC co-cultures and IFN-γ-stimulated hUTC or MSC co-cultures). **(B)** NK activation as measured by IFN-γ levels from NK: hUTC or MSC co-cultures in the presence or absence of the prostaglandin inhibitor, indomethacin (20 μM). **(C)** Transwell assay measuring NK activation (IFN-γ levels) from NK: hUTC or MSC co-cultures in the presence or absence of indomethacin. Results are representative of three independent experiments and are shown as the means ± SE of triplicate samples. $^{***}P \le 0.001$ (differences between activated NK cells and hUTC or MSC co-cultures) and $^{\#\#\#}P \le 0.001$ (NK-hUTC or MSC co-cultures compared with inhibitor-treated co-cultures).

absence of antagonists for PGE2, IDO and/or a neutralizing antibody for TGFβ1. Blocking IDO and PGE2 partially restored activation of NK cells in γhUTC or γMSC co-cultures (Fig. 3C). Neutralizing TGFβ1 only restored NK activation in γhUTC co-cultures but had no effect on γMSC co-cultures (Fig. 3C). Further investigation revealed that suppression of NK activation through PGE2/IDO was also contact independent, as activation of NK cells was unaffected by cell separation and was only influenced by the presence of the antagonists (Fig. 3D). However, activation of NK cells in γhUTC or γMSC co-cultures was never fully restored, raising the possibility that other factors may also contribute to suppression. In summary, IFN-γ stimulated MSC suppress NK activation through prostaglandins and IDO, while IFN-γ–stimulated hUTC use additional mechanisms involving TGFβ1.

Activated NK cells lyse nonstimulated hUTC or MSC but IFN-γ-stimulated hUTC or MSC are less susceptible to NK killing

NK cells are potent cytotoxic effectors that could influence hUTC or MSC survival in vivo. Therefore, NK-mediated killing of allogeneic hUTC or MSC was investigated. hUTC or MSC targets were labeled with CFSE and co-cultured with resting NK cells at different effector:to target ratios. Cytotoxic activity at 4 h was measured by flow cytometry. Previously, other groups have reported that resting NK cells were unable to kill MSC [23,26]; thus, it was not surprising that resting NK cells were also unable to lyse hUTC or MSC but instead killed the leukemia cell line K562 at high effector:target cell ratios (Fig. 4A). Moreover, IFN-γ stimulated hUTC or MSC were not lysed by resting NK cells (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). However, during an inflammatory response in vivo, NK cells might be activated at the site of injury or pathology by the cytokine milieu. Therefore, the capacity of activated NK cells to kill hUTC or MSC in vitro was examined. Labeled hUTC or MSC were co-cultured with IL-2/15-activated NK cells at different ratios, and cytotoxic activity was measured. Activated NK cells readily lysed K562 cells, and, to a lesser extent, MSC (Fig. 4B). In contrast, hUTC were also highly susceptible to NK killing (Fig. 4B), demonstrating that hUTC or human MSC are prone to deletion by activated NK cells, albeit at different sensitivities.

The role of IFN-γ licensing or stimulation in the cell populations was also explored. Stimulation of MSC by IFN-γ rendered these cells less sensitive to deletion and even protected them from lysis at some ratios (Fig. 4B, C). However, γhUTC were lysed by activated NK cells at comparable levels to K562 (Fig. 4B, C). Importantly, at a ratio of 5:1 and 1:1, γhUTC (Fig. 4B) were less susceptible to NK cell killing than hUTC (Fig. 4C). Thus, IFN-γ renders allogeneic hUTC and MSC more resistant to activated NK cytotoxicity.

NK inhibitory ligands are up-regulated in IFN-γ-stimulated hUTC or MSC, while activating ligands are reduced

Our observation that IFN-γ-stimulated hUTC or MSC were less susceptible to NK cytotoxicity than unstimulated hUTC or MSC led us to examine potential NK activating

