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Brief communication

Human CTLA4-Ig therapy can give false-positive anti-pig antibody results in primates after xenotransplantation

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ABSTRACT

Background: Measurement of serum anti-pig antibodies is an important parameter in immune monitoring after pig-to-nonhuman primate xenotransplantation. Pig aortic endothelial cells (pAECs) are commonly used for this purpose. However, human (h) CTLA4-Ig (abatacept/belatacept) could bind to pCD80/86 on the cells, and a secondary antibody (i.e., anti-human IgG) may recognize hCTLA4-Ig (in the absence of serum anti-pig IgG antibody binding to pAECs), potentially leading to misinterpretation of the results. Our aim was to determine whether hCTLA4-Ig binding to pAECs is associated with false-positive results.

Methods: Sera were obtained from (i) naïve baboons ($n = 3$) and (ii) baboons ($n = 2$) that had undergone pig artery patch transplantation with/without hCTLA4Ig therapy. Serum IgM and IgG binding to (i) AECs, (ii) red blood cells (RBCs), and (iii) $CD3^+$T cells in peripheral blood mononuclear cells (PBMCs) from an α1,3-galactosyltransferase gene-knockout pig expressing human CD46 (GTKO/hCD46) was measured by flow cytometry in the presence or absence of hCTLA-4Ig. Complement-dependent cytotoxicity (CDC) of wild-type (WT) pAECs by hCTLA4Ig was measured by flow cytometry.

Results: Sera containing hCTLA4-Ig demonstrated significantly increased IgG (but not IgM) binding to pAECs (relative geometric mean [rGM] = 1.8) compared to sera without hCTLA-4Ig (rGM = 1.3) ($p < .01$). In contrast, there was no increased binding to pRBCs or $CD3^+$T cells. hCTLA4-Ig did not result in cytotoxicity of WT pAECs.

Conclusions: pAECs might not be an optimal cell to investigate anti-pig IgG binding when hCTLA4-Ig is administered to the recipient, as a false-positive result may result from hCTLA4-Ig binding to the pAECs. $CD3^+$T cells would be preferable targets (compared to pRBCs) because they express both carbohydrate and MHC class I/II antigens.

1. Introduction

The availability of genetically-engineered pigs has prevented early xenograft rejection [1,2], but the prevention of T cell-mediated rejection is also essential for long-term xenograft survival [3–9]. Many immunosuppressive regimens have been used to regulate the immune response in recipients of xenografts [10]. In particular, blockade of the CD40-CD154 costimulation pathway, in combination with T cell lympho-depletion, results in prevention of xenogeneic sensitization in nonhuman primates [3–5,7,8,11,12], but these agents are not yet approved for clinical use.

Abbreviations: AECs, aortic endothelial cells; CDC, complement-dependent cytotoxicity; CTLA4, Cytotoxic T lymphocyte antigen-4; Gal, galactose-α1,3-galactose; GTKO/hCD46, α1,3-galactosyltransferase gene-knockout pigs transgenic for the human complement-regulatory protein, CD46; h, human; MHC, major histocompatibility complex; p, pig; PBMCs, peripheral blood mononuclear cells; RBCs, red blood cells; rGM, relative geometric mean; SLA, swine leukocyte antigen; WT, wild type

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Blockade of the CD28-B7 family (CD80/CD86) pathway has also been tested. Abatacept and belatacept are human (h) CTLA4-Ig fusion proteins that bind to both CD80 and CD86 on the surface of antigen-presenting cells, thereby blocking CD28 co-stimulatory signals, resulting in suppression of T cell activation [13–16]. Recently, we have shown that, in combination with other agents, blockade of the CD28-B7 pathway using hCTLA4-Ig prevented the adaptive immune response to genetically-engineered pig artery patch xenografts in baboons [17].

Measurement of anti-pig antibodies in recipients after xenotransplantation is an essential test to determine the response to pig antigens. Because pig aortic endothelial cells (pAECs) are the main target cells following pig organ xenotransplantation, they are commonly used for investigation of anti-pig antibodies in recipient sera [18]. However, hCTLA4-Ig (both abatacept and belatacept) can bind to pCD80/86 antigens [19,20]. We hypothesized that serum containing hCTLA4-Ig could confuse the result of antibody binding to pAECs.

The purpose of the present study was to determine the effect of hCTLA4-Ig when monitoring anti-pig antibody in vitro and in vivo after pig artery patch xenotransplantation in baboons.

2. Materials and methods

All animal care was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council (8th edition, revised 2011), and was conducted in an AAALAC-accredited facility. Protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (#20673).

2.1. Sources of primate sera

Blood was obtained from 3 baboons (Division of Animal Resources, Oklahoma University Health Sciences Center, Oklahoma City, OK). Three baboons were immunologically naïve, and two subsequently underwent transplantation with pig carotid artery patches into the abdominal aorta [17]. Both baboons with transplants received a similar immunosuppressive regimen, except that one (B3715) received hCTLA4-Ig, belatacept (20 mg/kg i.v. days −1, 0, 4, 7, 14 and then every 14 days) for 3 months followed by abatacept (25 mg/kg i.v every 7 days) for 3 months. The change from belatacept to abatacept was forced on us as belatacept became unavailable for research because of a shortage of supply. The other (B1915) received rapamycin instead of hCTLA4-Ig. Sera were obtained before and after (1–5 months) xenotransplantation. Decomplementation of serum was carried out by heat-inactivation for 30 min at 56 °C, and sera were stored at −80 °C until use.

2.2. Sources of pig cells

Red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs) from an α1,3-galactosyltransferase gene-knockout pig transgenic for the human complement-regulatory protein CD46 (GTKO/hCD46 pig; Revivicor, Blacksburg, VA) were isolated [21–23]. pRBCs (1×10^6) and PBMCs (1×10^5) were suspended in 100 μL phosphate-buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA) for surface antigen expression and serum antibody binding assay.

pAECs from a wild-type (WT, i.e., a genetically-*un*modified) pig and a GTKO/hCD46 pig were isolated and cultured, as previously described [24]. pAECs (1×10^5) were suspended in 100 μL staining buffer (PBS containing 1% BSA and 0.1% NaN3) for measurement of surface antigen expression and serum antibody binding [19,23].

For the complement-dependent cytotoxicity (CDC) assay, pAECs (5×10^4) were suspended in 50 μL CDC medium (RPMI 1640 culture medium [Invitrogen, Carlsbad, CA] containing 10% FBS [Sigma, St. Louis, MO], 1% HEPES buffer [Invitrogen], and 100 IU/mL penicillin-100 μg/mL streptomycin [Invitrogen]).

