Triple (GGTA1, CMAH, B2M) modified pigs expressing an SLA class I^low phenotype—Effects on immune status and susceptibility to human immune responses

Rabea Hein1 | Hendrik J. Sake2 | Claudia Pokoyski1 | Joachim Hundrieser1 | Antje Brinkmann1 | Wiebke Baars1 | Monika Nowak-Imialek2 | Andrea Lucas-Hahn2 | Constanca Figueiredo3 | Hans-Joachim Schuberth4 | Heiner Niemann2 | Björn Petersen2 | Reinhard Schwinzer1

1Transplant Laboratory, Department of General-, Visceral-, and Transplantation Surgery, Hannover Medical School, Hannover, Germany
2Department of Biotechnology, Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute, Mariensee, Neustadt, Germany
3Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany
4Immunology Unit, University for Veterinary Medicine Hannover, Hannover, Germany

Correspondence
Reinhard Schwinzer
Email: schwinzer.reinhard@mh-hannover.de
Björn Petersen
Email: bjoern.petersen@fli.de

Present address
Heiner Niemann, Department of Gastroenterology, Hannover Medical School, Hannover, Germany

Funding information
This work was supported by a grant from the German Research Foundation (DFG), CRC-TRR 127 (“Xenotransplantation”).

Porcine xenografts lacking swine leukocyte antigen (SLA) class I are thought to be protected from human T cell responses. We have previously shown that SLA class I deficiency can be achieved in pigs by CRISPR/Cas9-mediated deletion of β2-microglobulin (B2M). Here, we characterized another line of genetically modified pigs in which targeting of the B2M locus did not result in complete absence of B2M and SLA class I but rather in significantly reduced expression levels of both molecules. Residual SLA class I was functionally inert, because no proper differentiation of the CD8+ T cell subset was observed in B2M^low pigs. Cells from B2M^low pigs were less capable in triggering proliferation of human peripheral blood mononuclear cells in vitro, which was mainly due to the nonresponsiveness of CD8+ T cells. Nevertheless, cytotoxic effector cells developing from unaffected cell populations (e.g., CD4+ T cells, natural killer cells) lysed targets from both SLA class I^ wildtype and SLA class I^low pigs with similar efficiency. These data indicate that the absence of SLA class I is an effective approach to prevent the activation of human CD8+ T cells during the induction phase of an anti-xenograft response. However, cytotoxic activity of cells during the effector phase cannot be controlled by this approach.

KEYWORDS
basic (laboratory) research/science, immunobiology, major histocompatibility complex (MHC), T cell biology, xenoantigen, xenotransplantation

Abbreviations: 3H-TdR, tritiated thymidine; APC, antigen-presenting cell; B2M, β2-microglobulin; CD95L, CD95 ligand; CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; FcRn, neonatal Fc receptor; αGal, galactose-α1,3-galactose-α1,4-N-acetylgalactosamine-R epitope; GGTA1, glycoprotein α-galactosyltransferase 1; h, human; IL, interleukin; indel, insertion or deletion; ko, knockout; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; Neu5Gc, N-glycolyneuraminic acid; NK, natural killer; p, porcine; PBMC, peripheral blood mononuclear cell; pFb, porcine fibroblast; pFF, porcine fetal fibroblast; SCNT, somatic cell nuclear transfer; SLA, swine leukocyte antigen; TcR, T cell receptor; wt, wildtype.

Rabea Hein and Hendrik Johannes Sake share first authorship.
1 | INTRODUCTION

A major problem in transplant medicine is the growing imbalance between demand and availability of suitable human donor organs. Xenotransplantation, the use of porcine organs, tissues, or living cells in human transplantation, has emerged as promising to overcome the shortage of human organs. However, humans elicit a very complex immune response against porcine antigens. The complement-mediated early rejection is mainly caused by preformed antibodies directed against galactose-α1,3-galactose-β1,4-N-acetylgalcosamine-R epitope (αGal)\(^1,2\) and other non-Gal carbohydrate epitopes such as Neu5Gc\(^3,5\) or the glycan produced by porcine β1,4-N-acetyl-galactosaminyltransferase 2 (B4GALNT2)\(^6\) on porcine cells, which in turn rapidly destroys the porcine graft. The development of pigs deficient in αGal (GGTA1-Ko) and Neu5Gc (CMAH-Ko)\(^7,8\) and the transgenic expression of human complement regulatory proteins 9\(^-\)14 helped to overcome these barriers and shifted the research focus to the following cellular and late rejection processes against porcine tissue.

Differences in MHC molecules between donor and recipient are major inducers of immune responses against allografts. Mismatches in the MHC correlate negatively with long-term graft survival in solid organ transplantation due to the reactivity of donor-reactive anti-HLA antibodies.\(^15,16\) In addition, the incompatibility of MHC between donor and recipient activates donor-reactive T cells, especially from the CD8\(^+\) subset, which in turn trigger acute inflammation and destruction of the grafted organ by recognizing donor MHC class I molecules.\(^17,18\) Accordingly, several concepts are currently being tested to achieve immunity protection of allogeneic cells by reducing or deleting MHC class I expression.\(^19,20\)

As in alloreactivity, MHC molecules (swine leukocyte antigen [SLA]) of the porcine donor trigger the recipient's T cell responses after xenotransplantation.\(^21\)\(^-\)24\) Furthermore, porcine SLA molecules are targets of cross-reacting anti-HLA antibodies.\(^25,26\) Because of the possibility to genetically engineer the porcine donor, the generation of immune protected organs and tissues via the elimination of MHC molecules might be particularly beneficial in xenotransplantation rather than in allotransplantation.

