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Molecular events contributing to successful pediatric cardiac transplantation in HLA sensitized recipients

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ABSTRACT

Antibodies to HLA resulting in positive cytotoxicity crossmatch are generally considered a contraindication for cardiac transplantation. However, cardiac transplantations have been performed in children by reducing the Abs and modifying immunosuppression. To identify mechanisms leading to allograft acceptance in the presence of Abs to donor HLA, we analyzed priming events in endothelial cells (EC) by incubating with sera containing low levels of anti-HLA followed by saturating concentration of anti-HLA. Pre-transplant sera were obtained from children with low levels of Abs to HLA who underwent transplantation. EC were selected for donor HLA and exposed to sera for 72 h (priming), followed by saturating concentrations of anti-HLA (challenge). Priming of EC with sera induced the phosphatidylinositol 3-kinase/Akt mediated by the BMP4/WNT pathway and subsequent challenge with panel reactive antibody sera increased survival genes Bcl2 and Heme oxygenase-1, decreased adhesion molecules, induced complement inhibitory proteins and reduced pro-inflammatory cytokines. In contrast, EC which did not express donor HLA showed decreased anti-apoptotic genes. Primed EC, upon challenge with anti-HLA, results in increased survival genes, decreased adhesion molecules, induction of complement inhibitory proteins, and downregulation of pro-inflammatory cytokines which may result in accommodation of pediatric cardiac allografts despite HLA sensitization.

1. Introduction

Pediatric candidates for cardiac transplantation are often sensitized to human leukocyte antigen (HLA) [1–3]. Pre-transplant desensitization strategies have been used in adult renal and cardiac transplantation with varying success [4], but there is little experience to demonstrate efficacy in children awaiting cardiac transplantation, particularly in the presence of cryopreserved homografts (allografts) used for prior repair of congenital heart defects. Furthermore, infants and those who are hemodynamically unstable may not tolerate pre-transplant plasmapheresis. This has led some programs to undertake transplantation in highly sensitized pediatric heart candidates (with positive cytotoxicity crossmatch) by performing aggressive intraoperative plasma exchange (to remove Ab), followed by post-transplant plasmapheresis, and augmented immunosuppression, often with the addition of post-transplant intravenous immunoglobulin therapy [5–7]. Early results have been encouraging. It is presumed that these outcomes reflect the fact that donor vascular endothelium is initially exposed to low concentrations of donor-specific antibody (DSA) following conclusion of cardiology-pulmonary bypass with plasma exchange. These findings parallel the successful cardiac transplants that have been reported in the presence of low concentration and low strength of DSA (MFI < 2000) [8], as well as in renal transplant recipients following removal of Ab by immunoadsorption [9,10]. Solid phase approaches for detection of HLA sensitization is highly sensitive, so many more pediatric cardiac transplant candidates are identified as having low levels of sensitization to HLA, including to mismatched donor-HLA [3]. Historically, many of these children did well following transplantation without knowledge of the low-level sensitization and DSA at transplant.

Crosslinking of Abs to HLA can provide signals of cell cycle arrest leading to apoptosis [11–13]. Ligation of HLA class I by Abs on endothelial cells (EC) can induce phosphorylation of tyrosine of

Abbreviations: AMR, antibody mediated rejection; DSA, donor specific antibody; EC, Endothelial cell; HAEC, human aortic endothelial cells; HLA, Human leukocyte antigen; HO-1, Heme oxygenase-1; ICAM-1, Intercellular adhesion molecule; PECAM-1, Platelet endothelial cell adhesion molecule; PRA, Panel reactive antibody; qPCR, quantitative polymerase chain reaction; RT-PCR, real time polymerase chain reaction; VCAM-1, Vascular cell adhesion molecule

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intracellular proteins, thereby augmenting growth factor receptors leading to proliferation of EC [14]. We, and others, have previously demonstrated that crosslinking of HLA class I by sub-saturating concentrations of Abs to HLA can result in upregulation of the anti-apoptotic genes Bcl2, BAX, and heme oxygenase-1 (HO-1) and phosphorylation of BAD-triggered Phosphatidylinositol 3-kinase (PI3k)/Akt/PKA pathway, resulting in prevention of Ab/complement-mediated lysis [15,16]. We have also shown that donor hearts perfused with a sub-saturating concentration of anti-HLA can prevent antibody mediated rejection (AMR) of HLA-A2 transgenic murine cardiac transplants [17].