2.3. Sources of human CTLA4-Ig and human complement

Recombinant human CTLA4Ig was purchased from R&D Systems (Minneapolis, MN). Both abatacept (Orencia) and belatacept (Nulojix) were purchased from Bristol-Myers Squibb (Princeton, NJ). Commercially-available human complement was purchased from Innovative Research (Novi, MI).

2.4. Detection of surface antigen expression on human and pig cells by flow cytometry

Surface expression of MHC class I and class II on AECs, RBCs, and PBMCs ($CD3^+$ gated T cells) from humans and pigs was determined by LSR II flow cytometry (Becton Dickinson, San Jose, CA), and analyzed by FlowJo software (Treestar. Ashland, OR) [11,22,25].

2.5. Binding of serum IgM and IgG to pig cells by flow cytometry

Serum IgM and IgG antibody binding to pAECs, pRBCs, and pPBMCs ($CD3^+$T cells) was performed [18,22,23]. Following the antibody assays, PBMCs were stained with PerCP Cy5.5-conjugated anti-pig CD3 (Clone BB23-8E6-8C8, original, BD) for $CD3^+$ gated T cells. Binding was expressed as the relative geometric mean (rGM), which was calculated by dividing the geometric mean value for each sample by the negative control [22,26].

2.6. CDC assay by flow cytometry

CDC of pAECs by hCTLA-4Ig was determined by flow cytometry. WT pAECs (5×10^4 cells/50 μL CDC Medium) were incubated with hCTLA-4Ig at various concentrations for 45 min at 37 °C, followed by incubation with human complement (20% concentration) for 1 h at 37 °C. After incubation, the cells were washed and resuspended with 1 mL PBS followed by further incubation with 0.5 μL Live/Dead Aqua (Invitrogen), to detect dead cells, for 30 min at 4 °C. After washing, the cells were resuspended in staining buffer.

The percentage lysis of pAECs was calculated as follows:

$$\%\text{cytotoxicity} = ([A - C]/[B - C]) \times 100\%$$

where A represents the dead cells (target cells incubated with hCTLA-4Ig and human complement), B is the maximal dead cells (target cells lysed with 70% alcohol), and C is the minimal dead cells (target cells incubated with human complement only).

Cytotoxicity at varying hCTLA-4Ig concentrations was calculated, and a curve was generated for each sample.

2.7. Statistical analysis

Data are presented as mean and standard error of the mean (SEM) for all variables. The statistical significance of differences was determined by Student's *t*-test or nonparametric tests, as appropriate, using GraphPad Prism version 7 (GraphPad Software, San Diego, CA). A p value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Expression of MHC class I/II and CD80/86 molecules on AECs, RBCs, and $CD3^+$ T cells (Table 1)

Both hAECs and pAECs expressed MHC class I, but there was no or minimal expression of MHC class II (Fig. 1A, Table 1). Expression of the B7 family (i.e., CD80/86) on pAECs was detected by exposure to hCTLA4-Ig followed by staining with FITC-conjugated anti-human IgG antibody (Fig. 1A, right), but not with anti-human IgM antibody (data not shown). In contrast, hAECs did not express CD80/86 molecules. Neither hRBCs nor pRBCs expressed MHC class I/II or CD80/86 molecules (Fig. 1B, Table 1).

Table 1
Expression of known xenoantigens and immune molecules on human and pig cells.

	Carbohydrates (1)	MHC class I	MHC class II	B7 family (CD80/CD86)
hAECs	−	+	+/− (2)	−
pAECs	+	+	+/− (2)	+
hRBCs	−	−	−	−
pRBCs	+	−	−	−
hCD3$^+$T cells	−	+	+[a]	−
pCD3$^+$T cells	+	+	+[a]	−

(1) Pig antigens: galactose-1,3-galactose, N-glycolylneuraminic acid, Sda.
(2) MHC class II on AECs is expressed only after IFN-γ stimulation.
[a] The expression level of MHC class II on human and pig T cells was 70 and 20 times lower than those on B cells, respectively (data not shown).

CD3$^+$T cells were gated by staining PBMC with anti-CD3 antibody (Fig. 1C). The expression of MHC class I/II and CD80/86 molecules on them was determined by flow cytometry. Both human and pig CD3$^+$T cells expressed MHC class I/II, but *not* CD80/86 molecules (Fig. 1D, Table 1).

3.2. Anti-pig IgG binding to pAECs was significantly increased when serum contained hCTLA4-Ig

Since hCTLA4-Ig is an IgG class antibody, there was no detectable binding to pAECs when a FITC-conjugated anti-human IgM secondary antibody was used (Fig. 2A, left). In contrast, binding of hCTLA4-Ig was detected when a FITC-conjugated anti-human IgG secondary antibody was used (Fig. 2A, right). The level of binding was significantly increased (×2–3-fold) in the presence of hCTLA4-Ig (rGM 2.7 [hCTLA4-Ig 1 μg/mL]), compared to an absence of hCTLA4-Ig (rGM 1.0) ($p < .05$).

To confirm that serum containing hCTLA4-Ig demonstrated increased IgG binding to pAECs, several concentrations of hCTLA4-Ig were added to serum obtained from (i) a naïve baboon (Fig. 2B) and (ii) a baboon 4 months after a pig artery patch transplant (B1915) that received immunosuppressive therapy that did *not* include hCTLA4-Ig (Fig. 2C). Sera from both the naïve (rGM 2.9) and transplant recipient baboon (rGM 2.3) showed significant levels of anti-pig IgM antibodies against GTKO/CD46 pAECs (i.e., nonGal). The addition of hCTLA4-Ig in the serum was not associated with a further increase in IgM antibody binding to pAECs (Fig. 2B and C, left). In contrast, the presence of hCTLA4-Ig in the serum significantly increased the level of IgG binding to pAECs by approximately by 1.3 to 1.4-fold. (Naïve baboons w/o hCTLA4-Ig, rGM = 1.3, with hCTLA4-Ig [1 μg/mL] =1.8. B1915 w/o hCTLA4-Ig, rGM = 1.0, with hCTLA4-Ig [1 μg/mL] =1.4) (Fig. 2B and C, right), although, in serum containing hCTLA4-Ig, the increased level of IgG binding to pAECs was lower than when the assay contained no serum (Fig. 2A, right).

These results indicated that an increased level of anti-pig IgG binding was associated with the presence of hCTLA4-Ig in the serum.

3.3. IgG binding to pRBCs and pCD3$^+$T cells was not affected by the presence of hCTLA4-Ig in the serum

Since both RBCs (Fig. 1B) and CD3$^+$T cells (Fig. 1D) do not express CD80/86 molecules (Table 1), the presence of hCTLA4-Ig had no effect on the level of IgG antibody binding to either pRBCs (Fig. 3) or pCD3$^+$T cells (Fig. 4).

