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Multiple Receptors Trigger Human NK Cell-Mediated Cytotoxicity against Porcine Chondrocytes

Roberta Sommaggio,* André Cohnen,† Carsten Watzl,† and Cristina Costa*

Xenotransplantation of genetically engineered porcine chondrocytes may provide a therapeutic solution for the repair of cartilage defects of various types. However, the mechanisms underlying the humoral and cellular responses that lead to rejection of xenogeneic cartilage are not well understood. In this study, we investigated the interaction between human NK cells and isolated porcine costal chondrocytes (PCC). Our data show that freshly isolated NK cells adhere weakly to PCC. Consequently, PCC were highly resistant to cytolyis mediated by freshly isolated NK cells. However, the presence of human natural Abs in the coculture was often sufficient to trigger cytotoxicity against PCC. Furthermore, IL-2 stimulation of NK cells or activation of PCC with the proinflammatory cytokines TNF-α or IL-1β resulted in increased adhesion, which was paralleled by increased NK cell-mediated lysis of PCC. NK cell adhesion to PCC could be blocked by Abs against human LFA-1 and porcine VCAM-1. NKG2D and NKp44 were involved in triggering cytotoxicity against PCC, which expressed ligands for these activating NK cell receptors. Our data further suggest that Nkp30 and Nkp46 may contribute to the activation of NK cells by PCC under certain conditions. Finally, comparative studies confirmed that PCC are more resistant than porcine aortic endothelial cells to human NK cell-mediated lysis. Thus, the data demonstrate that human NK cells can kill pig chondrocytes and may therefore contribute to rejection of xenogeneic cartilage. In addition, we identify potential targets for intervention to prevent the NK cell response against pig xenografts. The Journal of Immunology, 2012, 188: 000–000.

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Address correspondence and reprint requests to Dr. Cristina Costa, Institut d’Investigació Biomèdica de Bellvitge (IDIBELL), Hospital Duran i Reynals, Gran Via de L’Hospitalat 199, L’Hospitalet de Llobregat, Barcelona 08908, Spain. E-mail address: csosta@idibell.cat

Abbreviations used in this article: ADCC, Ab-dependent cell-mediated cytotoxicity; aGal, galactose α1,3-galactose (αGal) Ag (1, 3); Transplantation of pig cartilage implants in nonhuman primates develops during weeks to months and is slow compared with that of vascularized organs (3). Nevertheless, xenogeneic cartilage is rejected by humoral and cellular mechanisms that target, among other molecules, the galactose α1,3-galactose (αGal) Ag (1, 3). Transplantation of pig cartilage induces a strong Ab response that comprises anti-αGal and anti-non-αGal Abs (1, 2). Regarding the cellular component, there is evidence that human T cells and macrophages participate in this process (1–3). Both CD4+ and CD8+ T cells are the predominant cell types found in the cellular infiltrate of pig cartilage grafted for 2 mo in cynomolgus monkeys (3). In this accordance, both CD4+ and CD8+ T cells participate in rejection in a pig-to-mouse model of cartilage xenotransplantation (1). Particularly, pig chondrocytes express swine MHC class I and CD86, and can provide strong costimulatory signals to Jurkat T cells (2). Up to 20% of the cellular infiltrates in cartilage xenografts can be macrophages (3). In support of a direct contribution of this cell type to rejection, the human monoblastic U937 cells adhere to pig chondrocytes and secrete IL-8 in response (2). In addition, at least two of the major cytokines produced by macrophages, TNF-α and IL-1β, lead to a proinflammatory phenotype of pig chondrocytes, and thus exacerbate cellular rejection (2). Despite these advances in the molecular characterization of xenogeneic cartilage rejection, it is unknown whether human NK cells can contribute to this process.

