Human NK Cell-Mediated Cytotoxicity Triggered by CD86 and Galα1,3-Gal Is Inhibited in Genetically Modified Porcine Cells

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Delayed xenograft rejection is a major hurdle that needs to be addressed to prolong graft survival in pig-to-primate xenotransplantation. NK cell activation has been implicated in delayed xenograft rejection. Both Ab-dependent and independent mechanisms are responsible for the high susceptibility of porcine cells to human NK cell-mediated cytotoxicity. Previous reports demonstrated a role of Galα1,3-Gal Ag in triggering the Ab-independent responses. We hypothesize that expression of CD80 and/or CD86 on porcine cells may also play a role in NK cell activation as human NK cells express a variant of CD82. Our initial analysis showed that porcine endothelial cells and fibroblasts express CD86, but not CD80. Genetic engineering of these cells to express hCD152-hCD59, a chimeric molecule designed to block CD86 in cis, was accompanied by a reduction in susceptibility to human NK cell-mediated cytotoxicity. The use of a specific anti-porcine CD86-blocking Ab and the NK92 and YTS cell lines further confirmed the involvement of CD86 in triggering NK cell-mediated lysis of porcine cells. Maximal protection was achieved when hCD152-hCD59 was expressed in H transferase-transgenic cells, which show reduced Galα1,3-Gal expression. In this work, we describe two mechanisms of human NK cell-mediated rejection of porcine cells and demonstrate that genetically modified cells resist Ab-independent NK cell-mediated cytotoxicity. The Journal of Immunology, 2002, 168: 3808–3816.

The possibility to obtain large quantities of pig cells, tissues, and organs under controlled conditions, in opposition to the shortage of human organs for allotransplantation, has generated interest in the development of new xenogeneic therapies (1–4). However, several immune barriers need to be addressed to achieve clinical success (1–4). Many porcine cell types and all vascularized organs transplanted into primate models are rejected by both humoral and cellular mechanisms (5, 6). Major advances have been made in abrogating the humoral component that leads to hyperacute rejection, mainly through complement inhibition (7–10). However, even in the presence of complement inhibitors and systemic immunosuppression, xenografts are rejected due to delayed xenograft rejection (DXR) (3, 6, 11–14). DXR is characterized by a strong humoral response, type II endothelial activation, and an acute cellular infiltrate comprised of NK cells and macrophages (6, 11–14). The importance of NK cell-mediated xenograft rejection has been demonstrated in small animal models (14–16), while its contribution to the rejection process in the pig-to-primate model is less defined (6, 17).

NK cells are a distinct population of lymphoid cells that recognize and kill a variety of tumorigenic, virus-infected, and allogeneic target cells (18, 19). NK cell cytolytic activity is mediated by granule exocytosis and release of perforin and granzyme B, and is influenced by a balance of triggering and inhibitory signals (18). The killer-inhibitory receptors in human NK cells are represented by the p58 (KIR2DL) and the p70 (KIR3DL) receptors from the Ig superfamily that provide dominant inhibitory signals through recognition of autologous MHC class I molecules (20). The triggering receptor best characterized is CD16 (FcγRIII), which mediates Ab-dependent cellular cytotoxicity (ADCC) (21). Another triggering receptor is NKG2D, which binds the MHC class I chain-related MICA, a distant homologue of HLA (21, 22). However, several other triggering receptors with non-HLA ligands have been recently discovered: NKp46, NKp30, and NKp44 (23). The ligands and function of these receptors are currently under active research (23). Although these triggering receptors can function independently, others such as 2B4 (CD244) and Nkp80 seem to act as coreceptors (23). In this regard, some of the costimulatory molecules described to play a role in T cell activation have recently been shown to be involved in NK cell triggering. This is the case for CD80/CD86 (24, 25), CD86/CD28 (24), and CD40/CD154 interactions (26).