MHC class I molecules are expressed on all nucleated cells. They are composed of a heavy α-chain, noncovalently bound to the light chain, β\(_2\)-microglobulin (B2M), and the presented peptide. Because B2M is required for expression of the MHC class I α-chain, targeting of B2M is an effective approach to induce the absence of MHC class I.\(^8,27\)

SLA class I-deficient pigs have already been generated, either by targeting directly the genes encoding for the heavy α-chain or by knocking out B2M.\(^8,29\) Phenotypic studies on the consequences of B2M/SLA class I α-chain targeting in pigs have already been described. However, so far it is not known to what extent porcine cells and tissues are protected against human immune responses by the elimination of SLA class I molecules. Using cells from B2M-targeted pigs and appropriate in vitro assays, we found that the induction/proliferation phase of human CD8\(^+\) T cells is significantly impaired. On the other hand, SLA class I-deficient porcine cells were not protected from lysis by human cytotoxic effector cells.

2 | MATERIALS AND METHODS

2.1 | Animals

Animal experiments were approved by the supervisory authority (LAVES, AZ 33.19-42502-04-16/2343) and conducted in compliance with the German animal welfare law, the German guidelines for animal welfare, and EU Directive 2010/63/EU. German Landrace pigs served as recipient animals for genetically modified embryos derived via somatic cell nuclear transfer (SCNT). A detailed description of the generation of (GGTA1, CMAH, B2M) triple-modified pigs is provided in supplemental Materials and Methods.

2.2 | Isolation of cells and cell culture

Human and porcine peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation (Biocrom GmbH, Berlin, Germany). Human CD8\(^+\) T cells were isolated by depletion of HLA-DR-CD14-CD56-CD4\(^+\) cells using an antibody cocktail and MACS (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany).\(^30\) Viability of separated cells was determined by flow cytometry (forward scatter characteristics) and by microscopic evaluation (trypan blue exclusion) and was usually 85% to 90%. PBMCs were cultivated at 5% CO\(_2\) in RPMI-1640 medium (Lonza, Basel, Switzerland), supplemented with 10% FCS, 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L sodium pyruvate, and 0.05 mmol/L β-mercaptoethanol. Porcine fibroblasts (pFB) were isolated and cultured as previously described.\(^31,32\)

2.3 | Antibodies and flow cytometry

The antibodies used and the staining procedures are described in detail in supplemental Materials and Methods. Data were acquired on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed with summit 5.1 software (Beckman Coulter, Brea, CA).

2.4 | Analysis of immunoglobulin and cytokine levels in porcine serum

The enzyme-linked immunosorbent assay (ELISA) is described in detail in supplemental Materials and Methods.

2.5 | Cell proliferation

A total of 1 × 10\(^5\) human cells (CD8\(^+\) T cells/PBMCs) was cocultured in triplicate with 2 × 10\(^3\) irradiated (30 Gy) porcine PBMCs in a total of 200 μL in microtiter plates. Tritiated thymidine (\(^3\)H-TdR; Perkin Elmer, Waltham, MA) was added after 5 days. After an additional
incubation of 16 hours incorporated $^3$H-TdR was measured in a MicroBeta scintillator counter (Wallac, Victoria, Australia).

### 2.6 | Cytotoxicity assay

The $^{51}$Cr release assays were conducted as described. Briefly, to generate anti-pig sensitized effector cells, human PBMCs were cocultured with porcine PBMCs (wildtype [wt] or B2M low, ratio 1:2) for 5 to 6 days. pFb were labeled with 100 μCi sodium $^{51}$Cr-chromate (GE Healthcare, Buckinghamshire, UK). A total of $1 \times 10^4$ porcine pFb per well were incubated with human effector cells with increasing effector:target ratios. After 4 hours, 25 μL cell supernatant was removed and radioactivity was measured (MicroBeta). Specific lysis was calculated as follows: % specific lysis = (experimental $^{51}$Cr release − spontaneous $^{51}$Cr release)/(maximum $^{51}$Cr release − spontaneous $^{51}$Cr release) $\times$ 100.

### 2.7 | CD107a degranulation assay

Human PBMCs were pretreated with 50 ng/mL human interleukin (hIL)-2 for 5 to 6 days to generate effector cells. Then, $3 \times 10^4$ wt or B2M low pFb was cultivated with $3 \times 10^5$ human effector cells for 2 hours. Cells were stained with the anti-human mAb CD107a-FITC (H4A3), CD3-PE (HIT3a), and CD56-APC (B159, all from BD Biosciences, San Diego, CA). Degranulation activity of human natural killer (NK) cells was assessed by flow cytometry based on the percentage of CD107a$^+$ cells in the CD3$^+$CD56$^+$ cell population.