NK cells are a distinct subpopulation of lymphoid cells that recognize and kill a variety of neoplastic, virus-infected, and nonself target cells (6). The activity of NK cells is regulated by a variety of activating and inhibitory surface receptors (7). The cytotoxic activity of NK cells is mediated by the directed release of perforin and granzyme B toward a locally attached target cell (8, 9). The contribution of NK cells to graft rejection is increasingly being acknowledged in allotransplantation (9, 10). Interestingly, a potential role of NK cells in the rejection of transplanted allogeneic chondrocytes has been suggested (11). However, recent findings indicate that NK cells may play a more prominent role in the rejection of xenografts than allografts (12, 13). In accordance to this, human NK cells adhere to and efficiently lyse porcine aortic endothelial cells (PAEC) in vitro (14–16). Moreover, NK cells can be found in histological samples of rejecting grafts in pig-to-baboon xenotransplantation models and in pig organs perfused with human blood (17, 18).

The incapacity of swine MHC class I molecules to signal through human NK cell inhibitory receptors is one of the reasons why porcine cells are susceptible to human NK cell-mediated lysis (19). Furthermore, human NK cells can adhere and lyse porcine endothelial cells both directly and by Ab-dependent cell-mediated
In this work, we wanted to investigate whether human NK cells can contribute to the rejection of pig chondrocytes/cartilage. We found that multiple conditions can render pig chondrocytes susceptible to human NK cell cytotoxicity. Although resting pig chondrocytes were highly resistant to lysis mediated by freshly isolated NK cells, the presence of XNA or the activation of either target or effector cells led to efficient cytolysis. Furthermore, we identified multiple NK receptors involved in the killing of pig chondrocytes.

Materials and Methods

Tissue harvest, isolation, and culture of porcine costal chondrocytes

Porcine ribs were collected after euthanasia under sterile conditions in animal facilities following procedures approved by the local ethical committee. The porcine costal chondrocytes (PCC) were isolated from pig cartilage and cultured as described previously (2). In brief, costal cartilage was dissected from the ribs and cut into small pieces washed in PBS supplemented with 1% penicillin/streptomycin and 50 μg/ml. The tissue was digested using collagenase (Invitrogen, Carlsbad, CA). Tissue was collected by centrifugation, sieved with a 100-μm nylon mesh (BD Biosciences, San Jose, CA), washed, and resuspended in Ham's F12 (Invitrogen)/20% FBS (Biomedical Technologies, Stoughton, MA). Fresh feeder cells were added on day 5 to restimulate NK cells.

Isolation and culture of human NK cells

PBMC were isolated from buffy coats or whole blood using Lymphocyte separation medium (PAA Laboratories GmbH, Pasching, Austria) by density gradient centrifugation. The blood was obtained from healthy donors at Heidelberg Blood Center (Heidelberg, Germany) with permission of donors and the local ethical committee. NK cells were enriched from human PBMC using the Dynabeads Untouched Human NK cells kit (Invitrogen) by depletion of non-NK cells. Populations were 90–99% CD3-, NKp46, and CD56+, as assessed by flow cytometry. The purified cells were resuspended in IMDM supplemented with 10% human serum (PromoCell, Heidelberg, Germany), 10% (v/v) nonessential amino acids (Invitrogen), 10% (v/v) sodium pyruvate (Invitrogen), and 200 μM penicillin/streptomycin. In this study, we used freshly isolated NK cells, NK cells stimulated overnight with hIL-2, and NK cells cultured for 3 wk. For overnight culturing, the NK cells were maintained in the described IMDM-based medium supplemented with 200 U/ml hIL-2 (National Institutes of Health cytokine repository). For 3-wk culturing, the NK cells were seeded in the same medium in microtiter 96-well plates with irradiated JY feeder cells, 0.5 μg/ml PHA-P (Sigma). 200 U/ml hIL-2, and 5 ng/ml hIL-15 (R&D, Minneapolis, MN). Fresh feeder cells were added on day 5 to restimulate NK cells.

Expression analyses by RT-PCR

For comparative studies, PAEC were obtained from the European Collection of Cell Cultures (Porton Down, U.K.) and cultured in DMEM/10% FBS supplemented with 50 μg/ml endothelial cell growth supplement (Millipore, Temecula, CA). An 8-h treatment with 10 ng/ml hTNF-α was included as control. To assess expression of pig ICAM (pICAM)-2 in PCC and PAEC, we generated first-strand cDNA by reverse transcription with Cloned AMV polymerase (Promega, Madison, WI) following the manufacturer’s recommendations in a 2720 thermal cycler (Applied Biosystems, Foster City, CA). We chose for pICAM-2 the primer pairs 5'-TCTCCAGAGACACCATCCT CCC-3' and 5'-AGCAGACAGCAGACGC CGG-3' following previous work (27) and used the β-actin control described previously (2). The PCR products were amplified for 30 cycles with 60°C annealing temperature.

