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Porcine UL16-Binding Protein 1 Expressed on the Surface of Endothelial Cells Triggers Human NK Cytotoxicity through NKG2D

Benjamin G. Lilienfeld,* Carmen Garcia-Borges,† Mark D. Crew,‡ and Jörg D. Seebach2*

Cellular rejection mechanisms, including NK cells, remain a hurdle for successful pig-to-human xenotransplantation. Human anti-pig NK cytotoxicity depends on the activating receptor NKG2D. Porcine UL16-binding protein 1 (pULBP1) and porcine MHC class I chain-related protein 2 (pMIC2) are homologues of the human NKG2D ligands ULBP 1–4 and MICA and B, respectively. Although transcribed in porcine endothelial cells (pEC), it is not known whether pULBP1 and pMIC2 act as functional ligands for human NKG2D. In this study, surface protein expression of pULBP1 was demonstrated by flow cytometry using a novel pULBP1-specific polyclonal Ab and by cellular ELISA using NKG2D-Fc fusion protein. Reciprocally, pULBP1-Fc bound to primary human NK cells, whereas pMIC2-Fc did not. Transient and stable down-regulation of pULBP1 mRNA in pEC using short-interfering RNA oligonucleotide duplexes and short hairpin RNA, respectively, resulted in a partial inhibition of xenogeneic NK cytotoxicity through NKG2D in 51Cr release assays. In contrast, down-regulation of pMIC2 mRNA did not inhibit NK cytotoxicity. Human NK cytotoxicity against pEC mediated by freshly isolated or IL-2-activated NK cells through NKG2D was completely blocked using anti-pULBP1 polyclonal Ab. In conclusion, this study suggests that pULBP1 is the predominant, if not only, functional porcine ligand for human NKG2D. Thus, the elimination of pULBP1 on porcine tissues represents an attractive target to protect porcine xenografts from human NK cytotoxicity.

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Porcine UL16-Binding Protein 1 Expressed on the Surface of Endothelial Cells Triggers Human NK Cytotoxicity through NKG2D

Organ shortage is a severe problem in transplantation medicine; therefore, pig-to-human xenotransplantation has become an important field of research to overcome this hurdle (1). Currently, insufficient long-term graft function prevents the successful clinical application of xenotransplantation (2). Nevertheless, advances in preventing hyperacute rejection in preclinical nonhuman primate models using genetically engineered pigs as organ source indicate that all coagulation disorders, Abs to non-Gal Ags, and cellular immunity play a role in xenogeneic responses (3–5). There are also several lines of evidence that NK cells may be a factor in delayed rejection of porcine xenografts (6). Pig organs perfused with human blood ex vivo are infiltrated by NK cells (7, 8), and NK cells are present in histological samples of graft rejection in concordant and discordant rodent and pig-to-baboon models (9–11). In addition, in vitro NK cells lyse porcine target cells both directly and, in the presence of human serum containing xenoreactive Ab, by Ab-dependent cell-mediated cytotoxicity (12). In contrast to hemopoietic stem cell transplantation, the involvement of NK cells in rejecting organ grafts has been sought to be of minor importance until recently. However, several new findings support the notion that NK cells also participate in the immune response against solid organ allo- and xenografts (13, 14). Thus, implementation of strategies to inhibit NK cytotoxicity and other yet to be defined NK cell responses such as cytokine release and enhancement of adoptive immune responses may promote both successful clinical xenotransplantation and allotransplantation.

NK cells are tightly regulated through signals mediated by inhibiting and activating receptors expressed on their cell surface (15). Many of the inhibitory NK receptors recognize MHC class I molecules, and therefore allow NK cells to discriminate between normal self, nonself, and altered cells (16). Porcine endothelial cells (pEC)3 are susceptible to human NK cell-mediated lysis possibly due to the inability of swine leukocyte Ag class I molecules to signal through human NK inhibitory receptors (17, 18). In contrast, the activation of human NK cells by potential ligands expressed on pEC may be equally, or more, important. Activating receptors on NK cells include NKP30, NKP44, and NKP46 (19), collectively named natural cytotoxicity receptors (NCR), and the C-type lectin receptor NKG2D (20). Although NKP30 and NKP46 are detected on all NK cells regardless of their activation status, NKP44 is selectively expressed by activated NK cells (21). There is a direct correlation between the surface density of NCR and the ability of NK cells to lyse various tumor cells (22), but the cellular ligands recognized by NCR remain unidentified. As recently reported, human NK cytotoxicity against pEC is mediated primarily through NKG2D and NKP44, whereas NKP30 and NKP46 are not involved (23).