### 2.8 | Immunohistological staining

Tissue samples were snap-frozen in liquid nitrogen. Cryostat sections of 5 μm were stained for SLA class I antibody (74-11-10, provided by A. Saalmüller, Vienna, Austria). Stained cells were detected with HRPO-coupled goat anti-mouse antibody (dianova GmbH, Hamburg, Germany) and were visualized with the use of 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich, St. Louis, MO). Sections were lightly counterstained with hematoxylin (Merck, Darmstadt, Germany).

### 2.9 | Statistics

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA). Significant values were determined using repeated-measure 1-way ANOVA when comparing 2 different conditions and 2-way ANOVA when comparing >2 conditions. For comparison of mean values from different number of donors or when comparing only 1 condition, untailed students T tests were performed. $P < .05$ were assumed significant.

### 3 | RESULTS

#### 3.1 | Generation of B2M, GGTA1, CMAH triple-modified pigs

Triple-modified pigs were generated by cotransfecting CRISPR/Cas9 plasmids simultaneously targeting the porcine B2M, CMAH, and GGTA1 genes, respectively, into PFFs, followed by counterselection for αGal-negative cells and SCNT. In total, 186 reconstructed embryos were transferred into 2 recipient animals. Both recipients became pregnant, were allowed to go to term, and gave birth to 3 and 5 liveborn piglets, respectively (Table 1). Tissue samples of these piglets were taken, and DNA was extracted followed by PCR amplification of the targeted loci. Deep sequencing of these amplicons revealed indel formation in all animals at the loci of interest, frequently leading to a frameshift mutation (Table S3). Specifically, 3 different genotypes could be identified among all animals, including not only 2 but 4 alleles for B2M as previously described. In 4 animals (genotype I: 707/1, 707/3, 708/1, 708/2), all 4 B2M alleles, and 1 CMAH allele were modified. Three other animals (genotype II: 707/2, 708/4, 708/5) showed the same biallelic single-basepair insertion in GGTA1 as genotype I. However, the position of the 4-bp deletion in B2M differed from genotype I as well as the 20-bp deletion in CMAH. Piglet 708/3 displayed a unique genotype, in which all 4 B2M alleles showed the same single-basepair deletion, a biallelic single-basepair insertion in CMAH and 1 and 3 bp were deleted in the 2 GGTA1 alleles, respectively (Table S3). An assignment of animals to the 3 genotypes is summarized in Table 2.

To study the effects of gene targeting on the protein and carbohydrate levels, the expression profile of B2M and the presence of carbohydrate epitopes αGal and Neu5Gc were analyzed in PBMC. B2M, αGal, and Neu5Gc were readily detected on cells from wt control pigs (Figure 1A). B2M was significantly reduced in all tested triple-modified pigs to minimal levels above background but was still detectable. αGal epitopes were absent, suggesting a complete knockout (ko) of the GGTA1 gene. Analysis of Neu5Gc expression of heterozygous ko piglets 707/1 and 708/1 showed no difference from wt controls. No Neu5Gc could be detected on cells from the homozygous ko piglet 708/5. The following characterization of cells and tissues from triple-modified pigs focuses on the biological consequences of B2M targeting in these pigs.

#### TABLE 1 | Embryo transfer of somatic cell nuclear transfer–derived embryos4

| Recipient | Transferred embryos (n) | Pregnant | Piglets liveborn|stillborn |
|-----------|-------------------------|----------|-----------------|
| 707       | 95                      | +        | 3|1 |
| 708       | 91                      | +        | 5|5 |
| Total     | 186                     | 2/2 (100%) | 8 (4.3%)$^b$ |

$^a$A total of 186 embryos were transferred into 2 synchronized recipient sows. Both animals conceived successfully, leading to the birth of 3 and 5 viable piglets, respectively.

$^b$Overall cloning efficiency.
animals. Because B2M was not completely absent, samples were not termed B2M-ko but rather B2M-low.

### 3.2 | B2M targeting results in decreased expression levels of SLA class I and other cell surface receptors

B2M is not only required for expression of the α-chain of MHC class I but also for expression of other proteins such as CD1 and the neonatal Fc receptor (FcRn). To analyze the effects of reduced B2M levels, we compared SLA class I and CD1 expression in PBMC from B2M-low pigs and wt controls. All tested B2M-low pigs showed significantly reduced expression levels of SLA class I on PBMCs, whereas complete absence was never observed (Figure 1B, left). Quantification of the SLA class I expression levels by comparing mean fluorescence intensity (MFI) in B2M-low and wt cells revealed a reduction by ~90% in cells from B2M-targeted pigs (Figure 1B, right). CD1 expression was not detected in B2M-low cells (Figure 1C). We did not further evaluate whether this was due to the defective expression of CD1 or the absence of the subpopulation of CD1+ dendritic cells.

### 3.3 | B2M-low pigs possess a distorted T cell compartment

MHC class I molecules are required for positive selection of CD8+ T cells during T cell differentiation in the thymus. We asked whether reduced expression of SLA class I in B2M-low pigs is sufficient to allow normal T cell differentiation. Thus, the relative fractions of CD4+ and CD8+ T cell subsets within the T cell population (CD3+ T cells) were determined in whole blood from B2M-low and wt control animals. All B2M-low pigs showed a nearly complete absence of CD8+ (CD8α+CD8β+) T cells (Figure 2). The remaining 2% might be cells expressing the γδ TcR, because these cells can also express the CD8αβCD8β heterodimer. As expected from the absence of CD8+ T cells, there was an increase in the relative proportion of CD4+ (CD4αCD8β-) T cells in B2M-low pigs. NKT cells could not be detected in B2M-low pigs (data not shown). This is, most likely, a consequence of the observed CD1 deficiency (Figure 1C) because NKT cells depend on CD1d for proper development.