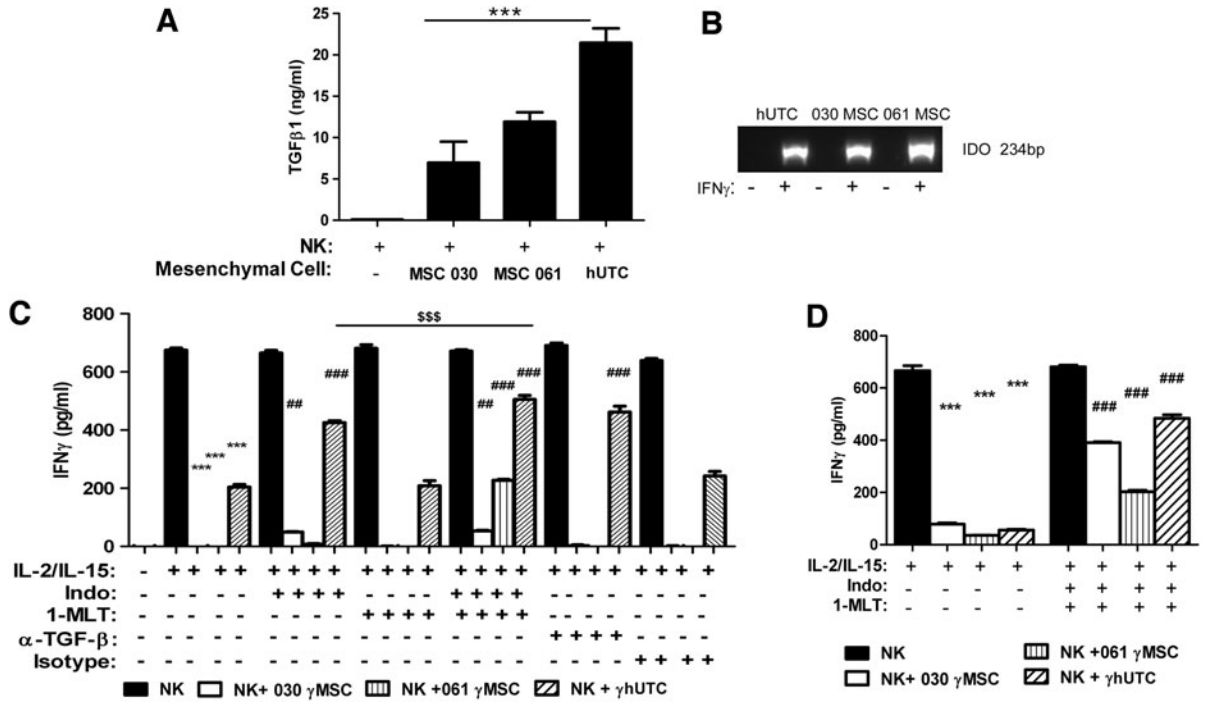

FIG. 3. Stimulated hUTC or MSC suppress NK activation through prostaglandin, IDO, or TGFβ. **(A)** TGFβ1 (pg/mL) is present in supernatant from activated (IL-2/15) NK cells co-cultured with IFN-γ-stimulated hUTC or MSC (1:0.2 ratio) for 48 h, detected by ELISA. Data represent means ± SE from three combined experiments of duplicate samples. $^{***}P \leq 0.001$ (difference between IFN-γ-stimulated hUTC compared with IFN-γ-stimulated MSC co-cultures). **(B)** IDO mRNA at 48 h was detected in nonstimulated hUTC or MSC or IFN-γ-stimulated hUTC or MSC by RT-PCR. **(C)** Activation (measured by IFN-γ) of NK cells co-cultured with IFN-γ-stimulated hUTC or MSC in the presence of indomethacin (20 μM), 1-MLT (20 μM), anti-human TGFβ1 (5 μg/mL), or control antibody (5 μg/mL). **(D)** as **(C)** but performed in transwell culture preventing NK/hUTC or MSC contact. Results are representative of three independent experiments and are shown as the means ± SE of triplicate samples. $^{***}P \leq 0.001$ (differences between activated NK cells and IFN-γ-stimulated hUTC or MSC co-cultures), $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ (NK-hUTC or MSC co-cultures compared with inhibitor-treated co-cultures) and $^{\$\$\$}P \leq 0.001$ (indo treated NK-hUTC co-cultures compared with indo + 1-MLT-treated NK-hUTC co-cultures). RT-PCR, reverse transcriptase–polymerase chain reaction.

ligands expressed in nonstimulated versus IFN-γ stimulated cells. Essentially nonstimulated hUTC or MSC showed comparable levels of expression for most NK ligands (Table 2 and Supplementary Fig. S2A–C). The exception was HLA-ABC (MHC Class I) expression, which was lower in hUTC than in MSC (Table 2). IFN-γ stimulation of both cells elevated this expression (Table 2). γMSC displayed increased HLA-E, the nonclassical MHC ligand for the NK inhibitory receptor CD94/NKG2A (Table 2), and both γMSC and γhUTC showed reduced levels of activating ligands ULBP1–3 (Supplementary Fig. S2A–C), recognized by NKG2D [20]. Therefore, prestimulation of hUTC or MSC with IFN-γ leads to increased expression of inhibitory HLA-ABC (MHC Class I) molecules and reduced expression of activating ligands for NKG2D.

