Establishment of T cell and B cell Flow Cytometric Xenocrossmatching test

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ABSTRACT

Aim; Genetically modified pigs have emerged as a potential source for organ transplantation. Developing effective crossmatch methods to detect humoral alloimmunity is crucial for successful xenotransplantation. In this study, we conducted flow cytometric xenocrossmatch using sera from HLA-sensitized patients, non-human primates (NHPs) with islet xenotransplants, and porcine peripheral blood mononuclear cells (PBMCs).

Methods; Donor PBMCs were isolated from heparinized whole blood collected from Pigs (WT and QKO; GGTA1, CMAH, β4galNT2, iGb3s genes knock-out) using density gradient centrifugation. Human sera were obtained from patients who underwent HLA antibody testing using single-antigen Luminex bead assay (SA). 2.5x10^5 PBMC and 50 µl serum were incubated with fluorochrome-conjugated anti-porcine CD3 epsilon, CD21 and anti-human IgG, IgM antibodies. Acquisition and analysis of flow crossmatch results were performed on a Cytek spectral flowcytometer. Median fluorescent intensity (MFI) values and MFI ratios (NHP; MFI after transplantation/ MFI before transplantation, Human sera; test MFI / negative control MFI) were compared across sera to quantify antibody levels.

Results; Human sera showed higher MFI towards wild-type pig cells compared to QKO pig cells. In NHPs experiencing rejection episodes, the T/B cell MFI ratio of IgG increased over time, with peak levels observed at day 56 post-transplantation (ratio 24.3 / 11.2). Human samples positive for HLA antibodies exhibited higher MFIs compared to negative samples. Additionally, human serum samples displayed higher MFI values for IgG compared to IgM, while NHP serum samples exhibited higher MFI values for IgM compared to IgG.

Conclusions; Elevated IgG MFI ratio represented a rejection episode in islet xenotransplantation. The optimized flow cytometric xenocrossmatch for T cells and B cells represents a valuable method for detecting humoral immunity following islet xenotransplantation.

CONCLUSIONS

The optimized flow cytometric xenocrossmatch detects humoral immunity after islet xenotransplantation. Human sera showed higher MFI against wild-type pig cells and HLA antibody-positive samples had higher MFI than negative ones.

MATERIALS and METHODS

- **Pig PBMC**; **WT (n=1), QKO (GGTA1, CMAH, β4galNT2, iGb3s genes knock-out) (n=3)**
- **Human sera (n = 61)**; **HLA antibodies** were determined using single-antigen Luminex bead assay (SA) (MFI >10,000). **Non-HLA antibody profiles** identified using LABScreen Autoantibody group 1 (32 targets) and group 2 (one target) kits (One Lambda).

Table 1. Classification of human sera according to the HLA and non-HLA antibodies

groups	HLA class I and II (+)	HLA class I (+)	HLA class II (+)	HLA Ab (-)	HLA Ab(-)/ non-HLA Ab (+)
NO. of sera	10	10	10	20	11

- **Flow Cytometric Crossmatch (FCXM)**; 2.5x10^5 PBMC(25ul) + 50 µl serum + **fluorochrome-conjugated anti-porcine CD3 epsilon for T cell, CD21 for B cell and anti-human IgG, IgM antibodies**. Acquisition and analysis performed using a Cytek spectral flowcytometer(Figure 1).

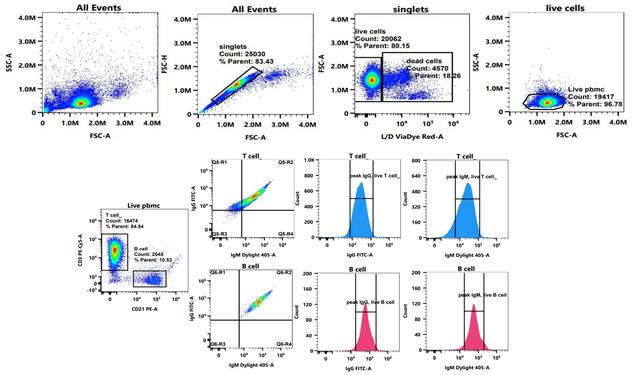

Figure 1. Flowcytometric xeno-crossmatching gating strategy.