Adhesion assay with NK cells

PCC were seeded in 96-well plates to reach confluence overnight and were left untreated or preincubated with 10 ng/ml hTNF-α, hIL-1α, or hIL-1β for 24 h. Triplicates for each assay condition were conducted. NK cells were washed and labeled with 0.5 μM CFSE (Vybrant Cell Tracker Kit; Invitrogen) in PBS for 30 min at 37°C in the dark. Immediately before the assay, the plate containing PCC was washed and kept at room temperature for 30 min with the anti-pig VCAM (pVCAM)-1 3F4 (Alexon Pharmaceuticals, Cheshire, CT), the anti-hICAM-1 15.2 (Abd Serotec, Oxford, U.K.), which cross-reacts with pICAM-1, and the isotype control Ab in designated wells at 2 × concentration (10 μg/ml). For assessing the participation of LFA-1, one labeled NK92 C1 cells were preincubated with anti–LFA-1 Ab (TS1/18; BioLegend, San Diego, CA) or the isotype control Ab for 30 min at 37°C. Then 5 × 10^4 PCC or PAEC were added to the plate at different E:T ratios. The start fluorescence was measured with cytofluorometer Victor3 (PerkinElmer, Turku, Finland) at 485/535 nm after spinning the plate for 2 s at 300 rpm without brake. The cells were then incubated together for 30 min at 37°C. For the conditions including Abs, these were present during the whole incubation period at a concentration of 5 μg/ml. The assay was terminated by washing the cells and adding 100 μl IMDM or HBSS to each well. Fluorescence was then determined with the cytofluorometer. The percentage of adhesion was calculated by dividing the end values with its corresponding start values and multiplying by 100.

Cytotoxicity assays with human NK cells

The cytotoxic activity of human NK cells, freshly isolated or IL-2 activated, and NK92 cells was assessed in 4-h [51Cr]-release assay. PCC and PAEC were detached from the culture flask using cell TripLE Express (Invitrogen), cell dissociation buffer (Invitrogen), or a combination of both. Then 5 × 10^4 PCC or PAEC were labeled for 1 h at 37°C with 100 μCi (3.7 MBq) [51Cr] as sodium chromate (Hartmann Analytic, Braunschweig, Germany). The target cells were subsequently washed twice with IMDM/10% FBS and added to the NK cells, which were previously washed to avoid any traces of human serum. Triplicates for each assay condition were set in round-bottom 96-well plates in a final volume of 200 μl/well. When multiple E:T ratios were used, the effector cells were plated in serial dilution before the labeled target cells were added. For assessing cytotoxicity mediated by Ab, the assay was identically conducted, but the target cells were preincubated for 30 min at room temperature with IMDM/10% heat-inactivated human serum (PromoCell) before labeling. For the blocking experiments, NK cells were preincubated for 30 min at room temperature with 10 μg/ml blocking Abs specific for NK22D (clone #149810; R&D Systems), NKp44 (p44/8, IOTest; Beckman Coulter GmbH, Krefeld, Germany), NKp30 (clone p30-15) (28), and NKp46 (Clone 9E2, IOTest; Beckman Coulter GmbH). During the coincubation of target and effector cells, the isotype control Ab was used alone at 10 or at 5 μg/ml when combined with the blocking Abs also at a final concentration of 5 μg/ml. The maximum release was determined by incubating the target cells with 1% Triton X-100, whereas the spontaneous release was assessed by in-
cubating the labeled PCC with medium alone. After 4 h of coculture at 37°C and 5% CO₂, the assay was stopped and the supernatants were collected. The [³⁵Cr] release was analyzed on a gamma counter, and the percentage of specific lysis was calculated as (experimental release − spontaneous release)/[maximum release − spontaneous release]) × 100.