3.4. Serum from a hCTLA4-Ig-treated baboon demonstrated significantly increased binding of IgG to pAECs, but not to pRBCs or pCD3$^+$T cells

Serum antibodies were measured in 2 baboons that had received GTKO/CD46 artery patch xenografts with or without hCTLA4-Ig treatment. Five months after the transplant, B3715 (that was receiving hCTLA4-Ig therapy) showed a significant increase in IgG binding to pAECs (rGM = 1.7) compared to pre-transplant (rGM = 1.0) ($p < .01$) (Fig. 5A, right). However, there was *no* increase of IgG binding to pRBCs (Fig. 5B) or pCD3$^+$T cells (Fig. 5C, right). In contrast, B1915, that did *not* receive therapy with hCTLA4-Ig, showed *no* increase in IgG binding to pAECs (Fig. 5A, right) or other cell types (Fig. 5B, C, right). There was no increase in IgM binding to any cell type in either baboon (Fig. 5, left). There were no histopathological features in the pig artery patch, or other immunological features, of antibody-mediated rejection in either baboon (not shown).

These results suggest that the increased level of IgG binding to pAECs is associated with the presence of hCTLA4-Ig in the serum, but *not* with sensitization to the pig cells.

3.5. hCTLA-4Ig in the serum is not cytotoxic to pig cells

Since hCTLA4-Ig can bind to pAECs, a CDC assay using WT pAECs was carried out to determine whether hCTLA4-Ig caused lysis of the pig cells (Fig. 6). Although all 3 types of hCTLA4-Ig bound to WT pAECs (Fig. 6A), there was *no* cytotoxicity associated with the presence of this binding, even at high concentrations (Fig. 6B).

4. Discussion

Measurement of serum anti-pig antibodies by flow cytometry is a standard method of monitoring the immune response in recipients after pig-to-nonhuman primate xenotransplantation [4,7,17,18]. In contrast to pig tissue transplantation (e.g., cornea, artery patch), it may be difficult to detect induced (de novo) anti-pig antibodies in organ (e.g., heart, kidney) xenograft recipients receiving immunosuppressive therapy because most of the antibodies bind to the pig organ. Although the level of anti-pig antibody is frequently lower after the transplant than pre-transplant (because of antibody adsorption on the xenograft), increased anti-pig antibodies can be detected in recipient sera if the recipients are sensitized against pig antigens [7].

Abatacept and belatacept, both forms of hCTLA4-Ig, are fusion proteins composed of a human IgG1 immunoglobulin Fc fragment of the extracellular domain of CTLA4, which is an important molecule in the regulation of T cell activation. Both forms of hCTLA4-Ig bind to the B7 family (CD80/CD86) molecules on antigen-presenting cells, and thus prevent the binding of CD28 (on T cells) to B7 on antigen-presenting cells, delivering a down-regulatory signal to the T cells, and blocking T-cell activation [14,27]. In combination with other drugs, hCTLA4-Ig may prevent T cell sensitization after pig-to-nonhuman primate xenotransplantation [11], although hCTLA4-Ig alone was found to be insufficient [11,28].

Although pAECs constitutively express CD80/86, there is conflicting evidence regarding human ECs. It has been shown that human aortic, umbilical vascular, and renal microvascular ECs do not express CD80/86 [19,29–31]. In contrast, human brain, intestinal, and cardiac microvascular ECs as well as islet ECs express CD80 and/or CD86 [32–35]. These discrepancies may reflect inherent differences between ECs derived from different tissues (e.g., kidney vs intestine) and/or phenotypes (e.g., macrovascular vs microvascular).

We have previously shown that hCTLA4-Ig including abatacept and belatacept cross-react with pig CD80/86 molecules, and suppress pig and primate T cell proliferation [19,20], suggesting hCTLA4-Ig is not species-specific.

In the present study, we demonstrated that the presence of hCTLA4Ig in the serum resulted in a false-positive increase in IgG binding when pAECs were used as target cells, but with no increase in IgM binding (because hCTLA4-Ig is the Fc portion of human IgG1, which is not recognized by secondary anti-human IgM antibodies). The secondary anti-human IgG antibody recognizes both baboon IgG binding to pAECs and hCTLA4-Ig binding to CD80/86 on pAECs.

The rGM of IgG binding to pAECs associated with the presence of hCTLA4-Ig was not very high (< 3), even when high doses of hCTLA4-