Activated NK cells kill hUTC or MSC through the NKG2D perforin/granzyme pathway

The potential NK (activating) receptors that may be involved in killing hUTC or MSC—NKp30, NKp44, and NKG2D were investigated. These activating receptors are known to be up-regulated in activated NK cells [20], and this was the case here also (data not shown). These receptors were blocked using previously validated monoclonal antibodies. Blocking NKp30 or NKp44 in activated NK cells did not reduce the killing of hUTC or MSC; however, neutralizing NKG2D significantly decreased cytotoxicity of both cells (Fig. 5A–C). Signaling through NKG2D activates the perforin/granzyme cytolytic pathway [21]. This pathway is enhanced by the ligation of adhesion molecules such as CD54 (ICAM-1) in target cells with LFA-1 in NK cells. hUTC expressed higher levels of CD54 than MSC (Fig. 5G–I). The simultaneous blocking of CD54 and NKG2D significantly reduced cytotoxicity of hUTC, compared with blocking with NKG2D alone (Fig. 5F). Blocking CD54 on MSC had no additional effect on reduced cytotoxicity. These results were supported by the treatment of NK cells with a granzyme B inhibitor. NK cell killing of MSC was abolished by inhibitor treatment (Fig. 5J). Taken together, these data suggest that NK killing of hUTC or MSC occurs via the NKG2D/Perforin/granzyme pathway and that this is supported by high ICAM-1/CD54 on hUTC.

MHC-Class I protects IFN-γ-stimulated hUTC or MSC from NK killing

IFN-γ stimulated hUTC or MSC were more resistant to NK cytotoxicity than nonstimulated hUTC or MSC (Fig. 4B, C). It was speculated that this protection may be linked to the

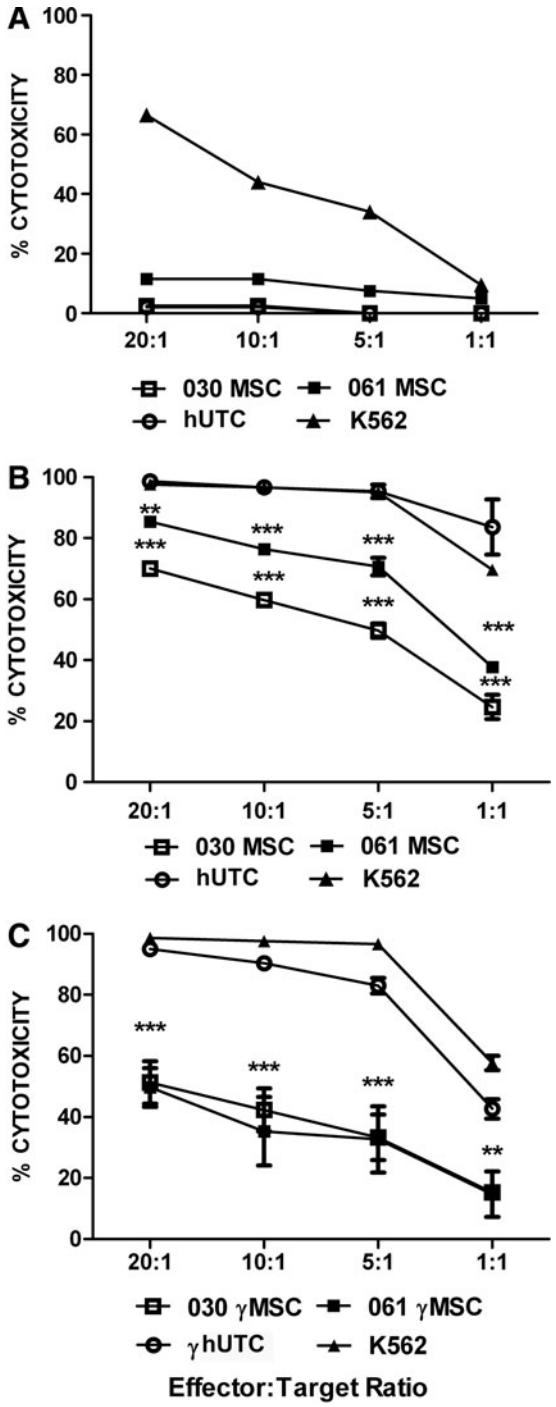

FIG. 4. NK cells lyse nonstimulated hUTC or MSC but IFN-γ renders hUTC or MSC less susceptible to NK killing. **(A)** Resting NK cells were used as effector cells against nonstimulated CFSE-labeled 030 MSC, 061 MSC, hUTC or K562 cells at different E:T ratios. Activated NK cells were used as effector cells against **(B)** nonstimulated CFSE-labeled hUTC or MSC or **(C)** IFN-γ-stimulated CFSE-labeled γhUTC or MSC at different E:T ratios. Cytolytic activity was measured by flow cytometry after 4 h of incubation. Data represent a combination of three independent experiments. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ (differences between NK-mediated killing of nonstimulated hUTC and MSC or NK-mediated killing of IFN-γ-stimulated hUTC and MSC). CFSE, 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester; E:T, effector:target.