The priming events in the EC by low levels of HLA that lead to successful transplantation remain unclear. In this study, we used an *in vitro* assay using EC to demonstrate that exposure of EC to low levels of Abs to HLA (priming) prevents cell death. Further, these primed EC upon challenge with saturating concentrations of anti-HLA resulted in increased expression of anti-apoptotic genes (e.g., Bcl2, BAX, and HO-1), complement inhibitory protein CD59 and significantly decreased expression of adhesion molecules.

2. Patients and methods

2.1. Patient sera and Abs to HLA

Pre-transplant sera were collected from 8 pediatric heart transplant patients participating in the Clinical Trials in Organ Transplantation in Children-04 study (CTOTC-04) [3]. We selected subjects who had low levels (MFI 2500 for HLA class I and 1500 for HLA class II) of pre-existing Abs to HLA (Table 1) and these sera were used for priming. Patients 3–9 did not demonstrate any DSA however we postulated that the sera may contain low levels of Abs to mismatched donor HLA. Therefore we tested the sera against EC expressing some of the donor HLA following dilution (1:5 and 1:100). These sera reacted to EC expressing a given donor HLA class I mismatched antigen with low MFI to the antigens in question > 500 (Pt 3 to HLA A3, Pt 4 to H LA B7, Pt 5 to A24, Pt 6 to A1, Pt7 to B44 and Pt 8 to A3). Pt 9 and Pt 10 sera were pooled and diluted to 1:5 and 1:100 and these sera also reacted to EC expressing HLA A24 and B7 with MFI below 500. HLA class I Ab W6/32 (IgG2a monoclonal Ab to HLA frame work) and high panel reactive antibody (PRA) sera (pooled human sera with > 90% reactivity to a panel of cells) were used for incubation with saturating concentrations of anti-HLA (challenge). Dilutions of patients' sera used for study were 1:5 and 1:100. We noted similar results using dilutions of 1:5 and 1:100 (data not shown) and, therefore, in all experiments we used 1:5 dilutions, referred to as sub-saturating concentration. For, the HLA class I Abs W6/32 and high PRA sera were used at 1:5 dilution which is referred to as saturating concentrations. W6/32 was also used at a higher dilution (1:100), referred to as sub-saturating concentration based on an earlier report [15].

2.2. Abs and reagents

Commercial Abs used are: β-actin (Sigma-Aldrich, St. Louis, MO), Bcl2 (eBioscience, San Diego, CA), Bcl-xl (Abcam, Cambridge, MA), BAX (Santa Cruz Biotechnology, Santa Cruz, CA), HO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and adhesion molecule ICAM-1, VCAM-1, PECAM-1, E-selectin, P-selectin (BioLegend, San Diego, CA).

2.3. Endothelial cells

Primary cultures of human aortic EC were purchased from Clonetics (San Diego, CA). EC were cultured in basal medium with supplements (EGM™-2 bullet kit, Clonetics Corp.) at 37 °C in a humidified incubator (5% CO_2, 95% air). Their HLA phenotypes were determined using low resolution molecular approach and EC was selected based on the HLA allele to which patients' sera had low levels of Abs (Table 1). As negative control, EC (HEAC 736), which did not express the endothelial HLA type, were used and incubated with the pre-transplant sera at different dilutions (1:5 saturation concentration; 1:100 sub-saturating concentration). Pre-transplant sera from patients 1 and 3 were pooled together and referred to as patient 9 and similarly patients 6 and 8 were pooled and referred to as patient 10 (Table 1).

2.4. Binding of HLA Abs to ECs

Cultured ECs (5×10^5) were harvested using 0.5 ml of cell dissociation buffer (Invitrogen). The ECs were washed with FACS buffer and incubated patient's sera and serum without anti HLA Abs (negative control) followed by FITC conjugated anti-human IgG for 30 mins at 4 °C. The ECs were subjected to FACScan flow cytometer (BD Biosciences), and data analysis was carried out using the FlowJo Software.