Abbreviations used in this paper: pEC, porcine endothelial cell; MIC A/B, MHC class I chain-related protein A/B; NCR, natural cytotoxicity receptor; PAEC, porcine aortic endothelial cell; pMIC2, porcine MIC2; pULBP1, porcine ULBP1; shRNA, short hairpin RNA; siRNA, short-interfering RNA.
NKG2D is constitutively expressed on NK, NKT, and CD8-positive T cells as a homodimeric receptor associated with the transmembrane adaptor molecule DAP10 (24). Over the past few years, several regulation mechanisms of surface expression have been reported (20, 25). Cellular ligands of NKG2D include the stress-inducible MHC class I chain-related proteins A/B (MICA/B) (26) and UL16-binding proteins (ULBP) (27). The latter were identified based on their ability to bind the human CMV glycoprotein UL16. Although these GPl-linked proteins are distantly related to members of the HLA class I family possessing α1 and α2, but not α3 domains (28), they are unable to present peptides (29). In contrast, both MICA and MICB are transmembrane proteins and possess all three α domains. Similar to ULBP, MICA and MICB do not bind β2-microglobulin nor present peptides (30).

Blocking of human NK cytotoxicity against porcine cells with anti-NKG2D mAb provided indirect evidence for cross-species interactions between human NKG2D and one or several different porcine ligands (23). However, little is known about the nature and function of these putative ligands. The porcine NKG2D gene has been cloned and revealed a 66% sequence identity with human NKG2D; transcripts are expressed in PBL, NK cells, macrophages, and monocytes (31). The sequence of porcine MIC2 (pMIC2), a homologue of human MIC proteins, has also been reported previously (32). It is comprised of six exons, and the predicted amino acid sequence displays characteristics similar to those of the human MIC genes, such as four N-glycosylation sites, three of which are apparent counterparts of the human glycosylation sites at positions 208, 235, and 263. In addition, several cysteine residues within the pMIC2 α2 and α3 domains may participate in the formation of disulfide bonds. Similarly to the human MIC genes, a consensus heat shock element, but no iron response element, was found upstream of the porcine exon one. Only recently, porcine ULBP1 (pULBP1) was cloned and characterized as a homologue of human ULBP (33). Phylogenetic analyses place pULBP1 evolutionarily close to the bovine ULBP-like genes MHCLA1 and MHCLA2. It exhibits 35–52% amino acid identity to human ULBP, including a relatively high level of conservation at positions predicted to make contact with human NKG2D (29). Southern blot analysis suggested that only one pULBP exists in the pig genome, which is in sharp contrast to the much higher number of ULBP genes, at least six, that were described in humans. However, considering hybridization conditions in these studies (33), the possibility that other more distantly related porcine ULBP-like genes exist cannot be fully excluded.

Because both pULBP1 and pMIC2 transcripts were detected in a PEC (33), they represent potential ligands for human NKG2D. Therefore, the aim of the present study was to test whether pULBP1 and pMIC2 can act as functional ligands for human NKG2D, resulting in xenogeneic human anti-pig endothelial cell NK cytotoxicity. Several lines of evidence are presented that suggest that pMIC2 is ineffective in binding to NKG2D, and that pULBP1 is the predominant, if not only, porcine ligand for human NKG2D.

Materials and Methods

Cells

The SV40-immortalized aortic PEC line PEDSV.15 was established and characterized in our laboratory (34). Porcine aortic endothelial cells (PAEC) were isolated from a normal pig following standard procedures (35) and cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS (PA Labaratories), 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential amino acids (100×), essential amino acids (50×), 20 mM HEPES (all Invitrogen Life Technologies). Isolation of PBMCs from healthy blood donors, purification of NK cells, and generation of polyclonal human NK cell populations have been described previously (18). Isolated NK cells with a purity of routinely >95% were either used directly or activated by culture in AIM-V medium (Invitrogen Life Technologies) supplemented with 10% human plasma obtained from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, essential amino acids (50×), nonessential amino acids (100×), 1% penicillin/streptomycin (Invitrogen Life Technologies), 20 mM HEPES, and 300 U/ml human IL-2 (Chiron). The immortalized human NK line NKG2 (a gift from C. Falk, University of Munich, Munich, Germany) was cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 15% FCS (PA Labaratories), 5% human plasma obtained from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% penicillin/streptomycin. The human embryonic kidney cell line HEK293 (a gift from J. Wittwer, University Hospital Zürich, Zürich, Switzerland) was cultured in the same medium as PAEC.