Further evaluation of the immune status of B2M-low piglets revealed strong IgG deficiency during the observation period (Figure 3A). IgM and IgA levels showed no differences between B2M-low and wt pigs (data not shown). The FcRn is involved in translocation of maternal IgG from piglet gut lumen into the blood. Because expression of FcRn depends on B2M, this receptor is likely also impaired in B2M-low pigs, which could account for the observed IgG deficiency. We also monitored cytokine levels in serum from B2M-low and wt control piglets within the first 4 weeks of life. As shown for day 26 after birth, several cytokines were significantly decreased in serum from B2M-low pigs compared with wt controls (Figure 3B).

### 3.4 | Priming of human PBMCs with B2M-low cells spares the CD8+ T cell subset

The human TcR can functionally interact with porcine SLA molecules leading to strong proliferation of human lymphocytes in human/pig MLR assays. Thus, we assumed that reduced levels of SLA class I in cells from B2M-low pigs should affect their stimulatory capacity in xenogeneic MLR. Testing this hypothesis, we used PBMCs from B2M-low and wt pigs as stimulators and human PBMCs as responder cells. Only a slightly decreased proliferation was observed after stimulation with B2M-low cells in comparison with wt cells (Figure 4A, upper panel). However, when purified human CD8+ T cells were used as responders, proliferation to B2M-low stimulators was nearly abolished (Figure 4A, lower panel). This was true for B2M-low stimulators carrying either genotype I (αGal Neu5Gc) or II (αGal Neu5Gc). We also used cells from a single B2M-modified pig expressing normal levels of αGal and Neu5Gc as stimulators. Similar to stimulation with triple-modified cells, CD8+ T cell proliferation was significantly impaired (Figure 4B). Together, these data suggest that the defective stimulatory capacity of triple-modified B2M-low cells mainly results from low-level SLA class I expression. The simultaneous absence of αGal and/or Neu5Gc had only marginal effects in decreasing the capacity of pig cells to trigger human CD8+ T cell responses.

### 3.5 | B2M-low cells are not protected from lysis by cytotoxic effector cells

We next asked whether in vitro priming of the entire human PBMC population with stimulator cells from B2M-low pigs generates cytotoxic activity despite impaired responses of the CD8+ T cell subset. Cells from 5-day MLR cultures were harvested and cytotoxicity against pFb...
was monitored by $^{51}$Cr-release assays. Priming of human PBMCs with cells from both wt and B2M$^{low}$ pigs resulted in significant cytotoxic activity. In wt and B2M$^{low}$ targets, the effector population generated by priming with B2M$^{low}$ cells was slightly less effective than when effectors had been primed by cells from wt pigs (Figure 5A). Together, these data indicate that, despite impaired CD8$^+$ T cell reactivity, cytotoxic cells can differentiate in human PBMC cultures primed with B2M$^{low}$ cells. These effector cells do not require SLA class I expression on targets to exert their cytotoxic activity.

To study the role of NK cells, we analyzed the reactivity of human CD3$^+$CD56$^+$ NK cells to pFb by measuring degranulation via CD107a expression. The pFb did not induce CD107a expression in resting NK cells (data not shown). When human PBMC, were activated by IL-2, approximately 20% of CD3$^+$CD56$^+$ NK cells expressed CD107a after being mixed with wt pFb (Figure 5B). The same amount of NK cells responded to pFb from B2M$^{low}$ pigs, suggesting that decreased levels of SLA class I are not associated with enhanced activation of human NK cells.
3.6 | SLA class I is almost absent in tissues from B2Mlow pigs

To evaluate the effects of B2M targeting on SLA class I expression in organs relevant for transplantation, we performed immunohistochemistry of cryosections from heart, kidney, and liver tissue (Figure 6). Strong SLA class I expression was observed in samples from wt pigs. In heart and kidney, staining of vessels was particularly pronounced, suggesting expression mainly in endothelial cells. Staining of liver samples resulted in a more homogeneous pattern. In samples from B2Mlow pigs, only weak staining of the anti-SLA class I antibody was observed with comparable patterns as in wt samples (heart, kidney: mainly endothelium; liver: broad).