2.5. Luminex assay

Diluted sera (1:5, 1:100) of pediatric lung transplant recipients were used to measure the Ab to human HLA class I and class II using a LABScreen mixed Ag kit (One Lambda, Canoga Park, CA) by Luminex assay according to the manufacturer's protocol. Briefly, 5 µl of beads coated with mixed HLA class I and Class II Ag was added to 20 µl of diluted serum samples (1:5, 1:100) in 96-well plates and incubated for 30 min at room temperature. The plates were washed and 100 µl of PE-conjugated goat anti-human Ab was added and incubated for 30 min. Plates were then washed and up to 100 beads were analyzed using the Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA). Negative controls (One Lambda, Canoga Park, CA) used for normalization.

Table 1
Selection of the HAEC cells based on Donor HLA type.

Sample ID	Donor HLA		EC were used based on HLA typing	CL I (MFI)	CL II (MFI)	DSA (MFI)
	A	B				
Pt 1	1, 34	**44**, 58	HAEC 90662 ***(44)***	3400	4800	B58 (1101)
Pt 2	2, 68	42, **7**	HAEC 358 ***(7)***	3400	8500	B42 (2100)
Pt 3	1, **3**	14, 8	HAEC 358 ***(3)***	7900	1500	No
Pt 4	1, 30	35, **7**	HAEC 358 ***(7)***	31,800	Neg	No
Pt 5	**24**, 25	55, 39	HAEC 365 ***(24)***	2800	Neg	No
Pt 6	**1**, 33	14:65, 8	HAEC 90662 ***(1)***	2500	Neg	No
Pt 7	24, 29	7, **44**	HAEC 90662 ***(44)***	5300	2300	No
Pt 8	**3**, 74	15, 15	HAEC 358 ***(3)***	118,300	7500	No
Pt 9			HAEC 736 ***(24,7)***			
Pt 10			HAEC 736 ***(24,7)***			

*Donor HLA type shown as **Bold** and HAEC cells as ***bold and italic***. As negative control HAEC 736 used which did not express donor HLA. Pre transplant sera patients 1 and 3 were pooled together and referred as Pt 9 and similarly patients 6 and 8 pooled and referred as Pt 10. Pt: Patient.