Table 2. Natural Killer Ligands Expressed by Nonstimulated or IFN-γ-Stimulated hUTC or MSC

	Expression (MFI)[a]			
Marker	*hUTC*	*γhUTC*	*MSC*[b]	*γMSC*
HLA-ABC	+ (80)	+ + (730)	+ (170)	+ + (870)
HLA-E	–	–	–	+ (18)
HLA-G	–	–	–	–
Nectin-2 (CD112)	–	–	–	–
PVR (CD153)	+ (40)	+ (40)	+ (60)	+ (70)
MIC-A/B	–	–	–	–

[a]Peak expression < 10 considered negative; 10–100 scored +; 100–1,000 scored + +; γMSC and γhUTC represent IFN-γ stimulated MSC and hUTC respectively.
[b]Data shown for donor 030, similar data seen for donor 061 and repeated thrice.
ND, not determined; hUTC, human umbilical tissue-derived cells; MSC, mesenchymal stem cells; IFN-γ, interferon gamma; PVR, poliovirus receptor; MFI, mean fluorescence intensity.

increased MHC Class I detected in stimulated γhUTC/γMSC (Table 2). Therefore, further neutralization studies were performed. Blocking MHC Class I (anti-HLA-ABC) in γhUTC/γMSC significantly increased cytotoxicity compared with control-treated cultures (Fig. 6A), confirming that MHC class I protects licensed hUTC or MSC from NK killing. In line with others [26], blocking MHC class I in nonstimulated hUTC or MSC did not alter the cytotoxicity profile of these cells (data not shown). Further investigation revealed that the concentration of IFN-γ influenced MHC Class I expression in hUTC or MSC, and this correlated to reduced cytotoxicity (Fig. 6B, C). However, high MHC Class I expression did not fully protect γhUTC/γMSC from NK cytolysis (Fig. 6B, C).

NKG2D and CD54 again played a role in NK killing of IFN-γ stimulated hUTC or MSC. Blocking NKG2D reduced killing of γhUTC and γMSC (Fig. 6D–F). Neutralizing CD54 in γhUTC (but not γMSC) reduced killing by NK cells but had no additional inhibitory effect on cytotoxicity mediated through NKG2D (Fig. 6D–F). Therefore, it is reasonable to assume that elevated MHC-Class I contributes to the protection of IFN-γ-stimulated hUTC and MSC from NK killing. The killing that is observed occurs via the NKG2D receptor and, in the case of hUTC, this is supported by CD54/ICAM-1 expression.

Discussion

Allogeneic MSC are potent immunomodulators [8,13], offering a promising cell therapy for the treatment of human diseases. However, the interactions between NK cells and these cell therapeutics are not well understood. hUTC have also been shown to exhibit significant repartive properties [1–4] and are a developmentally early cell type that can be expanded to greater extents before reaching senescence [1]. hUTC can be isolated with ease from umbilical tissue and represent a more readily available cell type than human bone marrow-derived MSC. In this study, the interactions between candidate human cell therapy technologies and NK cells were defined, and the mechanistic pathways were identified. hUTC or MSC suppress allogeneic NK activation, through prostaglandin secretion, without a requirement for direct cell

