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Brief communication

Modified glycan models of pig-to-human xenotransplantation do not enhance the human-anti-pig T cell response

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ABSTRACT

Genetically modified porcine models of pig-to-human xenotransplantation offer the most immediate answer to a growing shortage of available solid organs. Recently a modified porcine glycan model has been discovered that reduces human antibody binding to levels comparable with allograft standards. As this background provides an answer to the problem of acute humoral xenograft rejection (AHXR), it is important to consider the impact these modifications have on measures of cell-mediated rejection. The objective of this study was to examine the impact of currently relevant glycan knockout models of pig-to-human xenotransplantation in a lymphocyte proliferation assay. To accomplish these goals, genetically modified pigs were created through CRISPR/Cas9-directed silencing of the GGTA1, and CMAH genes. Peripheral blood mononuclear cells (PBMCs) and spleen cells were obtained from these animals and used as a source of stimulation for human responders in one-way mixed lymphocyte reactions. The response was tested in the presence and absence of clinically available immunomodifiers. Conclusions: Clinically relevant glycan knockout models of pig-to-human xenotransplantation do not enhance the human-anti-pig cellular response. Currently available and conventional immunosuppression has the capacity to mediate the human xenogeneic T cell response to these knockout cells.

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1. Introduction

At present, solid-organ xenotransplantation remains the most viable option for an adequate expansion of the donor pool. Given their short gestational cycle and physiologic similarity to humans, genetically-modified pigs have been identified as the best donor candidates. Unfortunately, wild-type swine cells exhibit a carbohydrate profile to which humans have preformed antibodies. Unmodified, these antigens cause acute humoral xenograft rejection (AHXR) in a pig-to-primate model. The creation of GGTA1$^{-/-}$ (GTKO) pigs in 2003 marked the first successful antigen reduction and legitimized the concept of pig-to-human xenotransplantation [1]. Over the past 13 years, many genetic modifications have been attempted to overcome AHXR. In 2015 the creation of a triple-knockout animal successfully lowered human antibody binding to levels comparable with currently-accepted allograft standards [2].

Since the development of the first GGTA1$^{-/-}$ pig, there has been uncertainty about the severity of the human-anti-pig T cell response and whether it can be adequately controlled using approved immunosuppressive therapies [3]. In vivo, pig-to-primate solid organ transplant has been sustained using a wide range of pharmacologic strategies [4]. Unfortunately, the enduring efficacy of these regimens has been difficult to ascertain, as graft rejection often occurred early in the context of high xenoantigen level and thrombotic microangiopathy [5]. As such, discussions of cell-mediated rejection have not commanded the same attention afforded to problems of AHXR.

Advances in genetic engineering strategies have dramatically sped the process of identifying and eliminating xenoantigens [6]. Within the porcine genome, the GGTA1 locus drives αGal glycan expression and the CMAH gene drives Neu5Gc glycan expression. Each of these gene products independently induces human antibody binding; when both genes are silenced, human IgG and IgM binding is dramatically lowered [7]. As these porcine cells now react similarly to human cells in crossmatch analysis, it is increasingly likely that xenograft AHXR can be effectively mediated by genetic donor modification; discussions of cell-mediated rejection pathways should now occupy a more prominent position within the study of xenotransplantation.

Though the dedicated study of glycoimmunology is relatively new, a growing body of evidence supports the ability of glycosylation variance to affect immune response [8]; modifications made to mammalian glycan profiles warrant careful consideration of the potential for immunogenic effects. Herein we describe the impact of successful glycan

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antigen-reduction models of pig-to-human xenotransplant in a cell-mediated proliferation assay. As these genetically modified cells have shown great promise by reducing antibody-mediated barriers of AHXR, their cell-mediated immunogenicity is of potential clinical importance.

2. Materials and methods

2.1. Modified porcine cell construction

Genetically modified pigs were created utilizing a CRISPR/Cas9 approach and somatic cell nuclear transfer as previously described by Li et al. [9]. Briefly, bicistronic CRISPR sgRNA expression vectors co-expressing the Cas9 gene were designed to bind and cleave the GGTA1 gene beginning at position 293,654,066 of NC_010443.4 (forward: CACCGCGAAAATAATGAATGTCAA) and CMAH gene beginning at position 210,555,038 of NCBI reference NC_010449.4 (forward: CACCGAGT AAGGTACGTGATCTGT). Porcine liver-derived cells were cultured, and transfected with targeted nuclease-expressing vectors using the Neon transfection system (Life Technologies, Grand Island NY, USA) as described by Lutz et al. [7]. After negative selection by flow cytometry, knockout cells were identified and somatic cell nuclear transfer (SCNT) was used to create clonal animals from modified porcine cells. Genetically modified animals were grown to maturity and. The Institutional Biosafety and Institutional Animal Care and Use Committee at Indiana University School of Medicine approved the use of animals in this research.

2.2. Preparation of stimulator populations

Porcine blood was obtained by venous sample from wild type (WT), $GGTA1^{-/-}$ (GTKO) or $CMAH^{-/-}$ $GGTA1^{-/-}$(DKO) animals. Human buffycoats were obtained from a local blood bank. PBMCs were isolated from these samples by Ficoll-Paque centrifugation (GE Health, Uppsala, Sweden) and assessed for viability using Trypan blue. These cells were then treated with mitomycin C at a concentration of 25 μg/ml/4E6 cells for 30 min at 37 °C. 10 μl/ml of DNase added for the last 5 min of incubation. After mitomycin C treatment, the cells were washed three times with HBSS, counted by hemocytometer and brought to a final concentration of 2E6/ml in AIM V media (Thermo Fisher, Grand Island, NY).