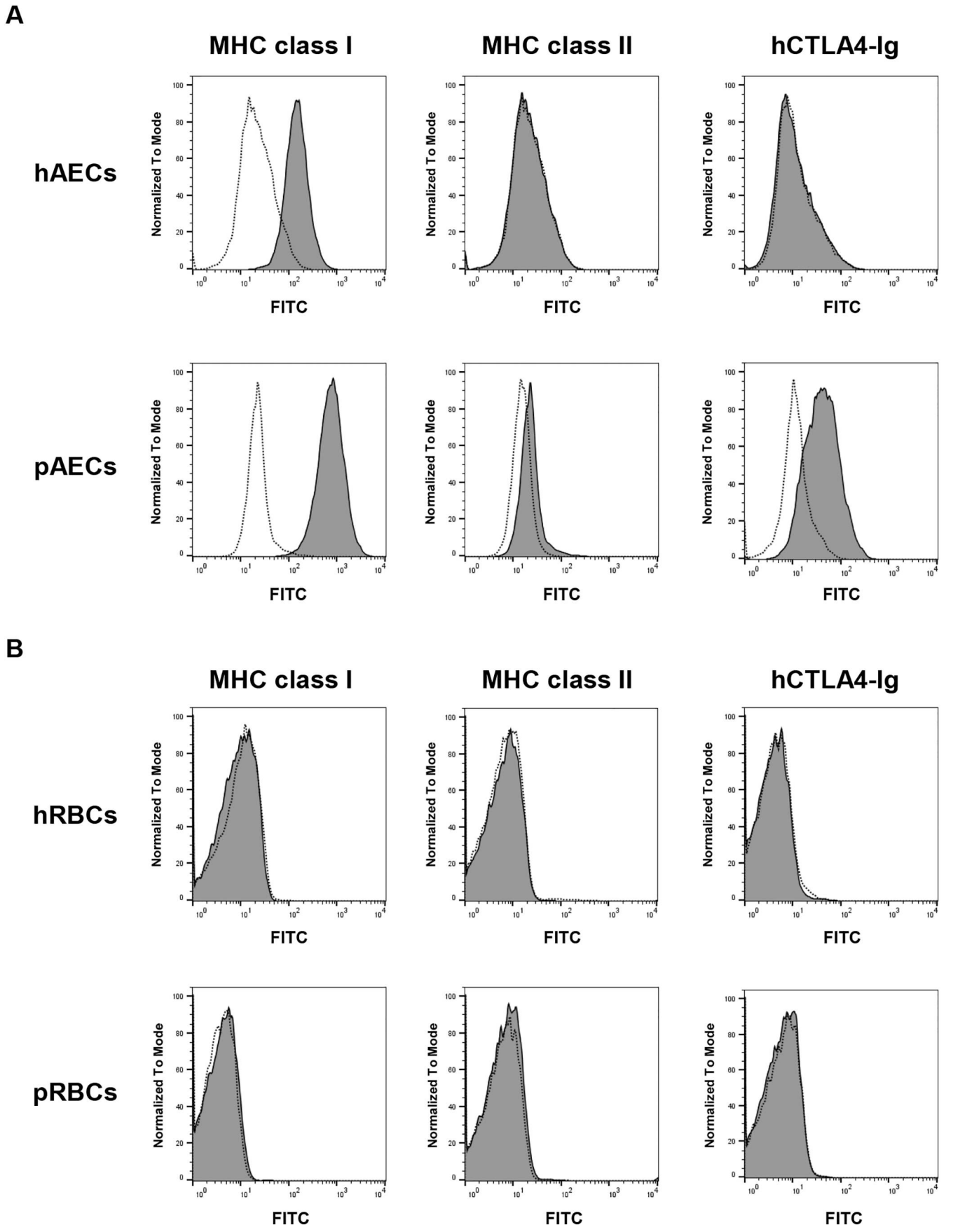

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Fig. 1. Expression of MHC class I and II and CD80/86 molecules on human and pig cells.
Surface expression of MHC class I and II, and B7 family (CD80/86) molecules on human (h) and pig (p) aortic endothelial cells (AECs) (A), red blood cells (RBCs) (B), and $CD3^+$ gated T cells in peripheral blood mononuclear cells (PBMCs) (C and D) was investigated by flow cytometry. Dotted lines represent isotype control or secondary antibody only. To detect CD80/86 molecules on the cells, hCTLA4-Ig (5 μg recombinant human CTLA-4 Fc chimera, R&D Systems) was added, followed by staining with FITC-conjugated goat anti-human IgG antibody.
(A) hAECs expressed MHC class I, but not MHC class II or CD80/86. pAECs expressed swine leukocyte antigen (SLA) class I and II (but class II at a low level), and a significant level of CD80/86. (B) Neither human nor pig RBCs expressed MHC class I or II or CD80/86. (C) Representative figure to demonstrate the gating of $CD3^+$T cells in PBMCs. Forward versus side scatter (FSC vs SSC) gating was used to identify the pPBMCs. Two-parameter density plot was used to distinguish T cells by creating a plot on SSC vs CD3. (D) Both human and pig $CD3^+$T cells expressed significant levels of MHC class I, and low levels of class II, but did not express CD80/86. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ig were added to the serum. Unlike hAECs, pAECs constitutively express CD80/86 molecules on their surface [19]. IgG binding to CD80/86 on pAECs should be saturated by hCTLA4-Ig at a concentration of 1 μg/mL. If the rGM of IgG binding to pAECs is increased > 3-fold, this would indicate genuine sensitization (i.e., binding of anti-pig IgG antibody) and not a false-positive result. Belatacept (20 mg/kg, every 2 weeks) was changed to abatacept (25 mg/kg, every week) 3 months after transplantation. The frequent administration of ababtacept might have been a factor in the increased IgG antibody binding to pAECs (as a false-positive result) associated with a high level of hCTLA4-Ig in serum. In

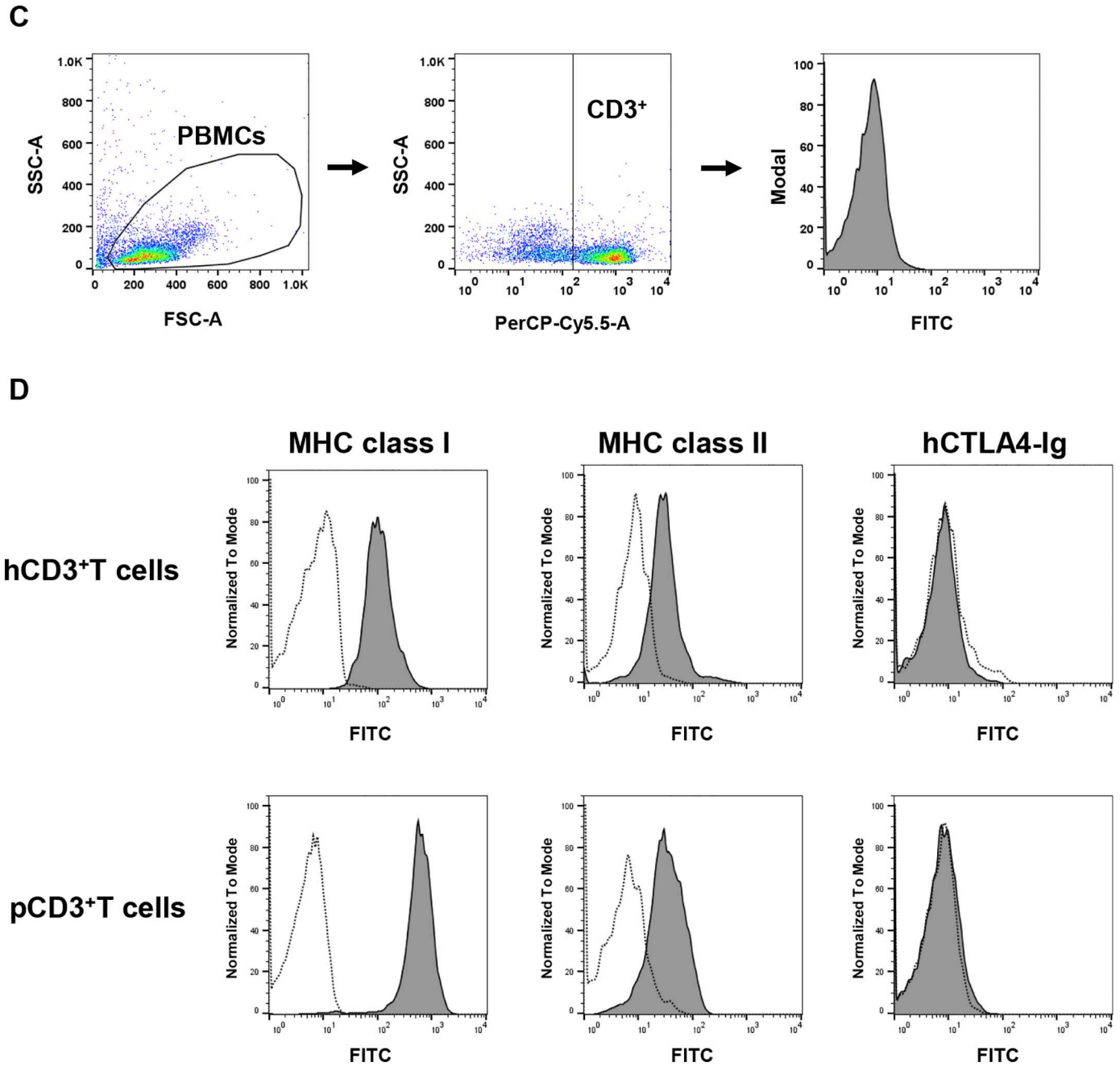

Fig. 1. (*continued*)