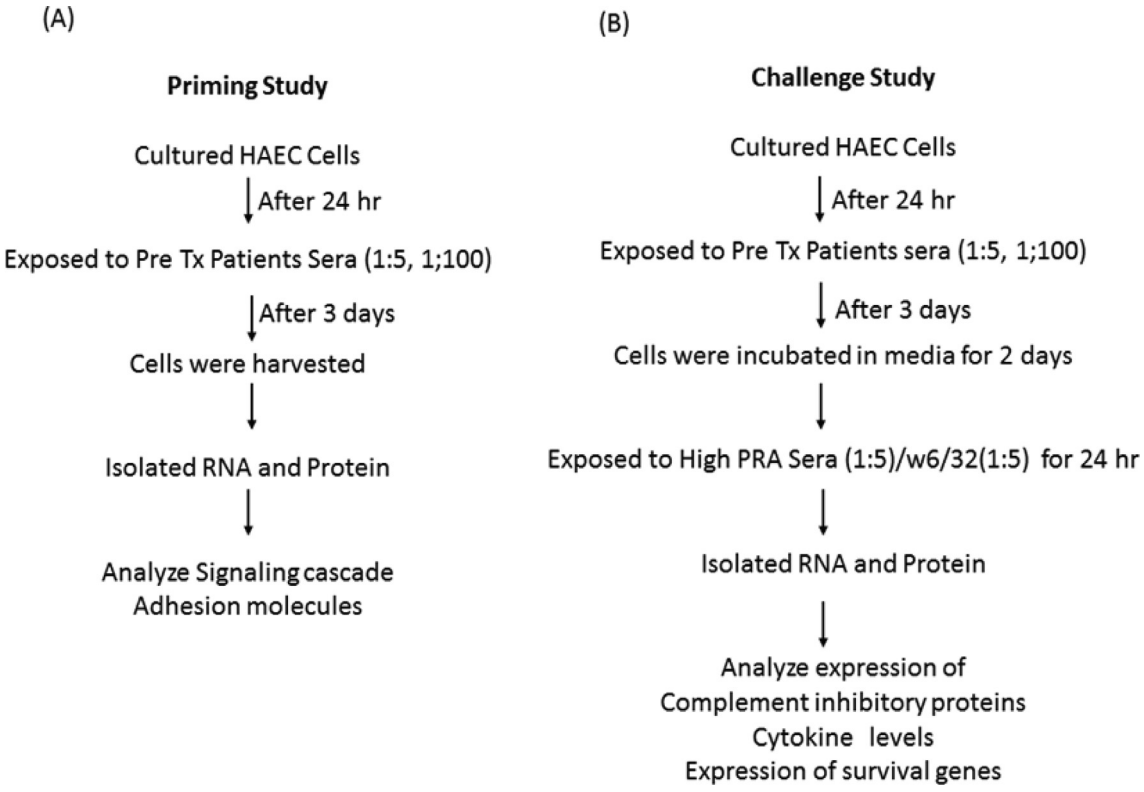

Fig. 1. Flow chart for priming and challenge studies.

2.6. Priming experiments

EC ($2x10^5$ cells) were exposed to patients' sera containing low levels of Abs to HLA (dilution 1:5 or 1:100) for three days. Cells were then harvested and washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and re-suspended in Trizol for RNA isolation. Concentration was measured using NanoDrop (ThermoScientific, Wilmington, DE). Schematic representation of priming experiment is given in Fig. 1A.

2.7. Challenge with saturating concentrations

EC primed, as described above, were re-exposed to high PRA sera (1:5 dilution) or 1:5 dilution of W632 for 24 h, and harvested using trypsin/EDTA buffer and washed twice with HEPES buffer. Cells were resuspended in Trizol for isolation of RNA. RNA concentration was measured using NanoDrop (ThermoScientific). Schematic representation of priming experiment is given in Fig. 1B.

2.8. Isolation of RNA

RNA from the EC incubated with sera was isolated using pure link Tm RNA minikit (Ambion, ThermoScientific, Wilmington, DE). In brief, EC were lysed and homogenized in buffer. One volume of 70% ethanol was added to the lysate mixed and transferred to the spin cartridge/column and centrifuged at room temperature, washed with wash buffer. RNA was eluted using elution buffer and yield and quality was analyzed and stored at −80 °C.

2.9. cDNA synthesis

1 μg of RNA was reverse-transcribed with SuperScript™ First-Strand Synthesis System for real time polymerase chain reaction (RT-PCR) (Qiagen, Hilden, Germany) using Oligo-dT primers. In brief, RNA and primer mix was incubated at 65 °C and then 2X reaction mix was added and incubated at 42 °C. Superscript II enzyme was added and incubated for 42 °C and then at 70 °C. Diluted cDNA was used for quantitative PCR (qPCR).

2.10. RT-PCR

qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) on Applied BioSystems 7900HT thermocycler as per the manufacturer's instructions. β-actin was used as a housekeeping gene. Fold change is calculated based on qPCR and data is normalized by control data. Further qPCR data was validated by western blot analysis. Primers used are listed in Table 2.

2.11. Western blotting

To determine the induction of apoptotic (Bax) or anti-apoptotic

Table 2
RT- PCR primers.