Fusion protein production

The vector encoding a pULBP1-Fc fusion protein (aa 1–219, 7-aa linker, Fc of human IgG1) under control of the CMV immediate-early promoter was constructed by standard techniques and was stably expressed in HEK293 cells selected with 1 mg/ml G418 (Invitrogen Life Technologies). After limiting dilution cloning, a clone secreting high amounts of fusion protein was selected and expanded. The pULBP1-Fc fusion protein was purified using a protein A column (Amersham Biosciences) and dialyzed against PBS. Protein concentration was determined photospectrometrically. The purity and specificity of the fusion protein pULBP1-Fc were confirmed with Western and Coomassie staining showing one specific band. A gene encoding a pMIC2-Fc fusion protein was similarly constructed and was transiently expressed in HEK293 cells, and pMIC2-Fc was purified from culture medium using a protein A column.

Polyclonal Ab production

Two rabbits were immunized with pULBP1-Fc by i.v. injection of 150 μg of protein in CFA, followed by three booster immunizations with 100 μg of protein in IFA (every 4 wk). Four weeks after the last boost, sera were collected, purified using a protein A column, and dialyzed against PBS. One of the rabbits elicited a specific immune response. Preimmune sera of the immunized rabbits were collected for control experiments.

Flow cytometry

Surface expression of NKG2D on human NK cells, and expression of pULBP1 on PEC was analyzed on a FACSScan (BD Biosciences) by indirect immunofluorescence. After resuspension of 1 × 10^6 cells/tube in staining buffer (HANKS, 0.1% BSA), cells were incubated for 30 min at 4°C with saturating amounts of Ab. As primary Ab, the mouse mAb 149810 (IgG1, anti-NKG2D, R&D Systems) and a polyclonal rabbit anti-pULBP1 obtained in our laboratory (see Results), respectively, were used. As secondary reagents, FITC-conjugated goat anti-mouse Ig Ab (Chemicon International) and FITC- or PE-conjugated goat anti-rabbit Ig Ab (Sigma-Aldrich) were used. Phenotypic analysis of NK cells was conducted by direct immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-B159 (anti-CD56) mAb (all from BD Pharmingen). An irrelevant, isotype-matched control mAb (MOPC21, mouse IgG1; Sigma-Aldrich) was used as control for mAb stainings, and normal rabbit IgG (R&D Systems) or preimmune sera were used as control for the polyclonal rabbit Ab. To exclude dead cells, propidium iodide gating was performed in all experiments. To compare the levels of surface expression, the geometric mean fluorescence intensity ratios were calculated by dividing the mean fluorescence intensity of each sample with the mean fluorescence intensity of the control Ab.

Cytotoxicity assays

The cytotoxic activity of freshly isolated and IL-2-activated human NK cells was tested in 4-h^1^Cr release assays in serum-free AIM-V medium, as described previously (35). Briefly, labeled target cells were added to triplicate samples of serial 2-fold dilutions of NK cells in round-bottom 96-well plates. Four E:T ratios ranging from 40:1 to 5:1 were used in each experiment. For blocking studies, NK cells were preincubated for 30 min at 4°C with 10 μg/ml of the following mAb: MOPC21, 3G8 (mouse IgG1, anti-CD16; Beckman-Coulter), and 149810. mAb were also present during the coinoculation of target and effector cells at a concentration of 5 μg/ml. A saturating amount of 10 μl of the polyclonal anti-pULBP1 Ab as well as normal rabbit IgG was used for blocking studies. After incubation for 4 h at 37°C, the assays were stopped, ^51^Cr release was analyzed on a gamma counter, and the percentage of specific lysis was calculated.
Short-interfering RNA (siRNA) oligonucleotides and short hairpin RNA (shRNA) vectors