2.3. Preparation of responder populations

Human buffycoats were obtained from a local blood bank and PBMCs were isolated from these samples by Ficoll-Paque centrifugation (GE Health, Uppsala, Sweden). Viability was assessed using Trypan blue. Isolated cells were washed three times with HBSS and brought to a final concentration of 2E6/ml in AMI V media. To control for inter-individual variability in human responders, three human samples were prepared and tested for each stimulator.

2.4. BrdU ELISA based mixed lymphocyte reaction (MLR) assay

A 96 well micro-titer plate (MTP) was used for mixed lymphocyte reaction over a 5 day period was set up and incubated for four days. All responder cells were added at a concentration of 200,000 PBMCs/well. All stimulator cells were added at a concentration of 200,000 PBMCs/well. Bromodeoxyuridine (BrdU) was then added for an overnight incubation and on day five a BrdU ELISA was performed utilizing a Roche ELISA proliferation kit following the manufacturers protocol (Roche Diagnostics, Indianapolis IN). Absorbance was read on a Dynex MRX plate reader at 450 nm (MTX Lab Systems, Vienna, VA). Allogeneic reactions consisted human PBMC responder cells with human mitomycin C treated PBMC stimulator cells. Xenogeneic reactions consisted of human responder cells with mitomycin C treated porcine PBMCs. Positive controls consisted of human or porcine PBMC responder cells with 2 μg/well of phytohemagglutinin (PHA). Negative controls consisted of human or porcine mitomycin C treated PBMCs. Simulation index was calculated by absorbance of proliferation response of experimental treatment/absorbance of proliferation of PBMCs alone. As a proof of concept for pharmacologic intervention, DKO-stimulated xenogeneic reactions were preformed in the presence of dexamethasone against an allogeneic control; in the absence of relevant clinical plasma levels for dexamethasone, concentration was chosen on the basis of previous in vitro study [10–12]. Each stimulator–responder pair was tested in triplicate; differences across groups were analyzed using a one-way analysis of variance.

2.5. Immunosuppressive treatment during MLR

A flow cytometric proliferative assay was performed to complement our DNA-synthesis measurements; by directly labeling responder cells, this approach directly examined proliferative potential over a broad time course in the presence or absence of clinically relevant pharmacologic intervention. To better characterize the effect of immunosuppression on the *direct* xenogeneic human T cell response to $GGTA1^{-/-}$ $CMAH^{-/-}$ (DKO) cells, human CD4 T cells were isolated using human CD4 T isolation kit (Miltenyi Biotec Inc., San Diego, CA, USA), and labeled with CFSE using CellTrace™ CFSE Cell Proliferation Kit (Life Technologies, NY, USA). Splenocytes from human or $GGTA1^{-/-}$ $CMAH^{-/-}$ (DKO) pigs were used as stimulating populations; they were labeled using CellTrace™ Far Red DDAO-SE (Life Technologies, NY, USA), and irradiated by Gammacell-1000 Irradiator at 3000 Gy. Human CD4 + responders at 1×10^5 cells/well in 96-well round-bottom plates (Corning, Lowell, MA, USA) were co-incubated with or without irradiated stimulators [13]. CFSE-labeled CD4 T cells were co-cultured with FarRed labeled splenocytes (R:S = 1:8) in serum-free AIM V® Medium (Life Technologies, NY, USA) in the presence or absence of immunosuppressive agents dosed for clinical relevance: 10 ng/ml FK-506 (FK, LC Laboratories); 10 ng/ml Rapamycin (RAPA, InvivoGen); 200 ng/ml Cyclosporin A (CsA, LC Laboratories). Analysis of proliferation was limited to live human T cells by light scatter gating and far-red exclusion of stimulator cells; a non-stimulated, non-suppressed human T cell control was included with each experiment. All tests were repeated in triplicate under identical conditions.

3. Results

3.1. Creation of modified porcine cells

CRISPR/Cas9-directed mutagenesis created targeted disruptions at the GGTA1 and CMAH loci (Fig. 1). All disruptions produced a null phenotype at the loci of interest, and have been previously published by Li et al. [9]. After phenotype selection and SCNT, these cells created healthy clonal animals. Animals used in this study were of a WT, $GGTA1^{-/-}$ (GTKO), or $CMAH^{-/-}$ $GGTA1^{-/-}$ (DKO), background. The viability of all PBMCs obtained was above 97% by Trypan blue staining.

3.2. BrdU ELISA based mixed lymphocyte reaction (MLR) assay

Human and porcine PBMC cultures showed positive PHA proliferative responses whereas mitomycin C treated PBMCs had stimulation indexes (SI) less than baseline PBMC cultures (SI < 1.0). Human responder PHA control groups exhibited a SI of 7.6, porcine PHA control groups exhibited SI ranging from 38.2–103.0. No evidence of overgrowth was noted throughout experimental wells. Allogeneic and xenogeneic responses to a one-way mixed lymphocyte reaction were comparable. The allogeneic response was positive with a stimulation index of 5.8. A xenogeneic response was seen for all tested cell stimulator populations with a stimulation index range of 5.5–7.1. Fig. 2 depicts stimulation indices for the tested stimulator populations. Differences between porcine stimulator cells failed to reach statistical significance ($p = .0529$). Although the WT xenogeneic reaction was 16% stronger than the

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