Flow cytometric analyses

To determine human natural Ab deposition, target cells were incubated with 2 and 10% heat-inactivated human serum from PromoCell for 30 min at 4°C, washed, and stained with goat anti-human F(ab′)₂ fragments conjugated with PE (Jackson ImmunoResearch Laboratories, Newmarket, U.K.). For detection of ligands for NK receptors on PCC, PCC were kept untreated or incubated with human cytokines as indicated. They were harvested using cell dissociation buffer, washed, and transferred to 96-V bottom-well plates to monitor cell-surface expression of NK ligands. The cells were incubated for 30 min at 4°C with 1 μg/ml NKp44-ILZ fusion protein or the negative control B7/H6-ILZ, 10 μg/ml fusion proteins NKG2D-Ig, NKp30-Ig, or the negative control CD99-Ig, all in PBS 1% FBS. All these fusion proteins were created and produced as previously described (28). The cells were subsequently washed and incubated with 5 μg/ml mouse anti-ILZ Ab (clone #1) (29) for the ILZ-based fusion proteins or 10 μg/ml goat anti-hIgG biotinylated Ab (Dianova, Hamburg, Germany) for the Ig fusion proteins (all in PBS 1% FBS). After 30 min at 4°C, the cells were washed and incubated with goat anti-mouse FITC-conjugated Ab (1:200; Invitrogen) for the ILZ fusion proteins or with PE-conjugated streptavidin (1:200; Jackson ImmunoResearch, West Grove, PA) for 20 min on ice. The acquisition and analysis was conducted with a BD FACSCalibur.

Statistical analysis

The indicated values are expressed as mean ± SEM. Statistical analysis was carried out using the Student t test when comparing two groups and ANOVA (applying Tukey testing) for multiple comparisons. Differences were considered statistically significant at p ≤ 0.05.

Results

Human NK cells adhere to PCC

To study how human NK cells react toward pig chondrocytes, we first assessed the capacity of NK cells to adhere to PCC. To this end, we performed a static adhesion assay in which confluent chondrocytes were cultured in 96-well plates with CFSE-labeled NK cells at different ratios and conditions. As shown in Fig. 1, all the NK cell populations tested adhered to PCC to a certain degree. Around 10% of freshly isolated NK cells bound to PCC at 1:1 E:T ratio, and the amount of cells adhered increased with higher E:T ratios. Overnight hIL-2–activated NK cells and NK cells activated for 3 wk with hIL-2 showed a similar pattern but demonstrated twice as much adhesion as the freshly isolated NK cells (23 and 24.5% of NK cells added at 1:1 E:T ratio, respectively; Fig. 1). Results with the NK cell line NK92 were comparable with those of 3-wk–cultured NK cells. As expected, the number of cells adhered duplicated from 1:1 to 2:1 E:T ratios and increased at least 5-fold at ratio 5:1, indicating that the system was not saturated under these conditions.

Human NK cells kill pig chondrocytes in an Ab-independent and -dependent manner

To assess whether pig chondrocytes are susceptible to lysis mediated by human NK cells, we determined the cytotoxic activity of freshly isolated NK cells, NK cells activated with hIL-2 overnight or for 3 wk, and NK92 cells against PCC. For the cytotoxicity assays, PCC were detached from the culture flasks using either trypsin or cell dissociation buffer, but we observed no major differences between treatments. Low-passage PCC cultured under resting conditions were highly resistant to lysis mediated by freshly isolated human NK cells from multiple donors (Fig. 2). Many fresh NK cell isolates were incapable of killing PCC, and a few showed low cytotoxicity toward PCC (1–5% specific lysis). Under these conditions, there was little correlation between the cytotoxicity levels and the E:T ratio. As an exception, one particular isolate from a single donor reached 20% lysis of PCC at 40:1 E:T ratio. In the same series of experiments, NK cells from the same donors were activated overnight with hIL-2 and assayed against PCC. As shown in Fig. 2, all activated NK cell populations displayed some degree of cytotoxicity that correlated with the E:T ratio. Moreover, NK cells that showed cytotoxicity toward PCC when freshly isolated also showed higher killing after overnight activation. In particular, the mean cytotoxicity of overnight hIL-2–activated NK cells was 7.3% (range 0–21.9) at 10:1 ratio and 19.8% (range 4.1–68.1) at 40:1 ratio. The 3-wk–activated NK cells displayed a mean cytolytic activity of 5.4, 8.8, 14.6, and 21.3% at ratios 5:1, 10:1, 20:1, and 40:1, respectively. Thus, overnight and 3-wk hIL-2 exposure led to similar NK cytolytic activity. In addition, NK92 cells also behaved like cultured NK cells (killing PCC up to 36%).
NK CELL-MEDIATED CYTOTOXICITY AGAINST PIG CHONDROCYTES