- **Complement-dependent Cytotoxicity (CDC) Assay**; 3X10^3 cells(1ul) + 1ul serum + rabbit complement (add 1% anti human kappa chain for AHG) + 5ul AO/EB, **CDC (+); >40% lysis at 1:4 dilution**
- **Pig to NHP islet xenotransplantation (4 cases)**; sera were collected 4-9 times over 20 weeks from four NHPs after islet xenotransplantation for FCXM analysis.

RESULTS

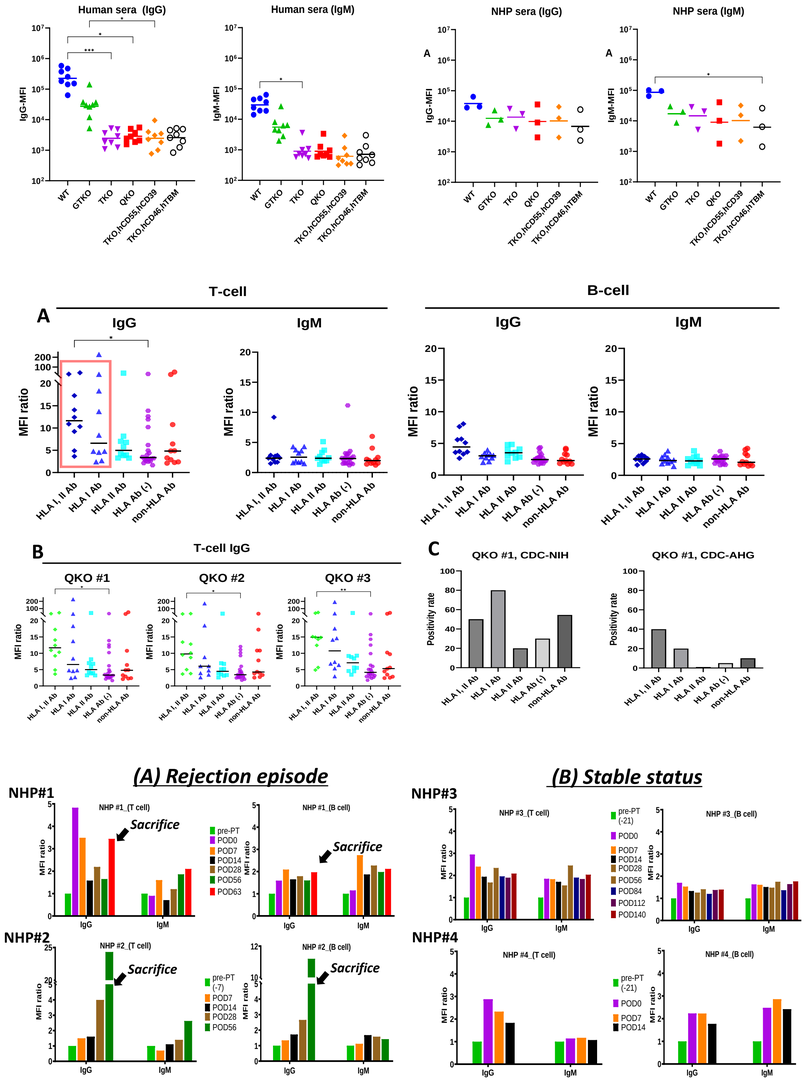

Figure 2. Comparison of FCXM results from human and NHP sera against pig cells with different gene types. Human sera had higher MFI for wild-type pig cells compared to QKO pig cells

Figure 3. Pig-to-human Flowcytometric xenocrossmatching results according to the HLA and non-HLA ab results.
HLA I, II Ab (+) samples exhibited higher MFI ratios for T cell IgG compared to HLA Ab (-) (A). In three QKO pigs, HLA I, II Ab(+) samples displayed a similar trend(B). HLA I, II Ab (+) samples also had higher positivity rate in both CDC-NIH and CDC-AHG compared to HLA(-) samples.

Figure 4. FCXM results from 4 cases of pig-to-NHP islet xenotransplantation

In NHPs (#1, #2), which experienced **rejection episode, the T/B cell IgG MFI ratio gradually increased** until sacrifice (NHP#1: 3.44/1.97 at POD63, NHP#2: 24.3/11.19 at POD56). The IgM results showed a similar trend, though not as pronounced as IgG results. In **stable NHPs (#3, #4), the T/B cell IgG/IgM MFI ratio stayed below ratio 3.0** until POD140(Figure 4B).