The mechanisms by which human NK cells recognize and kill porcine cells are currently under investigation (11). Lysis of porcine target cells by human NK cells is mediated by ADCC and non-ADCC mechanisms (27). IL-2-mediated activation of NK cells increases their cytotoxicity toward porcine cells (27, 28). Even if swine leukocyte Ag (SLA) class I may have a protective role through recognition of inhibitory receptors on human NK cells (29), its effect is clearly overridden by activating signals. In the absence of human serum, human NK cells lyse xenogeneic porcine aortic endothelial cells (PAEC) >2-fold more than allogeneic HUVEC (30, 31). The molecules involved in triggering Ab-independent NK cell-mediated cytotoxicity to porcine cells are not well defined. Several studies point to a role for the Galα1,3-Gal epitope expressed on porcine cells (30, 32). However, blockade of Galα1,3-Gal Ag is not sufficient to completely abrogate NK cell-mediated cytotoxicity (30, 31). Therefore, we hypothesized that expression of CD80 and/or CD86 on porcine cells may also trigger
human NK cell activation and killing. These molecules are interesting candidates because they are not expressed in HUVEC or human aortic endothelial cells (33), while they are expressed on porcine cells (33, 34). To test this hypothesis, we used a hCD152-hCD59 chimeric molecule expressed on the porcine cells to block CD80/CD86-mediated costimulation in cis. Our initial analysis indicated that PAEC and porcine fibroblasts constitutively express CD86 in the cell surface and do not express CD80 in resting conditions. Increasing levels of hCD152-hCD59 expression in these cells correlated with a reduction in both CD86 accessibility and susceptibility to lysis mediated by human NK cells in the absence of human serum. The use of a specific anti-CD86-blocking Ab and the NK cell lines NK92 and YTS further confirmed the involvement of CD86, but not CD80, in triggering NK cell-mediated cytototoxicity of porcine cells. To investigate the combinatorial role of CD86 and the carbohydrate epitope Galα1,3-Gal on NK cell activation, we expressed hCD152-hCD59 in PAEC and fibroblasts derived from our H-transferase (HT)-transgenic pigs (35). HT down-regulates Galα1,3-Gal Ag expression and generates fucosylated residues (H-Ag, the O blood group Ag) that are universally tolerated in humans (35, 36). Coexpression of high levels of HT and hCD152-hCD59 led to maximal protection from human NK cell-mediated cytolysis. These results demonstrate a role for porcine CD86 in triggering human NK cell activation. Strategies that combine inhibition of CD86 with carbohydrate remodeling have the potential to overcome DXR and improve the prospects of clinical xenotransplantation.

Materials and Methods
Isolation and culture of porcine cells
PAEC and fibroblasts from HT-transgenic and nontransgenic littermate pigs were isolated as described (35). Briefly, primary cultures of PAEC were obtained by scraping the aorta with a scalpel and culturing in DMEM/10% FCS. Primary cultures of fibroblasts were obtained by mincing ear tissue, or lung ear tissue in the case of HTAT21F, fibroblasts, and culturing in DMEM/10% FCS. PAEC and fibroblasts were cultured in DMEM/10% FCS supplemented with endothelial mitogen (50 μg/L; Biomedical Technologies, Stoughton, MA) for expansion, but the endothelial mitogen was withdrawn 24–48 h before the cytotoxicity assays to avoid interference.

Transduction of porcine cells
Viral supernatants from PA317 cells (murine amphotrophic packaging line from American Type Culture Collection, Manassas, VA) transfected with either pBABEpuro, vector alone (37), or pBABE-hCD152-hCD59 were used to infect control and transgenic PAEC and fibroblasts, as previously described (38). Transduced cells were selected with puromycin at concentrations of 3 μg/ml for PAEC and 4 μg/ml for fibroblasts. Single clones were selected from transduced PAEC, while pools of three to five colonies were obtained from infected fibroblasts for subsequent experiments.