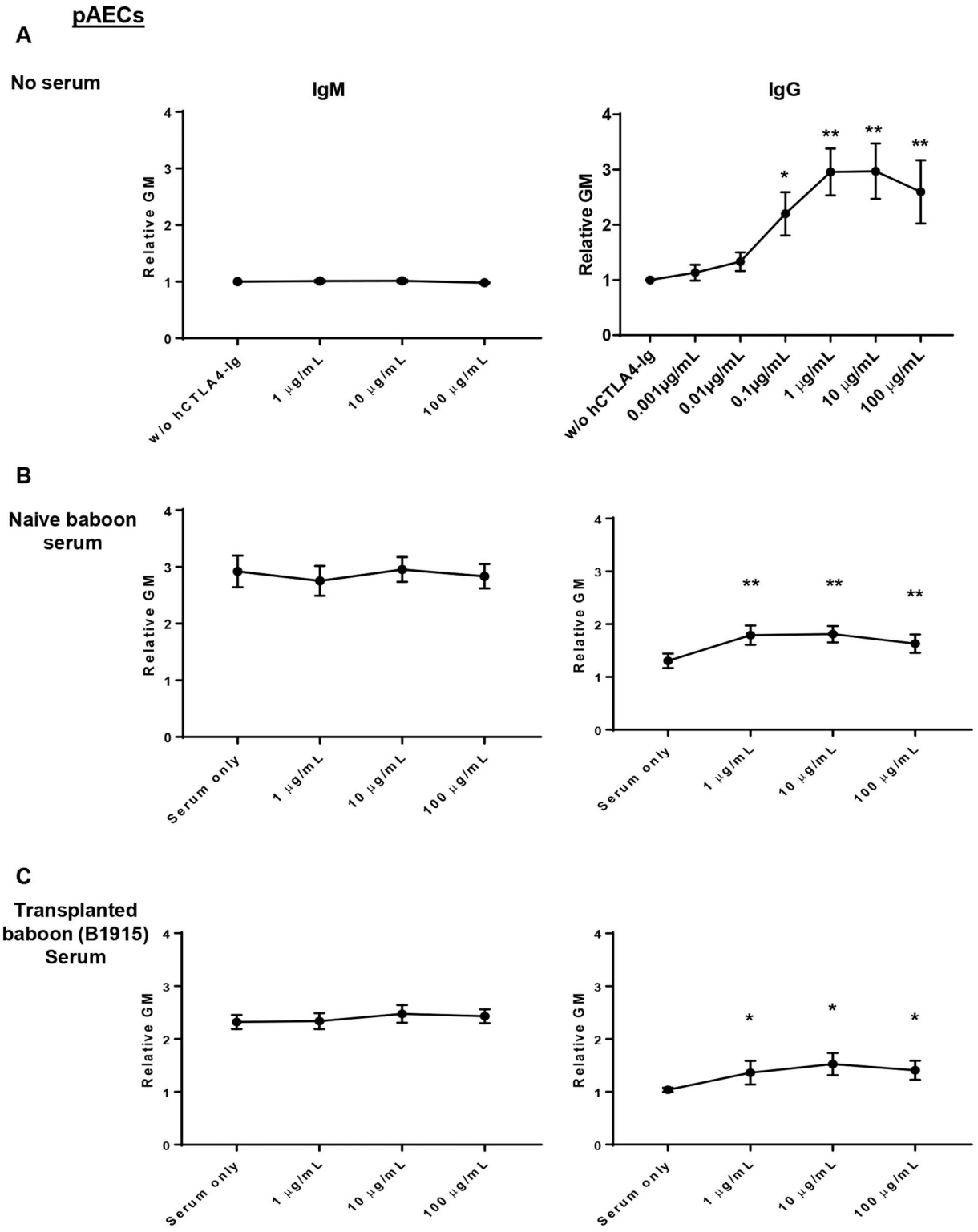

Fig. 2. Detection of binding of serum anti-pig IgM and IgG (with/without hCTLA4-Ig) to pAECs by flow cytometry.
(A) GTKO/CD46 pAECs were incubated with/without hCTLA4-Ig (abatacept and belatacept) at several concentrations (0.001 to 100 μg/mL), followed by staining with FITC-conjugated anti-human IgM (left) or IgG (right) antibody. Binding of hCTLA4-Ig to pAECs was determined by flow cytometry, and presented as relative geometric mean (rGM). Binding of hCTLA4-Ig to pAECs was detected by FITC-conjugated anti-human IgG antibody, but not by IgM antibody. There was no significant difference in the level of rGM with different concentrations of hCTLA4-Ig. Sera (20 μL) from naïve baboons ($n = 3$) (B) or from a baboon (B1915) that received a pig artery patch graft 4 months previously, and that was ***not*** treated with hCTLA4-Ig (C) (assay repeated ×3) were incubated with GTKO/CD46 pAECs with/without hCTLA4-Ig (abatacept and belatacept). The presence of hCTLA4-Ig in the serum did not affect the detection of anti-pig IgM antibodies (left), but did increase the level of IgG binding (right). The hCTLA4-Ig concentration was not associated with any significant difference in the level of rGM (*$p < .05$; **$p < .01$; vs without hCTLA4-Ig).