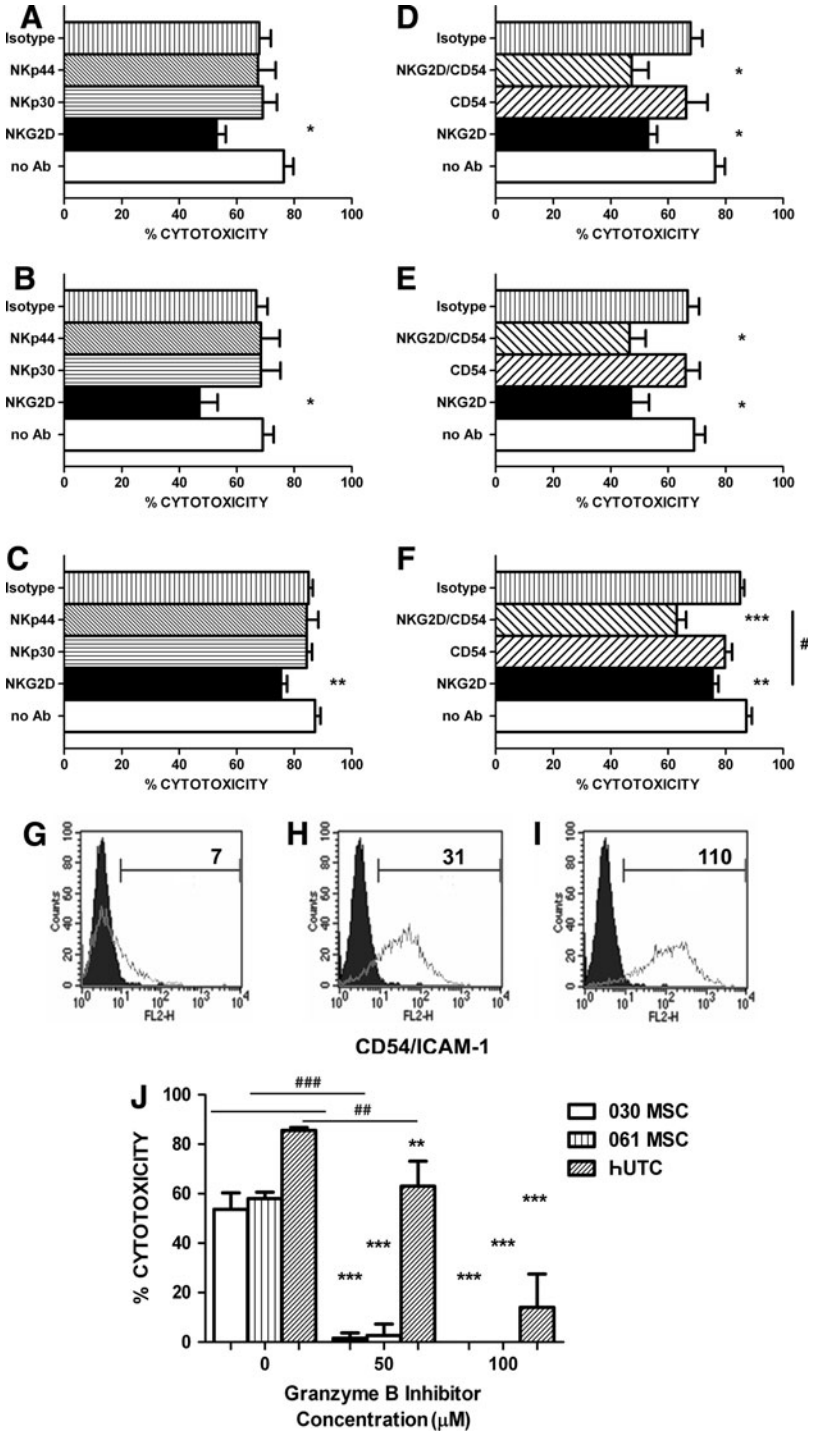

FIG. 5. NKG2D activation plays a role in NK-mediated cytotoxicity of hUTC or MSC. Cytolytic activity was measured by flow cytometry after 4 h of incubation of IL-2/15-activated NK cells cultured with 030 MSC **(A, D)**, 061 MSC **(B, E)**, or hUTC **(C, F)** at 1:0.2 (NK:MSC/hUTC) ratio. NK receptors **(A–C)** or NKG2D/CD54 **(E–F)** were blocked by neutralizing antibodies or isotype control. Data represent a combination of three to five independent experiments. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$ (differences between isotype control and neutralization antibodies), and $^{\#}P \le 0.05$ (difference NKG2D/CD54 vs. NKG2D alone neutralization). CD54 (*gray line*) expression by nonstimulated 030 MSC **(G)**, 061 MSC **(H),** or hUTC **(I)**; *solid* histogram represents isotype control. Fluorescence (peak MFI given on histogram) measured by flow cytometry at 48 h. **(J)** Activated NK killing of nonstimulated hUTC or MSC in the presence or absence of Granzyme B inhibitor. Data representative of triplicate experiments ± SE. $^{**}P \le 0.01$, $^{***}P \le 0.001$ (differences between NK cytotoxicity of MSC compared with hUTC) and $^{\#\#}P \le 0.01$, $^{\#\#\#}P \le 0.001$ (differences between granzyme B inhibitor-treated and nontreated cells). MFI, mean fluorescence intensity.