Name	(5′ → 3′)
H-Bcl-2 Fw	ATC GCC CTG TGG ATG ACT GAG
H-Bcl-2 Rev	CAG CCA GGA GAA ATC AAA CAG AGG
H-bax Fw	GGA CGA ACT GGA CAG TAA CAT GG
H-bax Rev	GCA AAG TAG AAA AGG GCG ACA AC
H-ICAM1-FW	GGCCGGCCAGCTTATACAC
H-ICAM1-Rev	TAGACACTTGAGCTCGGGCA
H-VCAM1-FW	TCAGATTGGAGACTCAGTCATGT
H-VCAM1-Rev	ACTCCTCACCTTCCCGCTC
H-E selectin-FW	CCCGAAGGGTTTGGTGAG
H-E selectin-Rev	TAAAGCCCTCATTGCATTGA
H-β-actin-Fw	TCT GGC ACC ACA CCT TCT ACA ATG
H-β-actin-Rev	AGC ACA GCC TGG ATA GCA ACG
H-P-selectin-FW	TGAGCACTGCTTGAAGAAAAAGC
H- P-selectin-REV	CACGTATTCACATTCTGGCCC
H-PECAM-1-FW	CTGAGGCTGAAGGTGATAGC
H-PECAM-1-REV	AGTATTTTGCTTCTGGGGAC

genes (Bcl2 and HO1), EC (2×10^5 cells) were exposed to sera containing Abs to HLA or w6/32 (positive control), then washed with EC media and harvested using trypsin/EDTA buffer. Cells were washed with HEPES buffer and lysed in radio immunoprecipitation assay buffer (RIPA buffer). 20-μg protein samples were electrophoresed and transferred to polyvinylidene difluoride membrane. The membranes were blocked and incubated with primary and secondary Abs. Blots were developed with an Amersham enhanced chemiluminescence Prime Western Blotting Detection Reagent kit (GE Healthcare Life Sciences, Pittsburgh, PA). β-actin was used as a loading control.

2.12. Cytokine production

Cytokines interleukin IL-1β, IL-6, and interferon-γ were measured using Luminex (Luminex, Austin, TX). Bio-Plex Luminex 100 was used to analyze the plates, and cytokine concentrations were calculated using Bio-Plex Manager 3.0 software with a 5-parameter, curve-fitting algorithm applied for standard curve calculations.

2.13. Statistical analysis

Statistical comparisons were carried out using two-tailed Student's *t*-test and with GraphPad 5.0 statistical software (GraphPad Software, Inc, San Diego, CA) with statistical significance set at $p < 0.05$.

3. Results

3.1. Binding of HLA with endothelial cells and detection of HLA Abs

Binding of HLA Abs (1:100) to endothelial cells was analyzed by flow cytometry. Our results showed mean channel shift in endothelial cells treated with 1:100 dilution in comparison to control (Cells alone and serum without HLA Ab 1:100 dilution (negative control)). Mean channel shift for cells alone was 988, cells treated with serum negative to HLA Abs (negative control) was 755, sera from P4 bound to EC with an MFI of 1928, Sera from P7 had an MFI of 1991 and sera from P8 demonstrated an MFI of 2410 (see Supplementary Fig. 1). We did not notice the cross reactivity when cells were treated with control.

Further, we detected low levels of HLA class I and II Abs in diluted sera (1:5 and 1:100) by Luminex. At 1:5 dilution, Pt 4 demonstrated HLA class 1 MFI = 1377, HLA class II = MFI 259; 1:100 dilution, HLA class 1 MFI = 454, HLA class II = MFI 63; Pt 7: at 1:5 dilution HLA class I MFI = 229, HLA class II MFI = 200 1:100 dilution HLA class I MFI = 306, HLA class II MFI = 190 and Pt 8: at 1:5 dilution HLA class I MFI = 1947, HLA class II MFI = 1312; and at 1:100 dilution HLA class I MFI = 835, HLA class II MFI = 593 (see Supplementary Fig. 2).