We next analyzed the contribution of CD16 to the NK cell-mediated killing of PCC. First, we demonstrated by flow cytometry the deposition of Ab on the cell surface of chondrocytes after incubating the cells with 2 and 10% heat-inactivated human serum (Fig. 3A). We subsequently conducted cytotoxicity assays in the absence or presence of 10% heat-inactivated human serum (Fig. 3B). In the case of freshly isolated NK cells, addition of Ab to the assay increased cytotoxicity for three of four isolates tested (one shown in Fig. 3B). NK cells from the same donors were also activated overnight with hIL-2 and assayed for cytotoxicity toward PCC. In this setting, all the NK cells tested killed the PCC to some degree, and the addition of the heat-inactivated human serum consistently increased the cytotoxicity to more than double in accordance with each E:T ratio (Fig. 3B). As a control, we conducted the same assay with NK92 cells, which lack the CD16 receptor, and observed no increase in lysis in the presence of human serum (Fig. 3C).

Human proinflammatory cytokines increase adhesion of human NK cells to pig chondrocytes through pVCAM-1 and pICAM-1

We have previously demonstrated that human proinflammatory cytokines, particularly hTNF-α, increase the expression of adhesion molecules pVCAM-1 and pICAM-1 on PCC (2). In the following experiments, we studied whether selected human proinflammatory cytokines augmented the adhesion of NK cells to PCC and assessed the involvement of pVCAM-1 and pICAM-1 in this process. The participation of pICAM-2 was not specifically assessed because we did not detect expression in PCC, whereas it was readily amplified in PAEC by RT-PCR (Fig. 4A). Treatment of PCC with hTNF-α resulted in a 2- to 3-fold increase in adhesion of overnight-activated and cultured NK cells (Fig. 4B). Similarly, the adhesion of freshly isolated NK cells to PCC was significantly elevated by hTNF-α (1.5-fold). The exposure of PCC to hIL-1α also increased binding by cultured NK cells, but to a lesser extent than with hTNF-α (Fig. 4B). In addition, the effect of hIL-1β on cell adhesion was negligible and did not reach statistical significance. Similar results were obtained using the human cell line NK92 (Fig. 5).

To determine the participation of pVCAM-1 and pICAM-1 in the binding of human NK cells to pig chondrocytes, we conducted static adhesion assays at 2:1 E:T ratio with NK92 cells (that lack the FcR CD16) and specific blocking Abs (Fig. 5). For the blockade of pVCAM-1, we preincubated the PCC, untreated or previously stimulated with human cytokines, with mAb 3F4 (Fig. 5A). To assess the involvement of pICAM-1, we inhibited its ligand on NK92 with an anti–hLFA-1 blocking mAb (Fig. 5B). In each case, we included the appropriate isotype-matched Ab controls for comparison. The blocking Abs had a minor reducing effect when tested in adhesion assays with resting or hIL-1β–stimulated PCC (ranging from a trend to low significant differences between experiments). On the contrary, the two adhesion molecules markedly contributed to the increase in adhesion to PCC after 24-h treatment with hTNF-α or hIL-1α. Both the anti-pVCAM-1 and the anti–hLFA-1 inhibited adhesion around 50–60% relative to the corresponding isotype control Ab in stimulated PCC when applied separately to the assay. An anti–hICAM-1 that cross-reacts with the pig molecule also led to a partial inhibition, although its effect was not as pronounced as the one observed with the anti–hLFA-1 mAb (Fig. 5C). As further confirmation of the contribution of both pathways, the combination of blocking Abs (anti–pVCAM-1 and anti–hLFA-1 or –ICAM-1) led to an additional decrease in adhesion to hTNF-α–stimulated PCC (Fig. 5C). No further reduction was observed by combining anti–hLFA-1 and anti–ICAM-1.