contact. Suppression extends to the two major KIR types of the NK cell identified. IFN-γ, which licenses MSC in other situations, stimulates hUTC and MSC expression of IDO, enhancing suppression of NK activation. Resting NK cells did not kill hUTC or MSC; however, activated NK cells killed these targets. Killing was mediated through the NKG2D-perforin granzyme pathway and was supported by CD54 (ICAM-1) expression by hUTC. IFN-γ stimulation protected MSC and, to a lesser extent, hUTC from NK killing, and this was associated with MHC class I expression by the cell. Data from clinical trials have highlighted the beneficial effects that mesenchymal cell-based therapies exert in the treatment of GvHD, Crohn's disease, and myocardial infarction [32–34]. The use of allogeneic products also enables scalable and controllable cell technologies to be developed [35], making them very attractive for large-scale manufacturing. Nevertheless, limitations remain to be addressed in relation to widespread clinical use, with the longevity of therapeutically administered cells in vivo being one contentious issue [36]. The most likely immune cells to confound early hUTC/MSC survival are NK cells. Their reported capacity to kill allogeneic stem cells [26,37] makes them a cell type that cannot be

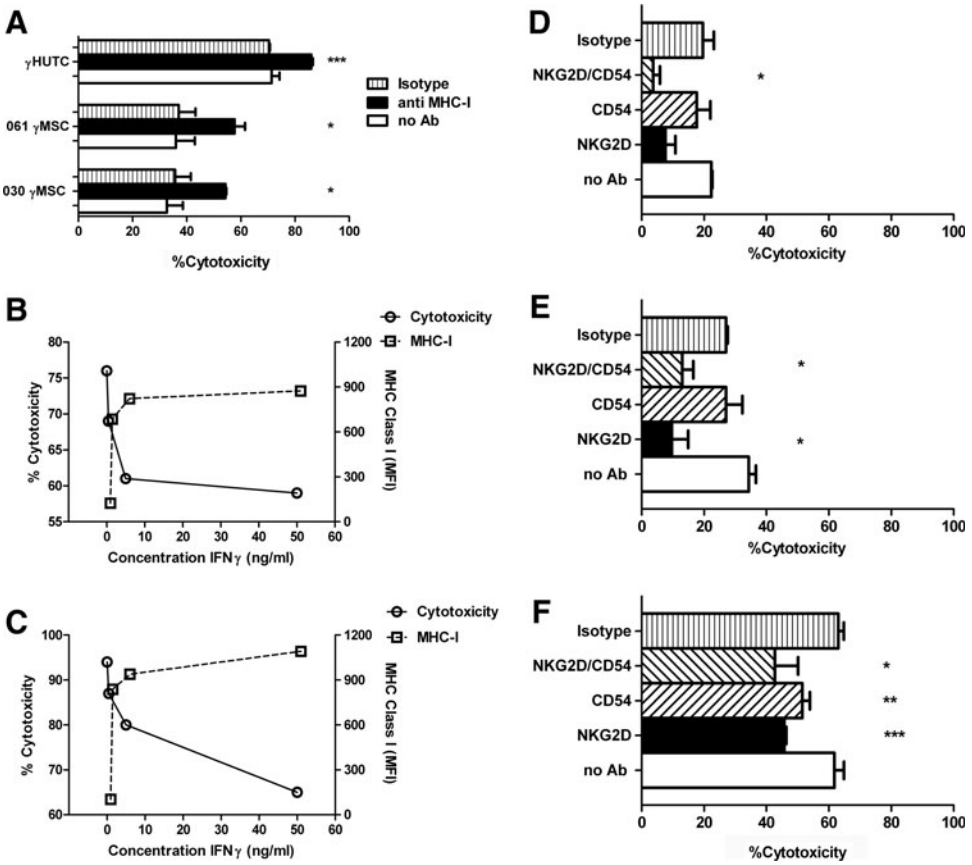

FIG. 6. MHC-Class I protects IFN-γ-stimulated hUTC or MSC from NK killing. Neutralizing MHC class I **(A)** on MSC or hUTC increases cytotoxicity compared with isotype control antibody. IFN-γ-stimulated hUTC or MSC (γhUTC or MSC) were labeled with CFSE and cultured alone (no Ab) or with saturating amounts of anti-human HLA-ABC, CD54, or mouse IgG1 control antibody before addition to cytolytic assays with activated NK cells (1:0.2 effector: target ratio). Killing of IFN-γ-stimulated 061 γMSC **(B)** or γhUTC **(C)** was also determined following varied levels of IFN-γ prestimulation (0, 0.5, 5, and 50 ng/mL) and correlated to HLA-ABC expression. Prestimulation was for 48 h before NK co-culture. Cytotoxicity by activated NK cells was also determined in the presence or absence of neutralizing antibodies for NKG2D, and/or CD54, or appropriate control antibody for IFN-γ-stimulated 030 γMSC **(D)**, 061 γMSC **(E),** or γhUTC **(F)**. Data represent mean ± SE of a combination of three independent experiments. $*P \le 0.05$, $**P \le 0.01$, and $***P \le 0.001$. Comparisons are with isotype control antibodies in all neutralization studies. MHC, major histocompatibility complex.