Flow cytometric analysis of porcine cells
Direct fluorescence of cell surface carbohydrate epitopes was performed with FITC-conjugated lectins: IB4 lectin isolated from Griffonia simplicifolia (EY Laboratories, San Mateo, CA) that detects Galα1,3-Gal (39), and UEAI lectin isolated from Ulex europaeus (EY Laboratories) that detects H substance (40). Indirect immunofluorescence of hCD59 and hCD152 was performed with the specific mouse mAbs BRA10G (Biosdesign International, Kennebunk, ME) and BNI3 (Immunotech, Marseille, France), respectively. SLA class I was detected with the murine mAb PT85A (VMRD, Pullman, WA). Porcine CD86 expression was detected with 4F9.86 and 5B9.88 mAbs, whereas pCD80 expression was assessed with the rabbit polyclonal Ab ALP61 (34). These last three Abs have been developed in Alexion Pharmaceuticals (Cheshire, CT). Goat anti-rabbit IgG and goat anti-mouse IgG, IgA, and IgM (Zymed Laboratories, San Francisco, CA) FITC-conjugated antisera were used to detect specific Ab binding. Cell surface expression was then measured by flow cytometry on a BD Biosciences FACSort (San Jose, CA).

NK cell isolation and culture
The human NK cell line NK92 (obtained from H. G. Klingemann, Rush University, Chicago, IL) was first described by Gong et al. (41). These cells were cultured in Myelocult H5100 medium (StemCell Technologies, Vancouver, British Columbia, Canada) supplemented with 100 U/ml human rIL-2 (Hoffmann-LaRoche, Nutley, NJ). The human YTS cells (obtained from G. Cohen, Massachusetts General Hospital, Boston, MA), a subline of the YT NK leukemia cell line (42), were cultured in IMDM Icove’s modified Dulbecco’s medium supplemented with 10% FCS and t-glutamine. Human NK cells were purified from PBL from different donors, as previously described (43). First, PBL were isolated from buffy coats by centrifugation with lymphocyte separation medium (ICN Biomedicals, Aurora, OH). NK cells were subsequently enriched by depletion of other cell types using the magnetic-activated cell sorter NK cell isolation kit (Miltenyi Biotec, Auburn, CA). Freshly isolated NK populations were 90–99% CD3 and CD56 by flow cytometric analysis. The IMDM plus 10% human serum, supplemented with rIL-2 (100 U/ml) and 10% purified human IL-2 (Hemagen, Columbia, MD), was used for culturing and expanding NK cells.

Flow cytometric analysis of NK cells
For surface staining, cells were incubated with the following Abs: anti-CD3 (UCHT1, -PE from BD Pharmingen, San Diego, CA), anti-CD56 (B159, -PE from BD Pharmingen), anti-CD28 (CD28.2, -FITC from BD PharMingen), and anti-CD28 variant (YTH913.12, -FITC from Serotec, Oxford, UK). Directly conjugated isotype-matched Abs (BD Pharmingen) were used as negative controls.

NK cell-mediated cytotoxicity assays
Cytotoxicity assays were performed as previously described (30). Briefly, target cells (PAEC or porcine fibroblasts) were seeded and grown overnight to confluence in 96-well plates. The next day, cells were washed in HBSS (BioSource International, Camarillo, CA), labeled with 35Cl (2–4 μCi/well; Amersham Biosciences, Piscataway, NJ) for 60 min, and washed extensively again before the assay. The assay was conducted 4 h after addition of the effector cells (NK92, YTS, or IL-2-activated NK cells) in the indicated E:T ratios in a 200 μl final volume. Data are presented as percentage of specific lysis and calculated as previously described (30). To block porcine CD86, target cells were incubated for 20 min at room temperature with the specific mAb 5B9.88 and washed before the addition of the NK92 effector cells.

Statistical analysis
The indicated values are expressed as the means ± SE. Statistical analysis was conducted using the Student-Newmann-Keuls test. Differences were considered statistically significant at p ≤ 0.05.