Pig chondrocytes exposed to human proinflammatory cytokines become more susceptible to human NK cell-mediated cytotoxicity

We further analyzed the effect of the proinflammatory cytokines on pig chondrocytes by assessing the NK cell-mediated lysis of...
cytokine-stimulated PCC. To this end, we preincubated PCC for 24 h with hTNF-α, hIL-1α, or hIL-1β and determined the killing mediated by freshly isolated NK cells, overnight-activated NK cells, 3-wk–activated NK cells, and NK92 cells (Fig. 6). Freshly isolated NK cells were unable to kill untreated PCC efficiently, but exposure to hTNF-α rendered the PCC more susceptible to lysis (Fig. 6A). Overnight-activated NK cells from the same donors displayed a similar pattern with higher overall killing. Likewise, the 3-wk–activated NK cells and NK92 cells displayed a more robust increase in cytotoxicity when the target cells were pretreated with hTNF-α (Fig. 6B). The stimulatory effect of hIL-1α was weaker and did not reach significance in all the assays, whereas hIL-1β failed to increase the PCC killing by any of the various human NK cell populations tested. Thus, the combination of factors such as cytokine stimulation of both effector and target cells had an additive effect in the level of killing attained. The mean cytolytic activity ranged from 5% with resting PCC and NK cells to close to 50% when both PCC and NK cells were activated.

Porcine chondrocytes express ligands for human NK cell-activating receptors that trigger cytotoxicity

To analyze potential ligands on PCC that may stimulate activating receptors on human NK cells, we assessed the binding of fusion proteins of human NKG2D, NKp44, NKp46, and NKp30 to resting and cytokine-stimulated PCC by flow cytometry (Fig. 7). Appropriate irrelevant fusion proteins were included as controls. Under resting conditions, we detected ligands for NKG2D (showing the highest reactivity), NKp30, and NKp44 on PCC, whereas the NKp46 fusion protein produced very little reactivity over background (Fig. 7A). Interestingly, when PCC were treated with hTNF-α, we observed an increase in binding of the NKp44 fusion protein and a similar trend for the NKp30-Ig (Fig. 7B). On the contrary, the expression of ligands for NKG2D and NKp46 did not substantially increase in cytokine-treated PCC. No significant effect was produced by hIL-1α or hIL-1β as indicated, were incubated with NK92 cells at 2:1 E:T ratio to determine adhesion. The mean ± SEM of four independent experiments is shown. Statistical differences were calculated between treated PCC and the corresponding untreated controls (*p < 0.05).
by 3-wk–activated NK cells by half and even more noticeably for overnight-activated NK cells (66%). Blocking NKp44 also had an inhibitory effect, although there were differences between the NK cell populations. The 3-wk–activated NK cells displayed an average of 88% reduction in killing when NKp44 was inaccessible, whereas the cytotoxicity mediated by overnight-activated NK cells was not compromised as dramatically (37%). In accordance with these findings, the combination of NKp44 and NKG2D blockade led to a greater inhibition of killing for 3-wk–activated NK cells (97%) than for overnight-activated NK cells (83%). Moreover, the NKp46 blockade also led to different responses depending on the NK cell conditions as cytotoxicity mediated by 3-wk–activated NK cells was ∼35% lower, whereas killing mediated by overnight-activated NK cells was reduced as much as 88%. In these experiments, the interference of NKp30 alone did not have much impact, although it produced a slight consistent inhibition for both NK cell populations.

To further clarify the NKp46 contribution to the cytotoxicity of cultured NK cells, we conducted another series of experiments at 20:1 E:T ratio (Fig. 8B). Under these conditions, the NKG2D and NKp44 blockades had very profound effects. Moreover, some intervention could also be attributed to NKp46 because its blockade was accompanied by significantly lower cytolysis.