The following targeting siRNA oligonucleotide duplexes were purchased from Proligo: pMIC2 (5'-ACAGCUCUACAGGAGAACA-3') and pULBP1 (5'-CACAUCGCGAUCAGCAAAA-3'). Transfection of PEDSV.15 cells was performed using X-tremeGene siRNA transfection reagent (Roche Applied Science). A transfection efficiency of >95% was determined by FACS analysis 8 h posttransfection using a 3'-fluorescein-labeled control siRNA (5'-CUACCCAUAUCUACATTT(Fluo)-3'). The shRNA expression vector pRNAT-H1.1 (Genscript) was used to construct the following vectors: pULBP1-targeting vector pRNAT-H1.1-U2 (encoding the shRNA ATGGAGGCAGACAGCAAAAG and pMIC2-targeting vector pRNAT-H1.1-M3 (encoding the shRNA ATUGUGCUUUACACGCCAAGCU UAUAUGCCGACAAGGCAGAACGU). PEDSV.15 cells were CaPO4 cotransfected with each of these vectors and pCDNA3.1-UGAUAUCCGGACAUGGCGUAAAGAGACCAAU). PEDSV.15 cells and the absorbance was measured at 405 nm.

RT-PCR and quantitative RT-PCR

RNA was isolated using TRIzol (Invitrogen Life Technologies), and the concentration was determined photospectrometrically. The RNA quality was verified by agarose gel electrophoresis. Reverse transcription was achieved using AMV reverse transcriptase (Promega) following the manufacturer’s protocol. The following primers were used in PCR: $\beta_2$-microglobulin specific (5'-AGTATATCCCAACTTTCCACGGCACTCAG-3' and 5'-ATAGATCTGGATTCATCCAACCCAGATGACGC-3'), pMIC2 specific (5'-GGTACAATCTCAGGATGTCGCC-3' and 5'-CACCGCGCTGGACACTGTGATTCCCGAGCGTGGACACTGTGATTCCC-3') and pULBP1 specific (5'-GGCGCTTGAGATCTGGATTCATCCAACCCAGATGACGC-3' and 5'-GAACGTGGTCAATCAGGCGTGGACACTGTGATTCCC-3'). PCR products were run on a 1% agarose gel. Quantitative PCR was performed using Absolute QPCR SYBR Green Mix (ABgene) on an ABI Prism 7700 (Applied Biosystems), according to the manufacturer’s protocol. Results were analyzed after the comparative threshold cycle method.

Western blot

PEDSV.15 and PAEC cells were lysed using Cell Lytic M (Sigma-Aldrich). Cell lysates were analyzed by Western blot using standard protocols, and normal rabbit IgG and affinity-purified anti-pULBP1 Ab were used as primary polyclonal Ab. An HRP-conjugated secondary goat anti-rabbit Ab (Bio-Rad) was added, and specific bands were visualized using Opti-4CN (Bio-Rad).

ELISA and cellular ELISA

Maxisorp ELISA plates (BD Biosciences) were coated 16 h before the assay using 1 $\mu$g of pULBP1-Fc or pMIC2-Fc protein in a volume of 100 $\mu$l. Rabbit anti-pULBP1 Ab was used as primary Ab with AP-conjugated goat anti-rabbit IgG Ab (Sigma-Aldrich) as secondary Ab. For cellular ELISA, 5 $\times$ 10^4 PEDSV.15 or 1 $\times$ 10^5 NK cells were seeded 16 h before the assay in 96-well plates. NK2D-Fc (R&D Systems), pULBP1-Fc, or pMIC2-Fc proteins were added (0.5 $\mu$g/well) and incubated at 37°C for 2 h. An AP-conjugated goat anti-human IgG Ab (Sigma-Aldrich) was used as secondary Ab. Then, 4-nitrophenyl phosphate (Merek) was added and the absorbance was measured at 405 nm.

Results

Human NK2D binds to pEC and to pULBP1

To test the ability of the activating human NK receptor NK2D to directly interact with a ligand on pEC, the pEC line PEDSV.15 was incubated with the chimeric fusion protein pM2D-Fc. NK2D-Fc bound to PEDSV.15 cells in a dose-dependent manner, as shown by cellular ELISA (Fig. 1A). Binding of NK2D-Fc was also observed using primary PAEC (data not shown). Accordingly, FACS analysis revealed staining of PEDSV.15 cells with NK2D-Fc, but not with an irrelevant Fc fusion protein (Fig. 1B). Preincubation of NK2D-Fc with anti-NK2D mAb abolished its binding to PEDSV.15, underscoring the specificity of the staining. Next, the binding of NK2D to pULBP1 and pMIC2 was examined by ELISA using Fc fusion proteins. Whereas pULBP1-Fc clearly bound to nNK2D, no binding of pMIC2-Fc was observed (Fig. 2A). The binding of pULBP1-Fc and pMIC2-Fc to NK2D expressed on human NK cells was further analyzed by cellular ELISA. Whereas pULBP1-Fc bound to both primary human NK cells as well as NK92 (data not shown), no binding of pMIC2-Fc was observed (Fig. 2B). Together, these ELISA and FACS results indicated that pULBP1, but not pMIC2, interacts with human NK2D.