Results
Porcine endothelial cells express constitutive levels of CD86 and no CD80 in the cell surface
We initiated our study by assessing the levels of CD80 and CD86 expression in our primary cultured PAEC by flow cytometric analysis (Fig. 1). To this end, we used a polyclonal Ab specific for porcine CD80 (34) and several mAbs specific for porcine CD86. Data generated with 4F9.86, an anti-CD86 Ab, are shown in Fig. 1. These experiments confirmed previous observations that PAEC express CD86 (33). However, in the standard culture conditions, we were unable to detect CD80 cell surface expression (Fig. 1). These results indicate that in resting conditions, PAEC express CD86, but not CD80.

NK cells and the cell line NK92 express receptors for CD80/CD86 and highly lyse porcine cells
To assess the susceptibility of porcine cells to NK cell-mediated cytotoxicity, we used freshly isolated NK cells, as well as two tumor-derived NK cell lines, NK92 and YTS. We chose to use NK92 and YTS cells because they express CD28 (41, 44). Whereas expression of CD28 has not been observed in human NK

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cells (24), Galea-Lauri et al. (25) have demonstrated expression of a CD28 variant in some NK cells that is detected with the mAb YTH913.12. We denote this molecule as CD28 variant. To determine the molecules involved in our system, we evaluated the expression of CD28 and CD28 variant in all NK cell populations assayed, as well as in NK92 and YTS cell lines (Fig. 1). We consistently observed reactivity toward CD28 variant in all preparations of IL-2-activated NK cells isolated from different donors, while no expression of CD28 was detected on these cells (Fig. 1). Our cell preparations contained from 60 to 90% of CD28 variant positive cells. The NK92 and YTS cell lines showed expression of both CD28 and CD28 variant, in which YTS exhibited the highest levels of expression for both molecules (Fig. 1).

NK cell-mediated cytotoxicity toward porcine cells was tested using IL-2-activated NK cells and NK92 and YTS cells in the absence of human serum (Fig. 2). Porcine cells were highly susceptible to lysis mediated by NK92 and activated NK cells in accordance with previous observations (11, 27, 28). Interestingly, YTS showed no cytotoxicity toward PAEC (Fig. 2). Similar results were obtained when these NK cell preparations were tested against HT-transgenic PAEC transduced with pBABE vector alone (Fig. 3). This protection from YTS-mediated lysis may be explained, at least in part, by the absence of CD80 on PAEC. Recent studies indicate that YTS triggering is mediated through CD80, and not CD86 (45, 46).

Expression of hCD152-hCD59 chimeric molecule on PAEC reduces CD86 accessibility and NK cell-mediated lysis

The hCD152-hCD59 chimeric was designed by M. C. Pizzolato and W. L. Fodor to block CD86 on the porcine cell. This chimeric molecule binds to CD86 in cis and inhibits costimulation provided by PAEC to human T cells.5 To study whether porcine CD86 contributes to human NK cell triggering and to assess the effect of CD86 blockade, we expressed hCD152-hCD59 in primary cultured PAEC from three different pigs. PAEC from the nontransgenic control 51 and two HT-transgenic pigs, 48 and 49, were transduced with pBABE-hCD152-hCD59. Several clones from each cell line were generated and selected based upon various levels of hCD152-hCD59 expression. We identified a low expressing clone, hCCh; a medium level expressor, hCCm; and a high level expressing clone, hCCl. The phenotypic characterization by flow cytometry of these selected clones and their controls is summarized in Table I. Expression of H epitope and the accompanying reduction of Galα1,3-Gal Ag was confirmed in the HT-transgenic cells by staining with UEAI and IB4 lectins, respectively (Table I). Both, BRA10G, specific for hCD59, and BNI3, specific for hCD152, detected comparable hCD152-hCD59 expression levels in the transduced clones. Most importantly, expression of hCD152-hCD59 correlated inversely with Ab reactivity toward CD86, as detected with the mAb 4F9.86 (Table I). CD86 was inaccessible to 4F9.86 in the high hCD152-hCD59-expressing clones (Table I), confirming the efficacy of this approach.5 The level of SLA class I expression was also assessed by staining with PT85A (Table I), given that SLA I may be involved in signaling to NK cells. Moreover, this parameter is a good indicator of the overall gene expression levels in the primary cultured cells. We observed a trend toward lower levels of SLA I expression in the transduced clones relative to nontransduced cells, but these differences were not significant. We conducted experiments that showed no effect of this variation, or the presence of pBABE vector alone, in the susceptibility of these cells to NK cell-mediated lysis (data not shown).