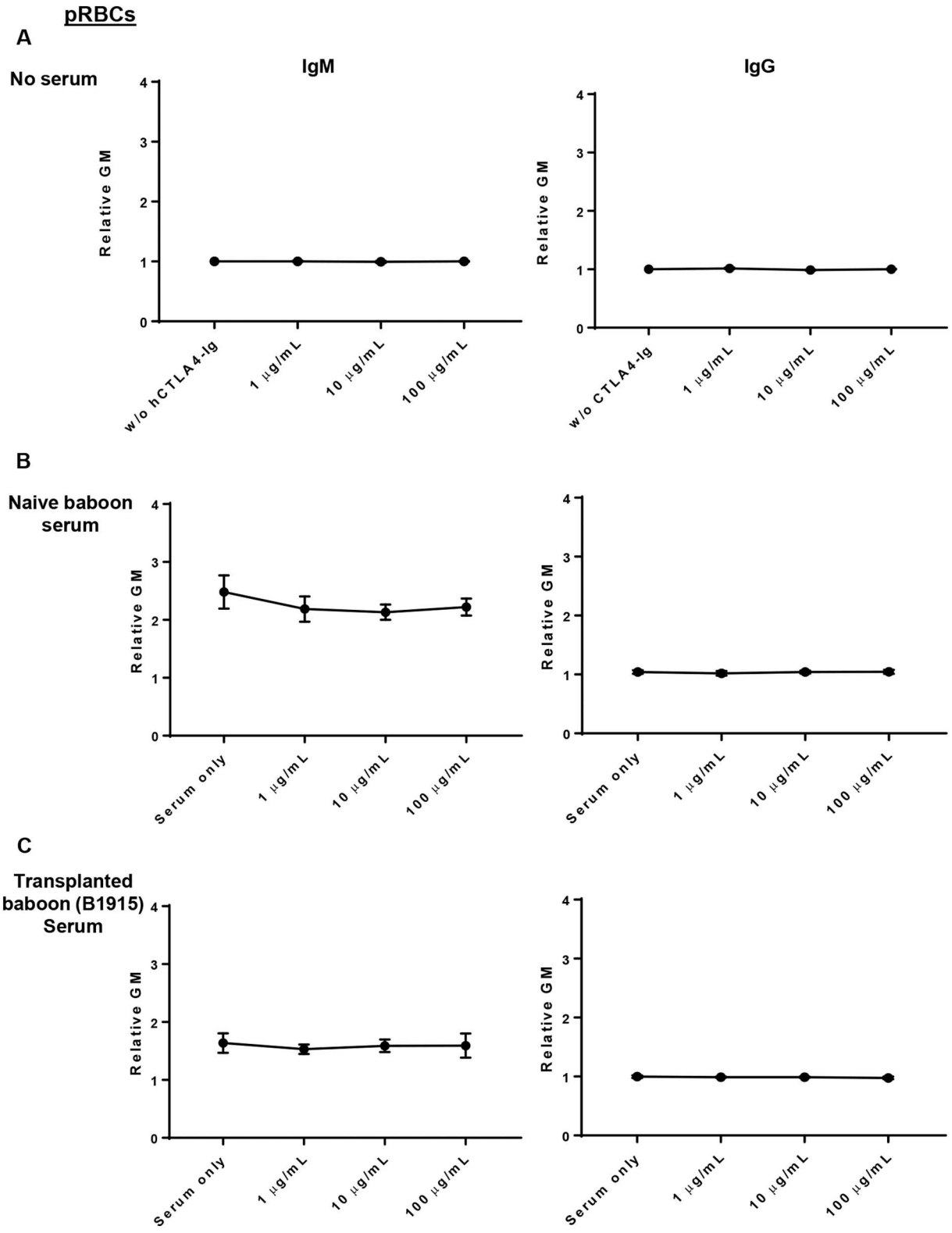

Fig. 3. Detection of binding of serum anti-pig IgM and IgG (with/without hCTLA4-Ig) to pRBCs by flow cytometry.
(A) GTKO/CD46 pRBCs were incubated with/without hCTLA4-Ig (abatacept and belatacept) at three concentrations (1, 10, and 100 μg/mL), followed by staining with FITC-conjugated anti-human IgM (left) or IgG (right) antibodies. Binding of hCTLA4-Ig to pRBCs was not detected by FITC-conjugated anti-human IgM (left) or IgG (right) antibodies, confirming no expression of CD80/86 on pRBCs. Sera (20 μL) from naïve baboons ($n = 3$) (B) or from a baboon (B1915) that received a pig artery patch graft 4 months previously, and that was *not* treated with hCTLA4-Ig (C) (assay repeated ×3) were incubated with GTKO/CD46 pRBCs with/without hCTLA4-Ig (abatacept and belatacept). The presence of hCTLA4-Ig in the serum did not affect the detection of anti-pig IgM (left) or IgG (right) antibodies. The hCTLA4-Ig concentration was not associated with any significant difference in the level of relative GM.