4 | DISCUSSION

GGTA1/CMAH/B2M triple-modified pigs were generated to provide cells and organs for clinical xenotransplantation. Because the beneficial effects of αGal and Neu5Gc absence on antibody binding/complement activation are well documented,7,14 the current experiments focused on the characterization of T cell reactivity. We assume that reduced proliferation of human CD8+ T cells mainly results from the SLA class I neg/low phenotype of porcine stimulatory cells. Thus, stimulatory cells from a pig with only B2M-ko but normal levels of αGal and Neu5Gc showed a similar defect to induce CD8+ T cell activation as triple-modified cells (Figure 4B). A minor effect of glycans on human
T cells is also supported by the observation that the human anti-pig T cell responses to cells from wt pigs and αGal- and/or Neu5Gc-deficient pigs are comparable. Similar to our experiments, porcine PBMCs were used as stimulators. However, other researchers using porcine endothelial cells as stimulators found that the absence of αGal is compatible with a low stimulatory capacity of cells. CRISPR/Cas9-mediated inactivation of GGTA and CMAH resulted in complete absence of the respective carbohydrate epitopes αGal and Neu5Gc (Figure 1). Residual cell surface expression of B2M, however, was detected in all triple-modified pigs (Figure 1) carrying either B2M genotype I, II, or III. B2M genotypes I and II have 3-bp deletions, which may leave a truncated protein. However, so far it is not clear why an identical B2Mlow phenotype was also observed in genotype III, which has a frameshift mutation in all B2M alleles. Unknown mutations make it difficult to tell if the phenotype observed is due to the known mutation introduced via CRISPR or the inadvertent changes caused by nonhomologous end joining or CRISPR inefficiency itself. Recently, a new method has been reported that can achieve a precise genotype-phenotype linkage. CRISPR/Cas9 was followed by laser microdissection and single cell genotyping. This protocol makes it possible to find out exactly what phenotypic changes the CRISPR-induced mutations caused in the cell studied and then to confirm the exact nature of the underlying DNA change that produced that phenotype.

Staining of cells from B2Mlow pigs with mAb PT85A detecting the α-chains of SLA class I revealed reduced expression compared with wt controls (Figure 1B). Because SLA class I genes were not affected by the CRISPR/Cas9 strategies that were used, low-level

**FIGURE 4** Proliferative responses of human lymphocytes after stimulation with porcine peripheral blood mononuclear cells (PBMCs). A, upper panel, 1 × 10⁵ human PBMCs were stimulated with increasing numbers of irradiated (30 Gy) wildtype (wt) or β₂-microglobulin (B2M)low porcine PBMCs. After 5 days of coculture, proliferation was measured by [³H]-thymidine incorporation for an additional 16 hours. Data are mean values obtained with samples from 6 different blood donors (±SD) stimulated with cells from 3 different wt or B2Mlow (707/1, 708/1, 708/5) pigs. A, lower panel, 1 × 10⁵ human CD8⁺ T cells were stimulated either with irradiated PBMCs from wt pigs or pigs carrying genotype I (Neu5Gc⁺) or genotype II (Neu5Gc⁻). Data are mean values (±SD) obtained in n = 4 (Neu5Gc⁺ group) and n = 2 (Neu5Gc⁻ group) independent experiments. B, 1 × 10⁵ human CD8⁺ T cells were stimulated with increasing numbers of irradiated PBMCs from a wt pig or a pig with B2Mko, GGTA1wt, CMAHwt genotype. Data are from a single experiment and represent mean cpm of triplicate cultures ± SEM. Similar patterns were observed using CD8⁺ T cells from a second blood donor. Statistical significance was analyzed by 2-way ANOVA with Sidak correction (*) \( P < .05 \), ** \( P < .01 \), *** \( P < .001 \), **** \( P < .0001 \).
expression can be explained by 2 possible mechanisms. First, the number of B2M molecules is too low to provide sufficient partner molecules for the association with the existing SLA class I $\alpha$-chains. Second, if B2M indeed is a truncated protein, aberrant association with SLA class I $\alpha$-chain may occur, which in turn affects stability of the complex. In any case, the few existing SLA class I $\alpha$-chain/B2M heterodimers expressed in B2M low pigs seem to be nonfunctional. This conclusion is based on the observation that 2 SLA class I–dependent processes—the development of the CD8$^+$ T cell subset in pigs (Figure 2) and the capacity to trigger proliferation of human CD8$^+$ T cells (Figure 4)—are significantly disturbed. Thus, B2M low pigs can be regarded as functional SLA class I ko animals.