The CD86 contribution to NK cell triggering was assessed with a series of NK cell-mediated cytotoxicity assays in which the different clones of PAEC were cocultured with the CD16-deficient NK92 cells or IL-2-activated human NK cells (Fig. 3). All assays
were conducted in the absence of human serum. Expression of hCD152-hCD59 led to some level of protection from cytotoxicity mediated by both NK92 and activated NK cells (Fig. 3). Blockade of porcine CD86 was more effective in reducing NK92 than NK cell-mediated lysis (Fig. 3). The reduction in lysis was more dramatic in the highest hCD152-hCD59-expressing clones, suggesting a role of CD86 in triggering NK cell-mediated cytotoxicity of porcine cells.

To further confirm our observations, we used the specific anti-pCD86-blocking Ab 5B9.88 in NK92-mediated cytotoxicity assays of PAEC (Fig. 4). This Ab has shown very similar reactivity by flow cytometry to all the PAEC lines assayed when compared...
Effect of CD86 blockade on NK 92-mediated lysis of control and HT-transgenic PAEC transduced with hCD152-hCD59

Table I. Flow cytometric analysis of control and HT-transgenic PAEC transduced with hCD152-hCD59

| Group       | UEA-1 | GS-IB4 | BRA10G | BNI3 | 4F9.86 | PT85A |
|-------------|-------|--------|--------|------|--------|--------|
| Control 51  | 2.7 ± 0.6 | 289.8 ± 63.5 | 0.4 ± 0.2 | 0.9 ± 0.3 | 27.2 ± 4.9 | 76.9 ± 22.1 |
| Control-hCCm| 1.7 ± 0.4 | 211.4 ± 59.6 | 85.6 ± 7.5 | 101.2 ± 12.3 | 9.3 ± 0.3 | 56.5 ± 7.9 |
| Control-hChc| 2 ± 0.8 | 236.6 ± 84.5 | 110.9 ± 21.3 | 111.8 ± 16.7 | 3.8 ± 0.5 | 40.4 ± 8 |
| HT 48-vector| 351.6 ± 91.9 | 124.6 ± 31.6 | 0.03 ± 0.01 | 0.08 ± 0.08 | 35.7 ± 4.8 | 43.1 ± 2.2 |
| HT 48-hCCh | 369.2 ± 35.3 | 64 ± 17.4 | 10.4 ± 2.2 | 10.2 ± 1.4 | 16 ± 1.9 | 47.3 ± 2.9 |
| HT 49      | 705.6 ± 397 | 190.6 ± 112.5 | 0.01 ± 0.01 | 0.3 ± 0.1 | 35.8 ± 12 | 113.4 ± 45.5 |
| HT 49-hCCm | 288.2 ± 92 | 29.8 ± 10.7 | 31.7 ± 7.9 | 30.9 ± 10.8 | 10 ± 2.4 | 39 ± 12.4 |
| HT 49-hCCh | 464.4 ± 162 | 63.1 ± 18.3 | 76.2 ± 7.6 | 82.6 ± 14.2 | 3.5 ± 0.3 | 34 ± 9.9 |