3.2. Priming with low levels of anti-HLA results in the activation of survival signals

To identify signaling events, we exposed human aortic EC (HAEC) with pre-transplant sera (MFI < 1000, 1:5 dilution) for 3 days. RNA was extracted from these EC for analysis. The EC that had been primed demonstrated significant increases in expression of survival signals MAPK1/ERK, TANK, WNT2, and BMP4 (9.1-fold, 4.83-fold, 4.59-fold, and 11.31-fold increases, respectively; $p < 0.01$) compared to the control and EC which had been incubated with saturating concentration of anti-HLA (1:5 dilution) (Fig. 2A). Western blot results confirmed increased expression of MAPK/ERK, WNT2, and BMP4 in the EC primed with sub-saturating concentrations of pre-transplant sera compared to EC which had been incubated with saturating concentrations of anti-HLA (Fig. 2C). These results demonstrate that exposure of EC with low levels of anti-HLA (sub-saturating concentrations) induces upregulation of the MAPK/ERK, WNT2, and BMP4 pathways. EC which did not express the donor HLA did not show expression of WNT 2 signaling pathways which also support our conclusion that EC with low concentration of specific anti HLA induces graft accommodation (Fig. 2D). Messenger RNA and protein association have shown very weak connection when compared at expression levels. We observed messenger RNA expression but not the protein expression due to post transcription modification.

3.3. Decreased expression of adhesion molecules after priming with low levels of anti-HLA

qPCR was used to determine the expression of adhesion molecules following priming with patients' sera. RNA was extracted from EC pretreated with patients' pre-transplant sera. Significantly decreased expression of the adhesion molecules ICAM-1, VCAM-1, PECAM-1, E-selectin, and P-selectin (3.6-fold, 5.4-fold, 2.7-fold, 4.9-fold, and 3.9-fold respectively $p < 0.005$) in cells treated with sub-saturated concertation of anti HLA (Fig. 3). In contrast, increased levels of adhesion molecules ICAM-1, VCAM-1, PECAM-1, E-selectin, P-selectin (6.6-fold, 7.4-fold, 7.2-fold, 6.9-fold, and 5.9-fold increases, respectively) were noted when cells were treated with saturating concentrations of anti-HLA. These results demonstrate that exposure of EC to sub-saturating concentrations of anti-HLA downregulates the adhesion molecules.

3.4. Challenge experiments

3.4.1. Downregulation of inflammatory signals after low levels of priming and challenge with saturating concentration of anti HLA Abs

To identify the inflammatory cytokines, we exposed HAEC with patients' pre-transplant sera (priming) for 3 days. EC were rested for 2 days in EC basal media, subsequently incubated with high PRA sera (challenge) overnight. Cell lysates were prepared and analyzed for the production of inflammatory cytokines using luminex. Cytokines IL-6, TNF-alpha, IL-8, MIP1-A and IL-1b were significantly reduced when the EC were primed with patient's pre-transplant sera followed by challenge with high-PRA sera ($p < 0.001$) (Fig. 4A). In contrast, incubation of EC with saturating concentration of anti-HLA alone demonstrated increased levels of all cytokines. This indicates that priming with patients sera with low levels of anti-HLA followed by challenge with higher levels of anti-HLA (saturating concentrations) leads to reduction in inflammatory cascade, which leads to less recruitment of cells involved in immune responses.

3.4.2. Low levels of priming and challenged with saturating concentration of HLA Abs increased expression of complement inhibitory proteins

Since EC expression of complement inhibitory proteins CD46, CD55, and CD59 has been associated with improved graft function even in the presence of DSA and complement deposition [18,19], we analyzed the expression of these proteins in EC primed with patients sera followed by challenge with high PRA sera. Significant increases in expression of CD46, CD55, and CD59 were observed in EC exposed to priming with patients' pre-transplant sera (2.7-fold, 3.4-fold, and 7.5-fold increases, respectively; $p < 0.002$) (Fig. 4B). In contrast, decreased expression of CD46, CD55, and CD59 (1.7-fold, 2.4-fold, and 1.5-fold respectively) were observed when cells were treated with saturated concentrations of anti-HLA (challenge with high PRA sera). Therefore, priming with sub-saturating concentrations of anti-HLA followed by challenge with saturating concentration of anti-HLA results in upregulation of complement inhibitory proteins, which we propose may be responsible for resistance of EC for lysis by Ab and complement.