It is well known that human CD4$^+$ and CD8$^+$ T cells can directly be activated via interaction of their TcR with SLA class II and class I, respectively.$^{21,23}$ Thus, porcine antigen-presenting cells (APCs) that express SLA class I and class II will induce activation of both human CD4$^+$ and CD8$^+$ T cells during the priming/sensitization phase of an antigrant response. It was, therefore, an expected finding that stimulatory cells from B2M low pigs did not induce proliferation of CD8$^+$ T cells (Figure 4). However, one has to keep in mind that the stimulating cell population (PBMCs) of these pigs contains cells expressing normal levels of SLA class II (eg, B cells, monocytes), thus facilitating effective activation of the human CD4$^+$ T cell subset. Following this line, we assume that cytotoxic CD4$^+$ T cells that develop during the priming of human PBMCs with B2M low cells are the major effectors in $^{51}$Cr-release assays (Figure 5A). This is also supported by earlier data showing that CD4$^+$ T cells play an important role in human anti-pig cytotoxicity.$^{43}$ The pFb that were used as targets in $^{51}$Cr-release assays did not express detectable levels of SLA class II (data not shown), which usually is the restriction element for the TcR of CD4$^+$ T cells. Thus, the question arises by which mechanisms porcine targets may be lysed. One possibility is that cytolytic activity of CD4$^+$ T cells is triggered by cross-reactivity of their TcR with low-level SLA class I expressed by pFb from B2M low pigs. On the other hand, cytotoxic mechanisms, independent from TcR/MHC interactions, may be involved. In fact, pFb strongly expressed the cell death–inducing CD95 (Fas) receptor (data not shown). Usually, CD4$^+$ T cells kill their targets in xenogeneic settings via expression of CD95 (Fas) ligand and not via release of perforin/FIGURE 5  Susceptibility of B2M$^{\text{low}}$ cells to lysis by human effector cells. A, Anti-pig sensitized effector cells were generated by in vitro priming (5 d MLR) of human peripheral blood mononuclear cells (PBMCs) with irradiated PBMCs from a wildtype (wt) pig or pig 708/5 carrying genotype II (B2M$^{\text{low}}$; aGal, Neu5Gc$^-$$^-$$^-$). Effector cells were harvested and titrated on $1 \times 10^4$ $^{51}$Cr-labeled Fb from wt pigs or pig 708/5 (B2M$^{\text{low}}$; aGal, Neu5Gc$^-$$^-$$^-$). After 4 hours, the amount of radioactivity in the supernatant was measured and specific lysis was calculated. Data are mean values from 2 different experiments. B, Degranulation of human natural killer (NK) cells. Interleukin-2–activated human PBMCs were cocultured with wt or B2M$^{\text{low}}$ pFb for 2 hours. CD107a expression of CD3$^-$CD56$^+$ NK cells was measured by flow cytometry. Left, Representative dot plots of one human blood donor. Numbers represent percentage of CD107a$^+$ cells. Right, Percentages of CD107a$^+$ NK cells from all tested individuals are summarized. Data are mean values (±SD) from 4 different human blood donors stimulated with pFb from 2 different wt or 4 different B2M$^{\text{low}}$ (707/1, 707/3, 708/1, 708/5) pigs.
granzyme vesicles.\textsuperscript{43} Thus, we favor the hypothesis that target cells from B2M\textsubscript{low} pigs are lysed in a TcR/MHC–independent manner by CD95/CD95 ligand interactions, which is supported by previous data pointing to a significant role of this pathway in xenogeneic killing of porcine cells.\textsuperscript{43-45}

In an allogeneic setting, MHC class I acts as an inhibitory ligand for NK cells.\textsuperscript{46} If this mechanism is functional also in pig-to-human xenotransplant, cells from B2M\textsubscript{low} pigs may be highly susceptible to lysis by human NK cells. To test this, we compared the activity of human NK cells triggered by pFb from B2M\textsubscript{low} and wt pigs. We found no difference in the responsiveness of NK cells to the 2 stimulator cell types (Figure 5B), which supports earlier findings suggesting that SLA class I is not an inhibitory ligand for human NK cells and that NK-mediated killing of porcine cells is MHC independent.\textsuperscript{47,48} Sequence comparison between porcine and human MHC class I molecules revealed that the binding regions responsible for interaction with NK cells differ between the 2 species.\textsuperscript{49}

\textbf{FIGURE 6} Analysis of swine leukocyte antigen (SLA) class I expression in porcine tissues. Cryosections of heart, kidney, and liver from a wildtype (wt) pig and \(\beta_2\)-microglobulin (B2M\textsuperscript{low}) pig 708/3 (genotype III) were stained with the anti-SLA class I mAb 74-11-10 or an irrelevant isotype matched control antibody (mouse IgG2b). Antibody binding was detected by an HRPO-coupled secondary antibody and incubation with AEC. Nuclei were counterstained with hematoxylin. Magnification is \(\times 100\). Similar patterns were observed using samples from pigs carrying B2M genotype I (707/3) or genotype II (708/5). As representative of this series, data obtained with cryosections from kidneys are shown in Figure S1.

\textsuperscript{B2M\textsubscript{low}} pigs expressed significantly reduced levels of SLA class I, which was associated with impaired development of CD8\textsuperscript{+} T cells (Figure 3). Furthermore, CD1 was completely absent (Figure 1C), which most likely is the reason for the lack of NK T cells in these animals (data not shown). In addition, we assume that the IgG deficiency in B2M\textsubscript{low} pigs (Figure 3A) also is somewhat related to B2M targeting. Maternal immunoglobulins are usually transported to the suckling blood system via the placenta or colostrum, which requires a functional FcRn that can only be expressed in association with B2M.\textsuperscript{39,50,51} Strongly reduced IgG levels have also been observed in B2M-deficient mice and humans.\textsuperscript{52,53} Thus, targeting B2M affected health and immune status of pigs in various ways. Presumably, the massive perturbations of the immune system in B2M-targeted pigs are a major reason for the development of acute feverish conditions and subsequent death, which occurred in 3- to 4-week-old pigs in recent experiments.\textsuperscript{29} These animals were kept under standard housing conditions. In contrast, the B2M\textsubscript{low} piglets reported here were kept
under strict hygienic housing conditions and were maintained completely separate from the rest of the herd. The animals of the present study developed normally up to an age of 6 months, when they were killed for sample collection. Thus, applying high hygienic standards, it is possible to successfully maintain B2M-deficient pigs for longer periods of time.