* All values are expressed as the mean ± SE of the mean fluorescence intensity (n = 4). The specificities of the lectins used are as follows: UEA-I/H-epitope, GS-IB4/1,3-Gal epitope. The specificities of the Abs used are as follows: BRA10G/hCD59, BNI3/hCD152, 4F9.86/pCD86, PT85A/SLAI. 
* Significant differences were detected between HT-transgenic and control cells, p ≤ 0.05. 
* Significant differences were detected between hCC-transduced and their corresponding control cells. 
* No differences were observed when comparing HT-transgenic and control cells. 
* A value of p ≤ 0.005. 
* A value of p ≤ 0.05.

with 4B9.86 Ab (data not shown), and has shown blocking activity in a human anti-porcine MLR (W. Fodor, unpublished observations). The highest hCD152-hCD59-expressing clones were also included in the assays to reconfirm that the reduction in CD86 accessibility was responsible for the observed protective effect. Whereas the addition of IgG1 isotype control did not affect the NK92-mediated lysis of control 51 and HT-transgenic 49 cells, incubation with 5B9.88 led to a reduction in lysis identical to that achieved by high expression of hCC (Fig. 4). Moreover, addition of either isotype control or 5B9.88 Abs did not alter the susceptibility to lysis of the high hCC-expressing PAEC (Fig. 4, A and B). These data demonstrate that CD86 ligation on porcine cells contributes to human NK cell triggering and that blockade of porcine CD86 confers partial protection from NK cell-mediated cytotoxicity. It is of interest to note that this triggering pathway differs from that described in the recognition of PAEC by human T cells via CD28 (47). In this case, the CD28 variant detected on NK cells is probably predominant.

Effect of carbohydrate remodeling on NK92 and NK cell-mediated cytotoxicity

CD86 blockade reduces, but does not completely abrogate NK cell-mediated cytotoxicity (Fig. 3). In accordance with previous studies (30, 31), there are probably multiple molecules controlling this process. Galα1,3-Gal and other carbohydrate Ags may also trigger signals that render PAEC more susceptible to human NK cell-mediated lysis than HUVEC. In this regard, we observed that the 2 HT-transgenic PAEC assayed showed a trend to be more resistant to lysis, especially the clones expressing high levels of hCD152-hCD59 (Figs. 3 and 4). This trend was also observed when the control, and the HT-transgenic PAEC were simultaneously assayed with NK92 cells (data not shown). Due to availability, the HT-transgenic PAEC we used were derived from our moderate expressing transgenic line HTAT20 (35, 48). As we have observed a marked reduction in Galα1,3-Gal Ag in fibroblasts isolated from the high expressing HTAT20 line (35), we proceeded to assay these cells in an NK cell-mediated cytotoxicity assay. Fibroblasts isolated from the two founder pigs, AT20 and AT21, from F1 descendants of each line, as well as a control pig, were included in these experiments. These cells could be distributed into four groups that differed in their expression levels of H and Galα1,3-Gal epitopes. Representative results from one cell line per group are shown (Figs. 5 and 6). Nontransgenic cells had the highest cell surface expression of Galα1,3-Gal epitope. In inverse correlation to H epitope expression, relatively high levels of Galα1,3-Gal epitope remained on HTAT21-transgenic fibroblasts (mosaic founder). HTAT21F1 cells expressed moderate levels, and HTAT20-transgenic fibroblasts had the lowest Galα1,3-Gal expression (Fig. 5). No significant differences were detected between these cells in CD86 and SLA I expression (Fig. 5). Expression of porcine CD80 was not detected (data not shown). The efficacy of HT expression in providing protection from NK cell-mediated cytotoxicity was confirmed when the transgenic fibroblasts were
challenged with NK92 (Fig. 6A) and IL-2-activated NK cells (Fig. 6B) in the absence of human serum. In all assays, control cells were the most susceptible to lysis, whereas cells from the HTAT20 line were the most resistant (Fig. 6). Cells from the HTAT21 line were intermediate in resistance (Fig. 6). This resistance is most likely due to the reduction in Gal\(\beta\)\(\beta\)1,3-Gal epitope expression on the transgenic cells (30).