Decreased susceptibility of PEDSV.15 to lysis by human NK cells following down-regulation of pULBP1 mRNA

To test whether pULBP1 and/or pMIC2 interact functionally with NK2D on human NK cells, cytotoxicity assays were performed following transient down-regulation of the respective mRNAs using siRNA. PEDSV.15 cells were transfected with siRNA oligonucleotide duplexes specifically targeting pULBP1 and pMIC2 and a nontargeting control siRNA. Compared with PEDSV.15 cells transfected with control siRNA, a reduction of 39 ± 23% (mean ± SD) for pULBP1 and of 58 ± 20% for pMIC2 mRNA was observed 48 h posttransfection by quantitative RT-PCR (data not shown). A reduced susceptibility of PEDSV.15 cells to NK2D-mediated cytotoxicity was observed in 51Cr release cytotoxicity assays using pULBP1 siRNA, whereas pMIC2 siRNA transfection...
slightly increased the susceptibility (data not shown). Because mRNA levels were only transiently down-regulated by siRNA, experiments were performed within 48–72 h posttransfection. There were many variable parameters in these siRNA assays, such as the unknown E:T ratios of NKG2D ligands on the surface of pEC and the exact duration and extent of mRNA down-regulation. Consequently, the optimal time point for functional assays was difficult to estimate.

Therefore, PEDSV.15 lines with stable down-regulation of mRNA were generated using shRNA-expressing vectors. The reduction of pULBP1 mRNA was 46 ± 11% (mean ± SD) in the PEDshU2 lane, and 87 ± 17% for pMIC2 mRNA in the PEDshM3 line, respectively, as demonstrated by quantitative RT-PCR (Fig. 3, A and B). NK cytotoxicity mediated by IL-2-activated human NK cells against PEDshU2 was reduced to a relative level of 70 ± 3% (mean ± SEM; n = 8) as compared with PEDsh control targets (Fig. 3C). Blocking with NKG2D mAb reduced NK cytotoxicity to a level of 40 ± 3% regardless of whether PEDsh control or PEDshU2 were used as target cells (Fig. 3C). In contrast, PEDshM3 target cells were more susceptible to NK cytotoxicity (119 ± 3%; n = 9), and blocking with NKG2D mAb reduced the lysis by 48 ± 3% and 52 ± 2%, respectively, in comparison with the respective isotype control (Fig. 3D). These observations were consistent with the results obtained by transient siRNA transfection. The reduced NK susceptibility of PEDshU2 was pULBP1 specific and corresponded to the level of remaining pULBP1 expression. The increased NK susceptibility of PEDshM3 was independent of NKG2D. Taken together, these results show that pULBP1, but not pMIC2, appears to act as a functional ligand for human NKG2D.

Generation of a polyclonal anti-pULBP1 Ab

In an effort to confirm pULBP1 surface expression on pEC by flow cytometry and to completely block activating signals elicited by pULBP1 in cytotoxicity assays, a polyclonal Ab against pULBP1 was generated. Following immunization of rabbits with pULBP1-Fc, a polyclonal pULBP1 Ab was purified from the serum of one rabbit. This Ab bound to pULBP1-Fc protein in ELISA, whereas purified rabbit Ig from preimmune serum did not (Fig. 4A). To further characterize the polyclonal Ab and to show pULBP1 specificity, PEDSV.15 and PAEC cell lysates were analyzed by Western blotting. As shown in Fig. 4B, a band of 55–60 kDa was detected by the affinity-purified polyclonal anti-pULBP1 Ab corresponding to the predicted size of pULBP1 dimers. In contrast, no band was seen with the control rabbit Ig obtained from preimmune serum (data not shown). Cell surface expression of pULBP1 on PEDSV.15 and PAEC cells was demonstrated by flow cytometry using the anti-pULBP1 polyclonal Ab (Fig. 4, C and D). Providing additional support for the specificity of the Ab, HEK293 cells stably transfected with full-length pULBP1 were positively stained by anti-pULBP1 polyclonal Ab, whereas untransfected HEK293 were negative (Fig. 4E). Finally, pULBP1 cell surface expression on the PEDshU2 cell line obtained by shRNA transfection (described above) was reduced by 50% as compared with PEDsh control cells (data not shown), supporting the results obtained by quantitative RT-PCR (Fig. 2B). These data show that
rabbit anti-pULBP1 polyclonal Ab specifically binds to pULBP1 expressed on the surface of porcine cells.