The absence of functional SLA class I makes B2M<sup>low</sup> pigs promising as donors for clinically used xenografts. However, it has to be taken into account that despite effective suppression of human CD8<sup>+</sup> T cells during the induction phase of an antigen response, the lack of functional SLA class I does not protect porcine cells and tissues from cytotoxicity during the effector phase. Nevertheless, susceptibility of porcine cells to lysis by human effector cells could be reduced by transgenic expression of the protective molecule A20. Furthermore, human inhibitory ligands like PD-L1<sup>30,55</sup> or HLA-E<sup>56</sup> have been shown to protect porcine cells.

ACKNOWLEDGMENTS

We would like to acknowledge the assistance of the Cell Sorting Core Facility of the Hannover Medical School supported in part by Braumann-Wittenberg-Herz-Stiftung and Deutsche Forschungsgemeinschaft. Sequencing data used or referred to in this publication were generated by the Research Core Unit Genomics (RCUG) at MHH. We are grateful for the competent and continuous support by Antje Frenzel, Petra Hassel, Roswitha Becker, and Maren Ziegler and the staff of the experimental pig facility.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the <i>American Journal of Transplantation</i>.

AUTHOR CONTRIBUTION

R. Hein and H.J. Sake designed and performed research, analyzed data, and wrote the manuscript. A. Brinkmann, W. Baars, Dr M. Nowak-Imialek, Dr A. Lucas-Hahn, and Dr H.-J. Schuberth performed the experiments. Dr C. Figueiredo performed experiments and analyzed data. Dr C. Pokoyski analyzed data. Dr J. Hundrieser discussed the experiments. Drs R. Schwinzer, B. Peterson, and H. Niemann designed research, analyzed data, and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Constanca Figueiredo https://orcid.org/0000-0003-2786-0388
Hans-Joachim Schuberth https://orcid.org/0000-0002-5904-5751
Heiner Niemann https://orcid.org/0000-0003-0282-9704
Björn Petersen https://orcid.org/0000-0002-1532-4863
Reinhard Schwinzer https://orcid.org/0000-0002-9226-4796

REFERENCES

1. Cooper DK, Good AH, Koren E, et al. Identification of alpha-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. <i>Transpl Immunol</i>. 1993;13(1):198-205.
2. Gallili U. Xenotransplantation and ABO incompatible transplantation: the similarities they share. <i>Transfus Apher Sci</i>. 2006;35(1):45-58.
3. Nguyen DH, Tangvoranuntakul P, Varki A. Effects of natural human antibodies against a nonhuman sialic acid that metabolically incorporates into activated and malignant immune cells. <i>J Immunol</i>. 2005;175(1):228-236.
4. Hurh S, Kang B, Choi I, et al. Human antibody reactivity against xenogeneic N-glycolylineuraminic acid and galactose-alpha-1,3-galactose antigen. <i>Xenotransplantation</i>. 2016;23(4):279-292.
5. Byrne GW, McGregor CG, Breimer ME. Recent investigations into pig antigen and anti-pig antibody expression. <i>Int J Surg</i>. 2015;23(Pt B):223-228.
6. Byrne G, Ahmad-Villiers S, Du Z, McGregor C. B4GALNT2 and xenotransplantation: A newly appreciated xenogeneic antigen. <i>Xenotransplantation</i>. 2018;25(5):e12394.
7. Estrada JL, Martens G, Li P, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGT1A/CMAH/ beta4GalNT2 genes. <i>Xenotransplantation</i>. 2015;22(3):194-202.
8. Wang Y, Du Y, Zhou X, et al. Efficient generation of B2 m-null pigs via injection of zygote with TALENs. <i>Sci Rep</i>. 2016;6:38854.
9. Kolber-Simonds D, Lai L, Watt SR, et al. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. <i>Proc Natl Acad Sci USA</i>. 2004;101(19):7335-7340.
10. Lai L, Kolber-Simonds D, Park KW, et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. <i>Science</i>. 2002;295(5557):1089-1092.
11. Nottle MB, Beebe LF, Harrison SJ, et al. Production of homozygous alpha-1,3-galactosyltransferase knockout pigs by breeding and somatic cell nuclear transfer. <i>Xenotransplantation</i>. 2007;14(4):339-344.
12. Tseng YL, Kuwaki K, Dor FJ, et al. alpha1,3-Galactosyltransferase gene-knockout pig heart transplantation in baboons with survival approaching 6 months. <i>Transplantation</i>. 2005;80(10):1493-1500.
13. Azimzadeh AM, Kelisahdi SS, Ezzelarab MB, et al. Early graft failure of GaITKO pig organs in baboons is reduced by expression of a human complement pathway-regulatory protein. <i>Xenotransplantation</i>. 2015;22(4):310-316.
14. Fischer K, Kramer-Scheiber S, Petersen B, et al. Efficient production of multi-modified pigs for xenotransplantation by ‘combineering’, gene stacking and gene editing. <i>Sci Rep</i>. 2016;6:29081.
15. Yamada Y, Langner T, Inci I, et al. Impact of human leukocyte antigen mismatch on lung transplant outcome. <i>Interact Cardiovasc Thorac Surg</i>. 2018;26(5):859-864.
16. Williams RC, Opelz G, McCarvey CJ, Weil EJ, Chakkeria HA. The risk of transplant failure with HLA mismatch in first adult kidney allografts from deceased donors. <i>Transplantation</i>. 2016;100(5):1094-1102.
17. Yap M, Brouard S, Pecqueur C, Degauque N. Targeting CD8 T cell metabolism in transplantation. <i>Front Immunol</i>. 2015;6:547.
18. Ascon M, Ascon DB, Liu M, et al. Renal ischemia-reperfusion leads to long term infiltration of activated and effector-memory T lymphocytes. <i>Kidney Int</i>. 2009;75(5):526-535.
19. Börger A-K, Eicke D, Wolf C, et al. Generation of HLA-universal iPSC-derived megakaryocytes and platelets for survival under refractoriness conditions. <i>Mol Med</i>. 2016;22:274-285.
20. Figueiredo C, Wedekind D, Müller T, et al. MHC universal cells survive in an allogeneic environment after incompatible transplantation. <i>Biomed Res Int</i>. 2013;2013:796046.
21. Yamada K, Sachs DH, Dersimonian H. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed
leukocyte reaction and for both direct and indirect pathways of recognition. *J Immunol.* 1995;155:5249-5256.