**Porcine chondrocytes are more resistant to human NK cell-mediated cytotoxicity than endothelial cells**

Because previous reports showed higher susceptibility of pig cells to human NK cell-mediated lysis (16, 26) than those obtained with PCC, we conducted simultaneous cytotoxicity assays for PCC and PAEC in the presence or absence of human serum (Fig. 9). As mentioned previously, PCC were hardly killed by resting NK cells unless they were preincubated with human serum. Using the same resting NK cells, we observed a low degree of cytotoxicity against PAEC that was only slightly enhanced by XNA (Fig. 9). Differences were more dramatic when testing overnight-activated NK cells (data not shown). In both cases, the percentages of cytolysis were much higher for PAEC than for PCC, and the difference was observed at most E:T ratios and for all three donors tested. Indeed, PAEC cytolysis was significantly higher than that of PCC when we compared the mean ± SEM (%) of 6 independent experiments with cultured NK cells from 7 different donors at 20:1 in the absence of XNA (24.4 ± 5.6 for PAEC and 3.8 ± 1.3 for PCC; p = 0.01). Moreover, ADCC was readily seen for both PCC and PAEC when assessing overnight-activated NK cells, reaching PAEC the highest levels of overall cytotoxicity (Fig. 9). ADCC was assessed correctly as it was lower when assaying the cultured NK cells and no increase in killing of NK92 cells was observed in the presence of XNA (data not shown).
Discussion
This work focused on elucidating how human NK cells could contribute to rejection of porcine xenogeneic chondrocytes. In particular, we have demonstrated that human NK cells adhere and lyse porcine chondrocytes in various degrees depending on multiple factors such as the activation level of the NK cells and PCC, and the presence or absence of XNA. These factors play a major role in triggering cytotoxicity against PCC as our data demonstrate that PCC are considerably more resistant to NK cell-mediated cytotoxicity than PAEC. In addition, we have identified some key molecules that participate in triggering PCC cytolysis in this setting. NK cells probably play a prominent role in rejection of solid organ xenografts because many studies support this concept (12, 13). However, it was unknown whether NK cells could contribute to rejection of xenogeneic avascular tissues such as cartilage. Although chondrocytes cannot be in direct contact with NK cells when embedded in the cartilage extracellular matrix, they could become accessible in transplant procedures of isolated cells and during resorption/rejection. Our results provide evidence that NK cells could participate in rejection of xenogeneic chondrocytes, in keeping with previous findings that support a role of NK cells in rejection of transplanted allogeneic chondrocytes (11). Nevertheless, the mechanisms of recognition of the xenogeneic cells are surely different.

Our data show that NK cells can adhere to PCC. This adhesion was, in part, dependent on LFA-1. Ligand binding of LFA-1 is regulated by inside-out signaling, which can increase the binding affinity of LFA-1 (30). Resting NK cells displayed only weak adhesion to PCC, which is in line with the fact that LFA-1 is in its low-affinity state on these cells (31). IL-2 stimulation can increase the binding affinity of LFA-1 (31), which would explain the elevated adhesion of IL-2-treated NK cells. Likewise, exposure of freshly isolated NK cells to a 45-min pulse with hIL-2 or hIL-15 increased their adhesion to Drosophila cells expressing hICAM-1 (32). We also demonstrated that the stimulation of PCC with hTNF-α, and at lesser extent with hIL-1α, increased the adhesion of human NK cells, and that pVCAM-1 and pICAM-1 were majorly involved in this process. Because we did not know whether the anti-hICAM-1 15.2 was a good blocking mAb for pICAM-1, we also targeted its most probable ligand, hLFA-1, and obtained robust reductions in adhesion. Furthermore, blocking the two pathways resulted in very low adhesion to both stimulated and unstimulated PCC. These results are consistent with our previous observations that pVCAM-1 and pICAM-1 are highly upregulated in porcine chondrocytes stimulated with hTNF-α and IL-1α (2). On the contrary, pCAM-2 (another LFA-1 ligand) is most likely not contributing to adhesion because no expression was detected by RT-PCR and its expression remains unmodified after hTNF-α treatment (27). Furthermore, the adhesion of human NK cells to porcine endothelial cells relies mainly on the interactions between hCD49d and pVCAM-1, and hLFA-1 and pICAMs (33, 34).