Anti-pULBP1 polyclonal Ab completely blocks human NK cytotoxicity against pEC mediated through NKG2D

The ability of rabbit anti-pULBP1 polyclonal Ab to block functional pULBP1/NKG2D interactions was examined in cytotoxicity assays. Incubation of PEDSV.15 cells with anti-pULBP1 polyclonal Ab reduced NK cytotoxicity mediated by freshly isolated and IL-2-activated human NK cells by 67 ± 13% (specific lysis ± SEM) and 41 ± 4%, respectively (Fig. 5, A and B). This level of inhibition correlated well with the blocking of NK cytotoxicity observed with anti-NKG2D mAb preincubation of freshly isolated and IL-2-activated NK cells: 67 ± 14% and 39 ± 5% inhibition, respectively. Finally, as compared with single Ab blocking, combined Ab blocking of pULBP1 and NKG2D on porcine PEDSV.15 and human NK cells did not further enhance the inhibition of NK cytotoxicity. Similar results were obtained in cytotoxicity assays using primary porcine target cells (Fig. 5, C and D). Together, these findings not only demonstrate specific functional interactions between pULBP1 and human NKG2D, but also strongly suggest that pULBP1 is the predominant, if not the only, functional ligand for human NKG2D on porcine cells.

Discussion

NK cell-mediated rejection mechanisms may represent one of the remaining obstacles preventing the clinical application of pig-to-human xenotransplantation. As shown in a series of previous studies by several investigators, human NK cells are able to adhere to and activate pEC (36–39). These interactions lead to morphological changes, the release of proinflammatory cytokines, and endothelial damage. Whether these in vitro phenomena correspond to tissue damage and consequently to the loss of xenografts in vivo is still a matter of debate (12). Human NK cytotoxicity against pEC might depend on incompatible cross-species interactions between porcine MHC class I molecules and inhibitory human NK receptors (17). This notion was supported by the expression of HLA class I molecules on pEC and their complete protection from NK clones expressing the respective HLA-specific inhibitory NK receptor. However, only partial protection from human NK cytotoxicity mediated by polyclonal NK populations was observed (40–43). Besides the lack of MHC class I inhibition, the recognition of putative porcine ligands by human activating NK receptors plays an important role in xenogeneic NK cytotoxicity. Recently, we demonstrated that lysis of pEC mediated by freshly isolated human NK cells is mainly triggered by NKG2D (23). In contrast, lysis mediated by IL-2-activated human NK cells depends on both NKG2D and NKp44. These results predicted the presence of one or several porcine ligand(s) for human NKG2D expressed on pEC.