22. Dorling A, Lombardi G, Binns R, Lechler RI. Detection of primary direct and indirect human anti-porcine T cell responses using a porcine dendritic cell population. *Eur J Immunol.* 1996;26:1378-1387.

23. Murray AG, Khodadoust MM, Pojer JS, Bothwell ALM. Porcine aortic endothelial cells activate human T cells: direct presentation of MHC antigens and costimulation by ligands for human CD2 and CD28. *Immunity.* 1994;1:57-63.

24. Shishido S, Naziruddin B, Howard T, Mohanakumar T. Recognition of porcine major histocompatibility complex class I antigens by human CD8+ cytolytic T cell clones. *Transplantation.* 1997;64(2):340-346.

25. Ladowski JM, Reyes LM, Martens GR, et al. Swine leukocyte antigen (SLA) class II is a xenoreactive antigen. *Transplantation.* 2018;102(2):249-254.

26. Martens GR, Reyes LM, Butler JR, et al. Humoral reactivity of renal transplant-waitlisted patients to cells from GGTA1/CMAH/B4GalNT2, and SLA class I knockout pigs. *Transplantation.* 2017;101(4):e86-e92.

27. Figueiredo C, Carvalho Oliveira M, Chen-Wacker C, et al. Immunoeengineering of the vascular endothelium to silence MHC expression during normothermic ex vivo lung perfusion. *Hum Gene Ther.* 2019;30(4):485-496.

28. Reyes LM, Estrada JL, Wang ZY, et al. Creating class I MHC-null pigs using guide RNA and the Cas9 endonuclease. *J Immunol.* 2014;193(11):5751-5757.

29. Sakai H, Frenzel A, Lucas-Hahn A, et al. Possible detrimental effects of beta-2-microglobulin knockout in pigs. *Xenotransplantation.* 2019;e12525. https://doi.org/10.1111/xen.12525.

30. Plege A, Borns K, Beer L, Baars W, Klempnauer J, Schwinzer R. Significant inhibition of human CD8+ cytotoxic T lymphocyte-mediated xenocytotoxicity by overexpression of the human decay Fas antigen. *Transplantation.* 2006;81(5):789-796.

31. Niemann H, Verhoeyen E, Wonigeit K, et al. Cytomegalovirus infection of porcine major histocompatibility complex class I knockouts at averted hyperacute rejection. *Transplantation.* 2004;77(9):1289-1293.

32. Petersen B, Ramackers W, Tiede A, et al. Pigs transgenic for human immunoglobulin G (IgG) antibody into the gastrointestinal tract are resistant to human anti-porcine T cell recognition. *J Immunol.* 2009;182(12):7319-7328.

33. Murat J, Remmert M, Petersen B, et al. Pigs expressing the porcine major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. *Am J Transplant.* 2003;35:47-51.

34. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol.* 2008;9(5):495-502.

35. Buchner C, Yatko C, Johnson EW, Edge AS. Human natural killer cells account for non-MHC class I-restricted cytolysis of porcine cells. *Cell Immunol.* 1997;175(2):171-178.

36. Besser TE, McGuire TC, Gay CC, Pritchett LC. Transfer of functional immunoglobulin G (IgG) antibody into the gastrointestinal tract accounts for IgG clearance in calves. *J Virol.* 1988;62(7):2234-2237.

37. Wani MA, Haynes LD, Kim J, et al. Familial hypercatabolic hypoproteinaemia caused by deficiency of the neonatal Fc receptor, FcRn, due to a mutant beta2-microglobulin gene. *Proc Natl Acad Sci USA.* 2006;103(13):5084-5089.

38. Steinecke A, Kurabayashi N, Hayano Y, Ishino Y, In TH. Vivo single-cell genotyping of mouse cortical neurons transfected with CRISPR/Cas9. *Cell Rep.* 2019;28(2):325-331.e4.

39. Yi S, Feng X, Wang Y, Kay TWH, Wang Y, O’Connell PJ. CD4+ cells play a major role in xenogeneic human anti-pig cytotoxicity through the fas/fas ligand lytic pathway. *Transplantation.* 1999;67:435-443.

40. Wani MA, Haynes LD, Kim J, et al. Familial hypercatabolic hypoproteinaemia caused by deficiency of the neonatal Fc receptor, FcRn, due to a mutant beta2-microglobulin gene. *Proc Natl Acad Sci USA.* 2006;103(13):5084-5089.