3.4.3. Low levels of priming and challenge with saturating concertation upregulate of anti-apoptotic genes

In order to define the mechanism of survival for EC that underwent priming with low levels of anti-HLA (patients' sera), we analyzed the expression of genes commonly involved in apoptosis: Bcl2, BAX, and HO-1. As shown in Fig. 5A, the anti-apoptotic gene Bcl2 and the anti-inflammatory gene HO-1 demonstrated significantly increased

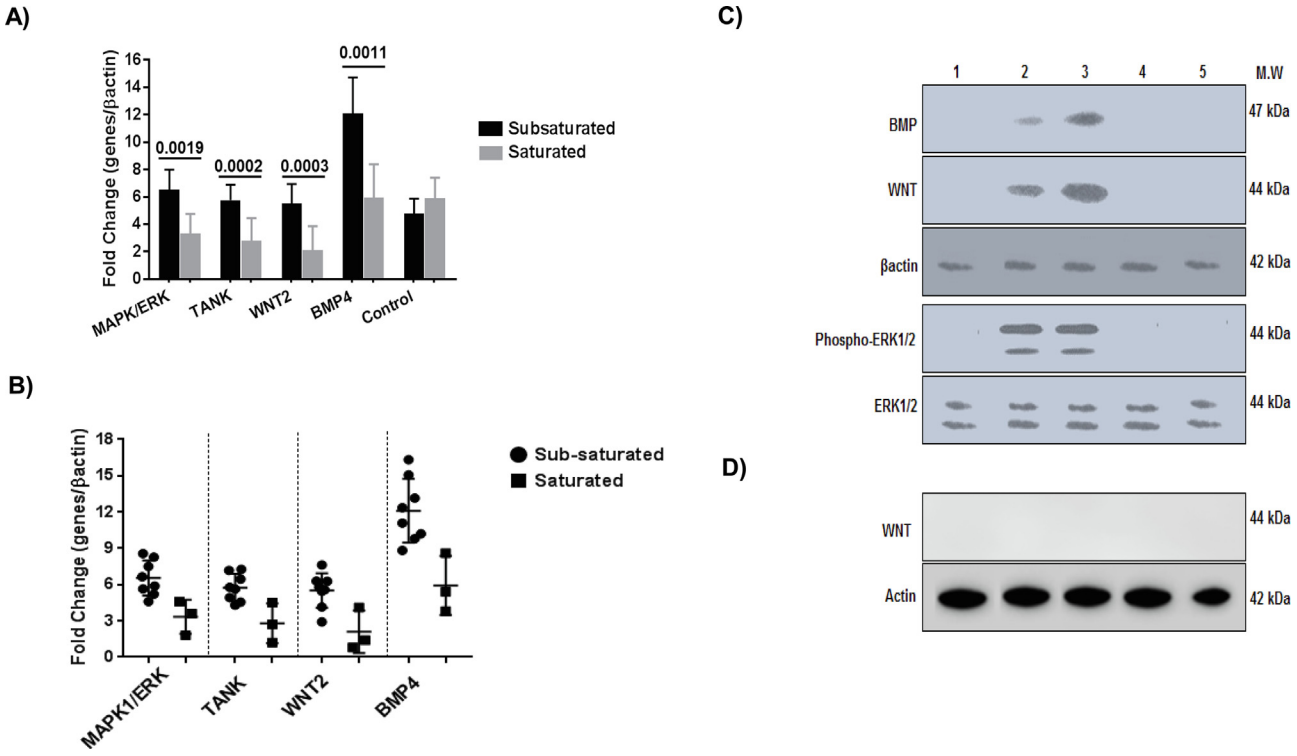

Fig. 2. Activation of survival signaling pathways after EC exposure to sera. HAEC were exposed to pediatric patients (n = 8) pre-cardiac-transplant sera (1:5 dilution) for 3 days. (A) and (B) Expression of signaling pathways by qPCR: HAEC were exposed to pediatric pre-cardiac transplant patient's sera (1:5) for 3 days. Expression of intermediates in signaling cascades were analyzed by qPCR p value < 0.05 consider as significant A): Data represented as average of all 8 patients; B) Individual patient's data is presented as dot blot. (C) and (D) Expression of signal cascade proteins by Western analysis: HAECs were exposed to pediatric pre-cardiac transplant patient's sera (1:5) for 3 days. Expression of intermediates in signaling cascades were analyzed by western blot using anti BMP, anti βactin, Anti WNT, anti Phospho ERK1/2 and anti ERK1/2 Abs Lane 1: control; lane 2: Pre-cardiac transplant serum (1:5); lane 3: Sub saturated (1:100) w6/32; Lane 4: Saturated (1:5) w6/32; Lane 5: High PRA serum (1:5). (D) EC which did not express donor HLA were also analyzed by western blot as a negative control. lane1: control; lane 2: high PRA sera at saturating levels (1:5); lane 3: high PRA sera at sub saturating concentration (1:100); lane 4: pre-transplant serum patient 9 (1:5); lane 5: pre-transplant serum patient 10 (1:5). All the genes p value < 0.05 consider as significant.