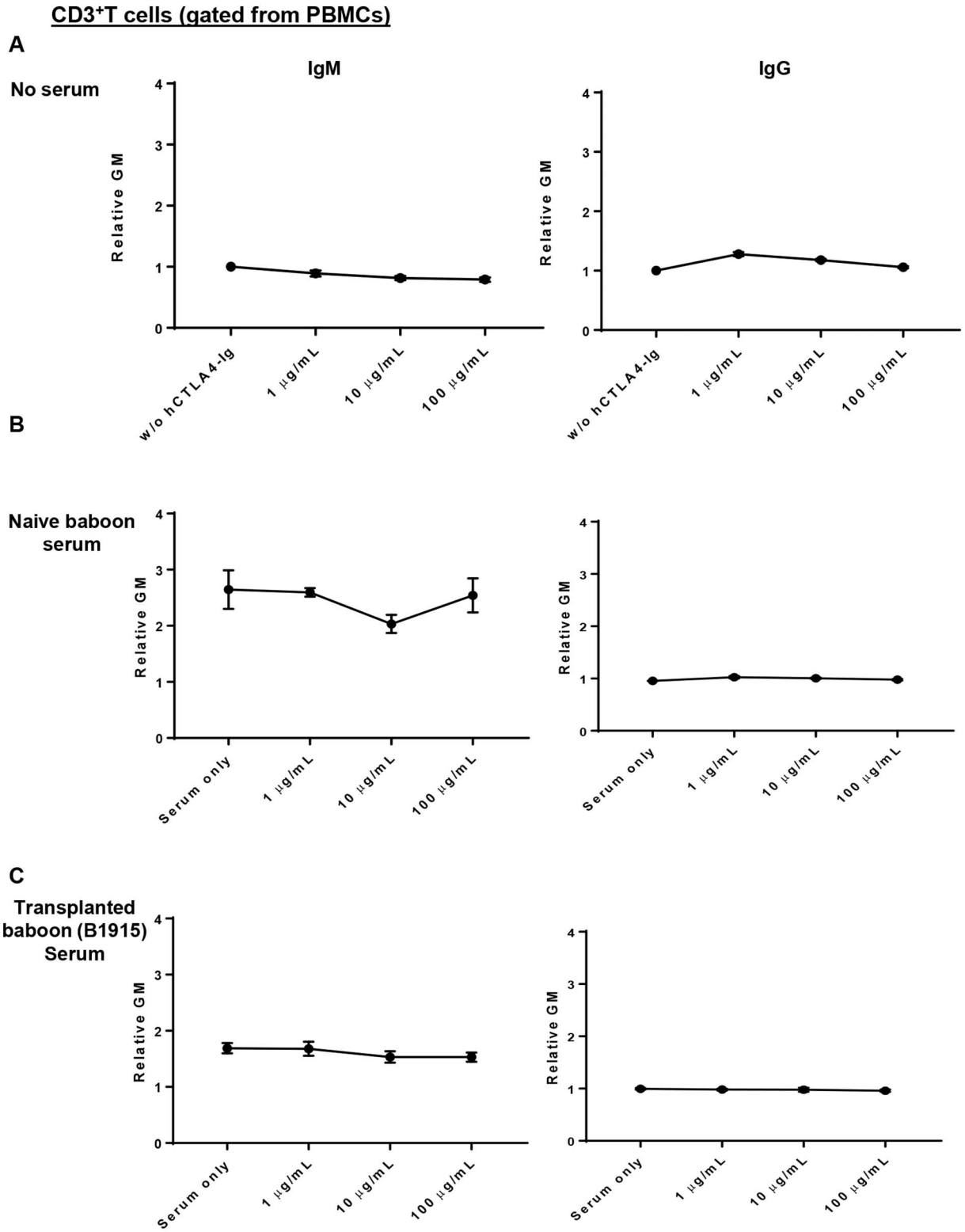

Fig. 4. Detection of binding of serum anti-pig IgM and IgG (with/without hCTLA4-Ig) to pCD3$^+$T cells by flow cytometry.
(A) GTKO/CD46 pPBMCs were incubated with/without hCTLA4-Ig (abatacept and belatacept) at three concentrations (1, 10, and 100 μg/mL), followed by staining with FITC-conjugated anti-human IgM (left) or IgG (right) antibodies. Binding of hCTLA4-Ig to CD3$^+$ gated T cells was not detected by FITC-conjugated anti-human IgM (left) or IgG (right) antibodies, indicating no expression of CD80/86 on pCD3$^+$T cells. Sera (20 μL) from naïve baboons (n = 3) (B) or from a baboon (B1915) that received a pig artery patch graft 4 months previously, and that was *not* treated with hCTLA4-Ig (C) (assay repeated x3) were incubated with GTKO/CD46 pPBMCs with/without hCTLA4-Ig (abatacept and belatacept). The presence of hCTLA4-Ig did not affect the detection of anti-pig IgM (left) or IgG (right) antibodies. The hCTLA4-Ig concentration was not associated with any significant difference in the level of rGM.

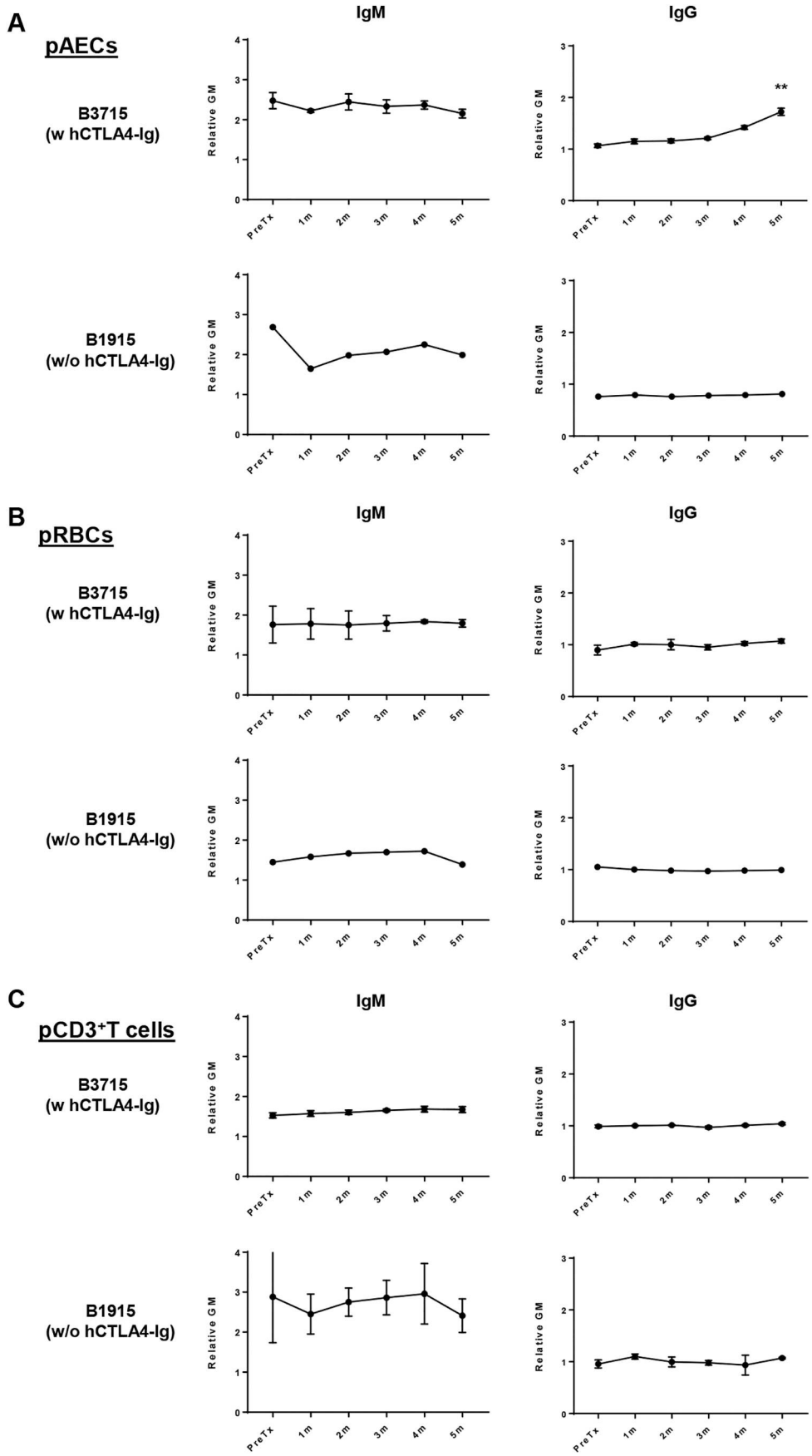

Fig. 5. IgM and IgG antibody binding to pig cells after pig artery patch transplantation in baboons.

Sera obtained from baboons that received (B3715) or did *not* receive (B1915) hCTLA4-Ig were tested to measure anti-pig IgM (left) and IgG (right) antibody binding to pAECs (A), pRBCs (B), or $CD3^+$T cells (C) following pig artery patch xenotransplantation. All analyses were carried out in triplicate. There was *no* increase in anti-pig IgM antibodies against all three type of cells (left), suggesting no sensitization of IgM to pig antigens. In B3715, when hCTLA4-Ig therapy had been administered, IgG binding to pAECs (but *not* to pRBCs or $CD3^+$T cells) was significantly increased 5 months after xenotransplantation compared to pre-transplantation (PreTx). There was no increase in anti-pig IgG antibody binding to any cells in B1915. (**$p < .01$ vs PreTx).