ignored when designing treatment regimens, as their influence could be critical to the success of cell-based therapies.

hUTC or human allogeneic MSC suppressed NK activation, and this extended to NK cells from donors of different KIR types. Suppression did not require direct cell contact but occurred through prostaglandin production by the non-hematopoietic cells. This is in agreement with some other reports [23,24,38], but, importantly, demonstrates that human polymorphisms in KIR alleles are unlikely to confound cell therapy. We observed that both IFN-γ-stimulated hUTC and MSC proved to be more potent suppressors of NK activation in vitro than nonstimulated cells. This study defines the role of trophic mediators in IFN-γ–licensed mesenchymal-like cell suppression of NK activation.

IFN-γ induced hUTC or human MSC expression of IDO, and this supported PGE2 in suppression of NK activation. This parallels our previous observation that MSC-derived kynurenine (the tryptophan breakdown product created by IDO action, which can be measured by HPLC detection) suppresses human T-cell activation [7]. Interestingly, in addition, IFN-γ-stimulated hUTC relied on TGFβ1 for this effect. Despite some similarities between hUTC and MSC, clear differences were seen between these cell types. Differences may be related to their source, intrinsic properties, and/or the isolation and expansion processes for these different cell types. This study defines suppression more precisely than heretofore; however, other soluble factors may be involved in licensed hUTC or MSC suppression of NK cells, as cytokine production was never fully restored in these co-culture blocking studies. One potential candidate is soluble HLA-G, which has been shown to be involved in induced pluripotent cell suppression of NK activation [38].

A number of previous reports have shown that MSC can inhibit NK cytotoxicity [23,24,39]. However, these examined resting NK cells, cultured with activating IL-2/15 in the presence of mesenchymal-like cells [23,24,38]. Our data suggest that in these scenarios the mesenchymal populations were able to exert their suppressive effects before the NK cells were activated. However, cell-based therapies are likely to be used to treat diseases that are associated with profound inflammation, and in environments in which NK cells are likely to be active. Accordingly, for effective therapy, hUTC

or MSC may need to survive clearance by activated NK populations. Importantly, we and others [25,26,40] have shown that IFN-γ–licensed allogeneic hUTC or MSC are superior to nonstimulated cells in avoiding cytolysis by activated NK cells. Here, we show that activated NK cell killing of nonstimulated hUTC or MSC involved the perforin/granzyme pathway and signaling via the NKG2D receptor. We observed some differences from Spaggiari et al. [26]; in our hands, NKp30 did not influence killing of hUTC or MSC. This difference is minor and likely due to heterogeneity in MSC. Indeed, we noted significant differences between nonstimulated hUTC and MSC. hUTC expressed higher levels of CD54 than MSC and contributed to NK cytotoxicity of hUTC, supporting the NKG2D effect. Interestingly, CD54 has been shown to play a role in the NK killing of other bone marrow stromal cells [37], but was not supportive of the cytotoxicity of our adult MSC. This has an important implication, as it suggests that cell therapy products which express high levels of CD54 (or selected for this to improve homing) may be more readily deleted by NK cells. Gotherstrom et al. showed that fetal MSC were more sensitive to NK killing than adult MSC [25]. However, they showed that fetal multipotent cells were killed through the TNF-related apoptosis inducing ligand (TRAIL) pathway, whereas adult multipotent cells were killed via FAS ligand [25]. In contrast, our results indicate that hUTC or MSC from different donors were killed via the perforin/granzyme pathway. Blocking TRAIL and/or FAS-L in activated NK cells of different KIR types did not significantly reduce cytotoxicity of our cells (data not shown). Multipotent cells are often considered precursor types to the cells here, and TRAIL/FAS-L have developmental roles that are consistent with these differences. However, we do not rule out other unidentified activators of the perforin/granzyme pathway contributing to NK killing of hUTC or MSC, as cytotoxicity was only partially blocked by neutralizing CD54 and NKG2D.