NK cell adhesion and cytotoxicity against PCC correlated nicely in our experiments. Adhesion to target cells is a first and essential step in NK cell cytotoxicity. This would explain the weak killing of PCC by freshly isolated NK cells and the increase in NK cell cytotoxicity against PCC after IL-2 stimulation. Furthermore, the
NCR ligands. Accordingly, we detected ligands for NKp30 and NKp44 with fusion proteins. Expression of NKp44 ligands on PCC probably contribute to triggering cytotoxicity of activated human NK cells, because cytolysis mediated by cultured NK cells was mainly dependent on NKp44. Regarding NKp30, we did not observe much effect in killing when we inhibited only this molecule, in agreement with previous studies with pig endothelial cells (16). However, we have preliminary data that provide evidence that NKp30 could enhance the cytotoxicity triggered by other receptors. The role of NKp46 in the NK cell-mediated killing of pig chondrocytes is not fully understood because we could not observe much reactivity with an NKp46-containing fusion protein by flow cytometry. However, this may be because of the low affinity for the ligand. In the context of cell-cell interactions, some molecules that display low-affinity binding can have strong effects (e.g., CD86/CD28). Thus, blocking NKp46 led to a significant inhibition of cytotoxicity when using activated NK cells, an effect that for overnight-activated cells was even more dramatic than that of blocking either NKp44 or NKGD2. The fact that NKp46 blockade did not have as much effect in cultured NK cells, despite that they did not lose NKp46 expression, could indicate that the cytotoxic activity of these NK cells against PCC is less dependent on NKp46 or could be because of changes in the signal transduction machinery after culture for several weeks. Forte et al. (16) demonstrated that NKp46 is not involved in the killing of pig endothelial cells by both freshly isolated and activated NK cells. Thus, the participation of NKp46 could be a particularity of chondrocytes in the pig. However, human NKp46 plays a critical role in triggering cytolysis of murine cells (38), indicating there may be conserved NKp46 ligands for humans, mice, and pigs.

Our study confirmed the contribution of CD16 to the NK cell-mediated killing of pig chondrocytes as the cytotoxicity increased in the presence of XNA for all NK cells tested with exception of NK92 cells (which lack CD16). We had previously demonstrated the deposition of human XNA on the surface of pig chondrocytes exposed to human serum, a high proportion of which recognize the αGal Ag (1). Interestingly, the increase in cytolysis was observed for both resting and activated NK cells, indicating that the presence of Ab is sufficient to trigger rejection. Our findings are consistent with previous results that show that human NK cells lyse porcine endothelial cells both directly and by ADCC (20). Notably, activation signals transmitted by CD16 are sufficient to induce efficient lysis of pig endothelial cells without additional signaling through other NK triggering receptors (16). In fact, we have results that support the same functional independence for PCC (data not shown). Although it is highly likely that the benefit conferred by expression of H transferase in cartilage implants was, in part, due to a reduction in ADCC (1), it remains to be evaluated whether pig chondrocytes that lack the αGal Ag are fully protected from ADCC mediated by human NK cells. Nevertheless, our results lead us to predict that a carbohydrate-remodeling approach that substantially reduces human natural Ab reactivity will have a major impact in protecting PCC from NK cell-mediated lysis.

Another factor that enhanced cytolysis was activation of the target cells with proinflammatory cytokines such as hTNF-α and hIL-1α. This finding was consistent with previous observations made with pig endothelial cells treated with hTNF-α (22). Adhesion certainly contributed to this effect, as it paralleled well the level of cytotoxicity observed under the multiple conditions tested. However, the upregulation of ligands for NKp44 and NKp30 in cytokine-stimulated PCC could also augment the killing of PCC by human NK cells expressing the corresponding receptors. This
would indicate that NK cells may contribute to the rejection of transplanted xenogeneic cartilage as the rejection process itself produces a proinflammatory environment. Our data show that different activating receptors are involved in this process. Because several ligands for these receptors are still unknown, it will be difficult to interfere with all these different stimuli to limit NK cell activation in a transplant setting. However, the combination of cell-surface carbohydrate remodeling to reduce XNA deposition with targeting adhesion and/or costimulation might be a promising strategy to avert NK cell-mediated cytotoxicity against pig chondrocytes.

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Disclosures

The authors have no financial conflicts of interest.

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