**Coexpression of high levels of hCD152-hCD59 and HT confers protection from NK cell-mediated lysis**

Finally to assess whether the combination of high HT expression and CD86 blockade confers maximal protection from NK cell-mediated lysis, we transduced HTAT20 fibroblasts with pBABE-hCD152-hCD59. HTAT20 cells transduced with the vector alone were also generated for controls. The pool of HTAT20-hCC-transduced cells showed good expression of hCD152-hCD59 (data not shown) and a 60% reduction in anti-CD86 4F9.86 Ab reactivity when compared with HTAT20-vector alone (mean fluorescence intensity 40.6 ± 1.6 vs 98.9 ± 1.5, respectively) as determined by flow cytometric analysis. To further study the effect of complete CD86 blockade, we also used the blocking Ab 5B9.88 in the cytotoxicity assays with NK92 cells (Fig. 7, A and B). Whereas the addition of 5B9.88 to control porcine fibroblasts provided partial protection from NK92-mediated lysis, this Ab completely abrogated NK92-mediated cytotoxicity toward HTAT20 transgenic cells (Fig. 7A). Complete resistance was also achieved when HTAT20 vector- or hCC-transduced cells were treated with the CD86-blocking Ab (Fig. 7B). Moreover, HTAT20 hCC-expressing cells showed intermediate resistance, correlating with the reduction in CD86 accessibility (Fig. 7B). This level of protection was reproduced when the HTAT20-hCC fibroblasts were challenged with different IL-2-activated NK cell preparations (Fig. 7C), indicating that this combinatorial approach confers resistance from human NK cell-mediated cytotoxicity.

**Discussion**

Research in pig-to-primate xenotransplantation aims to solve the increasing shortage of organs for human allotransplantation and develop new cell- and tissue-based therapies. A major hurdle that needs to be addressed to achieve engraftment of xenogeneic cells, tissues, and organs is DXR (11–14). The etiology of DXR is complex and needs further elucidation. We believe that the best approach to overcome DXR is to target each of the components that contribute to it, blocking first those processes resistant to current immunosuppression regimes. The CD28/CD80-CD86 pathway is of particular interest because it is resistant to cyclosporin A treatment (49, 50). Porcine CD86 on PAEC provides costimulatory function to human T cells (50, 33). We demonstrated in this work the involvement of porcine CD86 in triggering human NK cell-mediated cytotoxicity toward porcine targets. This pathway has
been discovered recently to play a role in triggering mouse and human NK cells by their CD80 and CD86 counterparts (24, 46, 51, 52). In the case of human NK cells, it involves a variant of CD28 (25). We confirmed the presence of this CD28 variant in all IL-2-activated NK cell preparations, as well as in the YTS and NK92 cell lines. To our knowledge, this is the first time that NK92 cells are shown to express both CD28 variants. The same observation was reported by Galea-Lauri et al. (25) in regard to the YT2C2 cell line that is related to the YTS cell line we used. It is important to point out that this variant of CD28 has not been implicated before in a xenogeneic setting. CD28-mediated T cell activation by PAEC has been blocked by either Fab fragments of 9.3 mAb anti-CD28 (47), which do not bind to this CD28 variant, or CD152-Ig (47, 53). CD152-Ig surely would play a competitive role for both variants, as we have observed that high expression of the chimeric molecule hCD152-hCD59 blocks CD80/CD86-mediated NK cell activation. Moreover, this CD28 variant may well be involved in the activation of human T cells, as the YTH913.12 Ab also binds to T cells (25). Expression of hCD152-hCD59 on PAEC also inhibits human T cell activation with the same efficacy as CD152-Ig.\(^5\)