IFN-γ stimulation of hUTC or MSC increased expression of inhibitory MHC Class I and down-regulated activating ligands (ULBPs) in hUTC or MSC. Furthermore, MHC Class I (HLA-ABC) was essential for the protection of IFN-γ-stimulated hUTC or MSC, and increasing levels of MHC Class I inversely correlated to cytotoxicity. It is now clear that hMSC have the capacity to migrate to damaged tissues and exert immunomodulatory effects on an array of immune cells in target tissues via paracrine and/or cell-to-cell contact mechanisms [30,41,42]. Infusion of IFN-γ-stimulated MSC in animal models of GvHD, allergic airway disease, and chronic obstructive pulmonary disease (COPD) have shown improvements in the efficacy of the cell-based therapy [17,43,44]. These beneficial effects have been linked to IFN-γ-stimulated MSC immunomodulators, including prostaglandins, TGFβ1, IL-10, and IDO [6,7,45,46], and establishing the exact factors involved will be critical to their use in vivo.

In demonstrating the parameters and mechanisms of hUTC or MSC suppression of NK activation, the susceptibility and mechanisms of killing, and the key role played by IFN-γ, this study helps resolve the conflicting reports concerning MSC/NK interaction. For example, while some reports have suggested a role for the FAS/FASL pathway [25], the present study confirms a previous study that the main pathway involved in NK cytolysis of adult MSC requires perforin/granzyme mobilization [38]. This leads us to propose the following model for NK/MSC interaction (Fig. 7). A complex two-way interaction occurs between NK cells and allogeneic MSC (or hUTC). Resting NK cells do not kill allogeneic MSC regardless of the treatment of MSC. In contrast (IL-2/15), activated NK cells kill resting MSC via the NKG2D/Perforin/Granzyme pathway; in addition, the killing of hUTC is supported by CD54/ICAM-1 engagement and occurs more readily. IFN-γ (a product of NK activation) "licenses" or renders MSC or hUTC more resistant to killing, and this is, in part, due to expression of MHC class I (HLA-ABC) by the MSC or hUTC. In the opposite direction, MSC affect NK activation. MSC block IL-2/IL-15 driven activation of NK cells through prostaglandin secretion. This is enhanced by IFN-γ stimulation of MSC to express the tryptophan degrading enzyme, IDO, which affords an alternative mechanism of NK suppression. Thus, while NK cells can kill MSC, this is limited and dependent on the activation status of both cell types. The implications for cell therapy of this model are that hUTC or MSC entering a site of inflammation will likely be licensed and rendered resistant to NK deletion unless high numbers of activated NK cells are present. In this scenario, hUTC or MSC will tend to block further NK activation and have an anti-inflammatory effect. In contrast, in the absence of systemic inflammation, hUTC or MSC that encounter activated NK cells at non-inflamed sites will be deleted through lysis. Thus, the persistence of hUTC or MSC cell therapy will vary with the nature of inflammation and the site examined.

In conclusion, our data reveal the intricate relationship that exists between allogeneic hUTC or MSC and NK cells. It deciphers the mechanisms employed by IFN-γ–stimulated hUTC or MSC in mediating NK suppression, as well as evading NK deletion. Since there have always been issues regarding the efficacy of cell-based therapies [35,47], this study suggests that simple manipulation of hUTC/MSC during culture and cell source selection in the ligands identified here

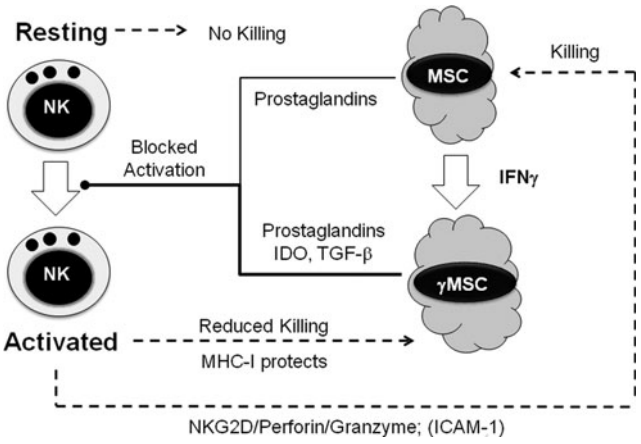

FIG. 7. Proposed model for the interaction between NK cells and hUTC or MSC. Resting NK cells do not kill hUTC/MSC effectively, whereas IL-2/IL-15-activated NK cells kill resting cells via an NKG2D pathway. IFN-γ–stimulated hUTC/MSC are less susceptible to this killing due to elevated MHC-I expression. IFN-γ is a product of NK cell activation (not shown for clarity). However, hUTC/MSC also affect IL-2/IL-15-driven NK cell activation through prostaglandin production. This is enhanced by IFN-γ stimulation, which induces IDO and further suppresses NK activation.

may result in the development of more effective cell-based therapies that avoid the confounding effects of NK deletion.

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