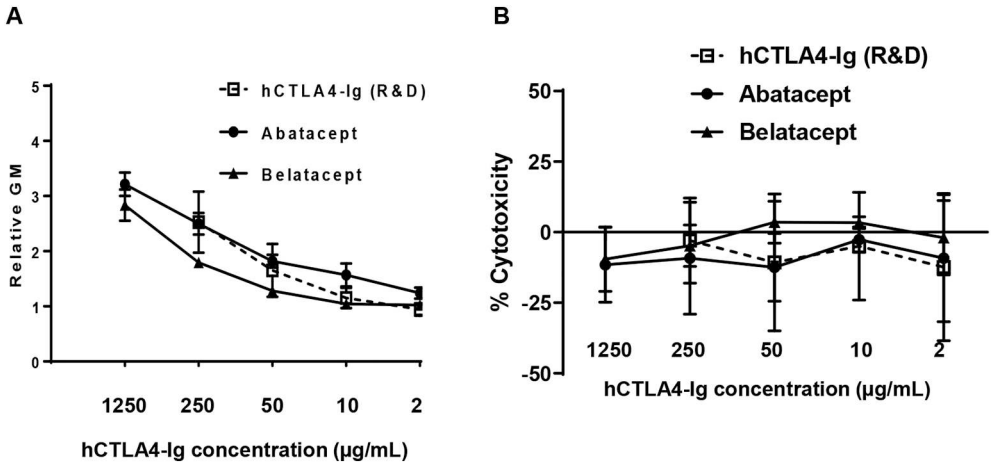

Fig. 6. Complement-dependent cytotoxicity of CTLA4-Ig.
Three type of hCTLA4-Ig were tested to determine their binding (A) and cytotoxicity (B) to pAECs. Wild-type (WT) pAECs were incubated with different concentrations of hCTLA4-Ig, followed by adding human complement. The highest concentration of hCTLA4-Ig (from R&D) was 250 µg/mL. Lysis of pAECs was determined by flow cytometry. Complement only (without hCTLA4-Ig) was used as a negative control.
Although all three hCTLA4-Igs bound to pAECs without significant difference (A), no lysis of WT pAECs by any of the 3 hCTLA4-Igs was detected (B).

clinical kidney transplantation, 5 or 10 mg/kg belatacept is administrated either every 2 weeks or 4 weeks (the serum trough level of hCTLA4-Ig is approximately 5–10 µg/ml) [36,37]. One of the limitations of the present study was that we could not measure the concentration of hCTLA4-Ig in serum because of a technical difficulty in developing the assay. It is important to note, however, that the binding of hCTLA4-Ig to pAECs does not induce complement-mediated cytotoxicity. Similar results were found by others [38].

pRBCs can be used as target cells to investigate sensitization after xenotransplantation as well as for screening the recipient antibody level against pig carbohydrate antigens before xenotransplantation [17,22,26,39]. pRBCs express the 3 known pig carbohydrate xenoantigens (i.e., Gal, Neu5Gc, Sda) [26,39], but, because they lack nuclei, do *not* express MHC (swine leukocyte antigen [SLA]) class I and II) (Table 1). Furthermore, pRBCs do not express CD80/CD86 molecules. If immunosuppressive therapy is inadequate, recipients of pig xenografts could become sensitized to both carbohydrate antigens and SLA (especially SLA class I). pRBCs are therefore not suitable for studies designed to investigate sensitization to SLA class I and II.

PBMCs contain antigen-presenting cells, e.g., B cells and monocytes, that express MHC class I and II and costimulatory molecules (e.g., CD80/86). Therefore, PBMCs are not suitable for the measurement of serum anti-pig antibody binding if hCTLA4-Ig has been administered (for the same reason as pAECs). In contrast, pCD3$^+$T cells express the 3 known carbohydrate xenoantigens and SLA class I and II, but *not* CD80/86 molecules (Table 1). However, quiescent AECs do not normally express MHC class II until stimulated by IFN-γ [25]. Both PBMCs and pAECs obtained from a donor pig could be used as donor-specific target cells. However, pAECs obtained from the thoracic/abdominal aorta as well as PBMCs, might have different antigenicity from organ-specific endothelial cells, such as kidney microvascular endothelial cells. It is important to note the effect of xenogeneic serum-containing medium (e.g., fetal bovine serum) on cultured cells. For example, expression of Neu5Gc glycans can occur on nonNeu5Gc-expressing cells (e.g., human endothelial cells or pAECs which do not express Neu5Gc by knocking out of CMAH) [40]. To standardize an assay for detecting anti-pig antibody binding that is stable, simple, repeatable, and inexpensive, PBMCs might be the ideal cells.

In this study, we found that human CD3$^+$T cells expressed MHC class II (Fig. 1D). Although it has been shown that some populations of circulating human peripheral blood T cells (e.g., activated T cells, regulatory T cells) express HLA class II [41–44], we have further investigate the expression of HLA class II on CD3$^+$T cells in human PBMCs (n = 9). As we expected, all human express HLA class II on CD3$^-$CD20$^+$ B cells. The average of HLA class II positive cells in CD20$^+$B cells was 100% (data not shown). Human also express HLA class II on CD3$^+$CD20$^-$T cells. The average of HLA class II positive cells in CD3$^+$T cells were 22% (range: 10% to 40%). However, the fluorescent intensity (i.e., the level of expression) of HLA class II on CD3$^+$T cells was 70 times lower than to CD20$^+$ B cells (data not shown). These results indicated that human CD3$^+$T cells express HLA class II, although the expression level was lower than on human CD20$^+$ B cells. Several groups [18] [4,45–47] use PBMCs as target cells to detect anti-pig antibody. Since CD3$^+$T cells in PBMCs express both carbohydrate and MHC class I and II on their surface, but do *not* express CD80/86 molecules, these cells might be appropriate target cells to investigate the development of anti-pig antibodies when hCTLA4-Ig has been administered to the recipient. However, we could consider gating both CD3$^+$T cells and CD21$^+$B cells (as in the clinical setting) to perform a cross-match test if hCTLA4-Ig will not be administrated in the recipient.

We conclude that serum containing hCTLA4-Ig can be associated with a false-positive result when pAECs are the target cells and IgG binding is detected by flow cytometry. The selection of the pig target cell is therefore important when trying to detect de novo anti-pig antibodies, and CD3$^+$T cells would be the preferable cells to use. The study is a reminder that drug therapy may lead to misinterpretation of a result that indicates antibody binding.

Declaration of Competing Interest

David Ayares is an employee of, and receives a salary from, Revivicor, Blacksburg, VA, and provided the pigs and pig cells used in the study. However, he had no role in the study design, data collection and analysis, preparation of the manuscript (except to review and approve it), or in the decision to submit it for publication. The other authors declare that they have no conflict of interest.

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