In this study, we analyzed two recently identified porcine homologues of human NKG2D ligands and demonstrate that pULBP1, but not pMIC2, functionally interacts with human NKG2D. Using an algorithm to score potential ligands (29), pULBP1 was predicted to bind human NKG2D using both the crystal structure of human NKG2D/human ULBP3 and human NKG2D/mouse Rae-1β interactions as template, whereas pMIC2 was predicted to bind NKG2D only using the structure of human NKG2D/human ULBP3 as template (data not shown). Intriguingly, a previous study showed no binding of pULBP1-Fc to the human NK cell line NKL by flow cytometry, whereas binding to porcine PBMC was revealed (33). It was concluded then that pULBP1 does not interact with human NKG2D. The discrepant data regarding pULBP1 binding to human NKG2D might be explained by different experimental conditions. First, the relatively low affinity of Fc fusion proteins used in the previous report may prevent positive staining in flow cytometry assays. In the present study, pULBP1 binding to human NK cells was demonstrated by cellular ELISA. Second, primary human NK cells as well as the NK92 cell line were used, which may exhibit different surface molecule expression patterns as compared with the NKL cell line. Finally, the inability of the soluble Fc fusion proteins to bind to human NK cells may be due to aberrant folding as compared with the membrane-bound form on the cell surface.
However, these binding results did not shed light on the functionality of human NKG2D/pULBP1 interactions, which we further explored by down-regulation of mRNA by stable transfection of shRNA-expressing vectors (44). These experiments added further evidence that pULBP1 acts as a ligand for human NKG2D. The fact that not a complete abrogation of the NKG2D-mediated cytotoxicity was observed was probably due to the remaining pULBP1 mRNA (Fig. 3B) and surface protein (data not shown). In contrast, the reason that down-regulation of pMIC2 rendered PedsV.15 cells more susceptible to NK-mediated killing remains unclear. Because pMIC2 is closely related to MHC class I, we tested whether a protecting signal was delivered by pMIC2 through one of the MHC-specific inhibitory NK receptors on human NK cells. In such a situation, down-regulation of pMIC2 would interfere with these inhibiting signals, leading to an enhanced NK cytotoxicity. However, blocking receptor-ligand interaction using specific mAb against several MHC-specific inhibitory NK receptors (KIR2DL2, KIR2DL3, KIR3DL1, ILT2) did not provide proof for this hypothesis (data not shown). Another possible explanation for the observed increase in lysis of pEC following pMIC2 down-regulation are potential RNA interference off-target effects that were reported by Jackson et al. (45). Further studies are necessary to understand how pMIC2 down-regulation, independent of NKG2D signaling, increases susceptibility to lysis by human NK cells.

Complete inhibition of xenogeneic NK cytotoxicity triggered by pULBP1 was achieved by polyclonal anti-pULBP1 Ab blocking. The relative inhibition was stronger when freshly isolated NK cells were used as compared with IL-2-activated NK cells. This finding was expected, because only the latter express Nkp44, which is also involved in xenogeneic NK cytotoxicity observed using freshly isolated NK effectors indicated the presence of yet unidentified interactions between porcine ligands with human activating NK receptors. The identification of these ligand/receptor pairs as well as the porcine ligands for Nkp44 is currently being addressed to completely overcome NK-mediated xenograft rejection.

This study suggests that pULBP1 is the predominant, if not the only, functional ligand for human NKG2D on porcine cells. pEC express at least one other ULBP-like transcript (pULBP2), although at the mRNA level its expression is ~20-fold less than pULBP1. Also, preliminary analysis of a porcine bacteria artificial chromosome clone suggests several additional loci encoding ULBP-like proteins (our unpublished observations). In this regard, pigs appear similar to humans, in which several ULBP and MIC proteins serve as ligands for NKG2D (26, 27), and also mice, cattle, and primates, which express several NKG2D ligands (46, 47). The redundancy of the NKG2D system within a species might be driven by immune evasion mechanisms of pathogens such as CMV. However, considering that no evolutionary pressure acted on interactions between human and porcine molecules, the lack of redundancy across the species barrier (i.e., pULBP1 being the only ligand for human NKG2D) is not very surprising and sheds more light on the molecular incompatibilities between humans and pigs already identified. In contrast, the molecular compatibility between human NKG2D and pULBP1 might help to prevent zoonoses and infections of porcine xenografts by human pathogens. Therefore, future studies need to address the important question of whether the elimination of pULBP1 might pose a risk by interfering with anti-infectious NK cell responses.

In conclusion, similar to hyperacute xenograft rejection, which has been largely overcome using organs derived from αGal knock-out pigs, the elimination of pULBP1 on porcine tissues might have important implications as a complementary approach to protect porcine xenografts from human NK cell responses. In this study, we focused on direct human NK cytotoxicity against pEC, but there are other potentially harmful interactions between human NK cells and pEC. These include direct cellular activation of pEC, the release of chemokines and cytokines such as porcine TNF-α and IL-8 leading to further EC activation, and the recruitment of additional human leukocytes to the graft. Moreover, the proposed protection of pEC from human NK cytotoxicity by interfering with pULBP1/human NKG2D interactions indicates a possible use to facilitate hemopoietic stem cell xenotransplantation, which has been proposed as a method to induce xenogeneic tolerance (48, 49). Finally, the potential of pULBP1 to induce additional xenogeneic human NK cell responses such as IFN-γ secretion by triggering NKG2D remains to be considered.

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Disclosures

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