In this work, we also showed that the triggering signal is provided by CD86, and not by CD80, which is not expressed in resting porcine endothelial cells and fibroblasts. A specific anti-CD86-blocking Ab provided as much protection from NK92-mediated lysis as high expression of hCD152-hCD59, which blocks CD80/CD86 in cis. Moreover, the lack of cytotoxic activity of YTS cells toward porcine endothelial cells also indicates that CD80 is not involved. Our results provide further confirmation that YTS cells are specifically triggered by CD80, and not by CD86. Others have made a similar observation using different systems (45, 46). We provide no explanation for the CD80 restriction by YTS cells, despite showing the highest expression of both variants of CD28. We can only speculate that YTS cells may have alterations in their
CD28 variants or defects in a molecule(s) involved in downstream signal transduction events. Previous work by Tadaki et al. (54) failed to prove the involvement of porcine CD86 in human NK cell triggering. Several factors may have influenced this discrepancy, but we believe that it was most probably due to the nature of their reagents. They used anti-human CD80 and CD86 Abs that failed to block direct costimulation provided by the porcine cells. Moreover, they used the whole Ab instead of Fab fragments. By contrast, we conducted NK92-mediated cytotoxicity assays using anti-CD86 Abs with proven blocking activity and developed specifically against the porcine molecule. Moreover, we used the chimeric molecule hCD152-hCD59 in the NK cell-mediated cytotoxicity assays to block CD86 without triggering CD16-mediated ADCC. Finally, the combination of two inhibitory strategies allows for a better appreciation of their effect in the complex NK cell-triggering system (55).

Similar reasoning could be applied to explain the lack or mild effect of blocking Galα1,3-Gal epitope on NK cell activation by PAEC observed by Sheikh et al. (31). Our results are in accordance with a role of Galα1,3-Gal Ag in mediating NK cell triggering by porcine cells (30, 32). Nevertheless, our results also agree with previous observations indicating the involvement of other molecules apart from Galα1,3-Gal Ag in this process (30, 31). Expression of HT in porcine target cells has previously been shown to reduce NK cell-mediated lysis (30). Our work further refines this protective effect using cells isolated from HT-transgenic pigs. This probably has some relevance, as it is not clear that cells cultured and modified in vitro can completely replicate the in vivo carbohydrate phenotype. Moreover, the transgenic modifications may eliminate the need for further in vitro alterations or in vivo therapies in the xenotransplant setting. Although we cannot rule out that high expression of HT may cause modifications other than Galα1,3-Gal reduction that may affect NK cell triggering. Initial analysis found no alterations in sialylation that may explain a reduction in NK cell triggering (data not shown). Identification of the triggering receptor that binds Galα1,3-Gal Ag is still pending. One or more of the newly discovered NK-triggering receptors (NkP46, NkP44, or NkP30) may be involved. YTS cells, which were completely unable to lyse porcine cells, may lack expression of such a receptor(s).

A marked reduction in Galα1,3-Gal Ag expression on porcine cells together with CD86 blockade led to complete protection from human NK cell-mediated lysis. We achieved complete inhibition of activation signals in the NK cell cytotoxic machinery. Other studies showed the involvement of the adhesion molecules CD2 and CD49d in NK cell triggering by porcine cells (17), but these may be secondary events to triggering mediated by Galα1,3-Gal and CD86. This protective effect would also be in accordance with observations from Kwiatkowski et al. (29) that SLA I can play an inhibitory role through some killer Ig-related receptor. It would be interesting to assess these pathways within our system in a future study. Moreover, blocking NK cell triggering should definitively be beneficial to combating DXR. NK cells play a direct role in DXR by inducing endothelial cell activation, procoagulant function, and lysis (56, 57). They can also promote a humoral response by stimulating resting and activated B cells (58). This approach has already been shown to address different DXR components, as HT expression reduces Ab reactivity and hCD59 inhibits complement activation (48). This effect may also contribute to protection from ADCC mediated by NK cells, monocytes/macrophages, and granulocytes, which all have receptors for both Ab and complement (59). We would like to assess the efficacy of our approach in inhibiting DXR in future in vivo studies. In summary, this discovery provides further insight in the understanding of DXR, and more specifically in the mechanism of human NK cell triggering by porcine cells. Inhibiting this process may have important implications in the development of xenogeneic therapies.

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