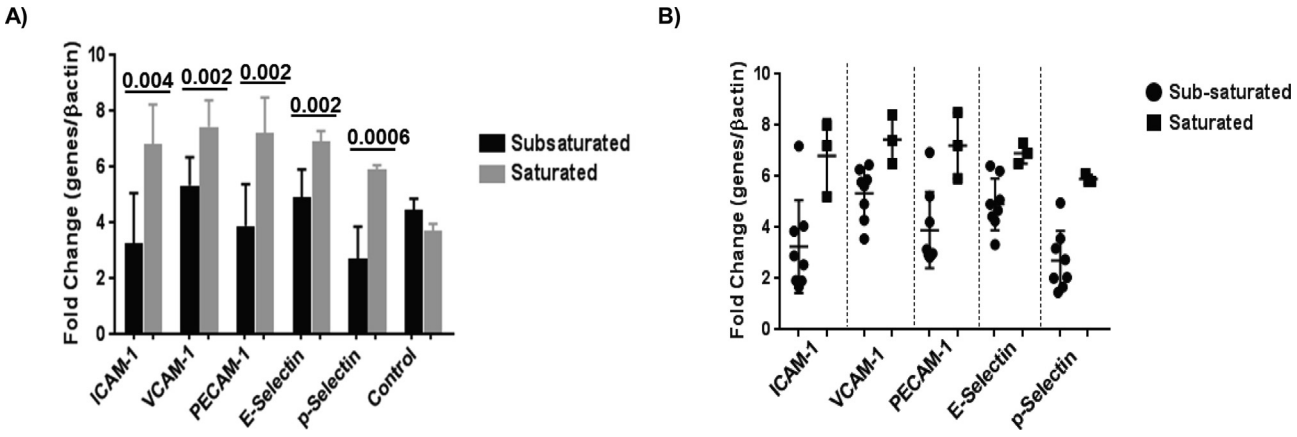

Fig. 3. Downregulation of adhesion molecules after exposure to sera. HAEC were exposed to pediatric patients (n = 8) pre-cardiac transplant sera for 3 days. After exposure, cells were allowed to rest in EC media. Expression of adhesion molecules was analyzed by qPCR. B-actin was used as a housekeeping gene. Changes were calculated in comparison to β-actin expression. A) is showing all 8 patient data together. B) Individual data. All experiments were repeated twice and p value < 0.05 consider as significant.

expression (9.5-fold and 6.25-fold increases, respectively; $p < 0.002$) in the EC primed with sub-saturating concentrations of patients' pre-transplant sera followed by challenge with higher levels of anti-HLA. In contrast, increased expression of the apoptotic gene BAX was observed (5.9-fold increase) and decreased expression Bcl2 and HO-1 genes were noted in EC treated with saturated concentrations of anti-HLA.

Results obtained using qPCR were validated by Western blotting, which confirmed increased expression of Bcl2 and HO-1 and decreased expression of BAX (Fig. 5C) in EC primed with sub-saturating concentrations of anti-HLA followed by challenge with higher levels (saturating concentration) of anti-HLA.

Further to validate our finding, we chose EC lines which did not express the donor HLA and were incubated with sub saturating and saturating concentrations of sera and high PRA sera. Western blot analysis showed that anti apoptotic gene Bcl2 expression is decreased in cells whereas apoptotic gene BAX expression is significantly increased

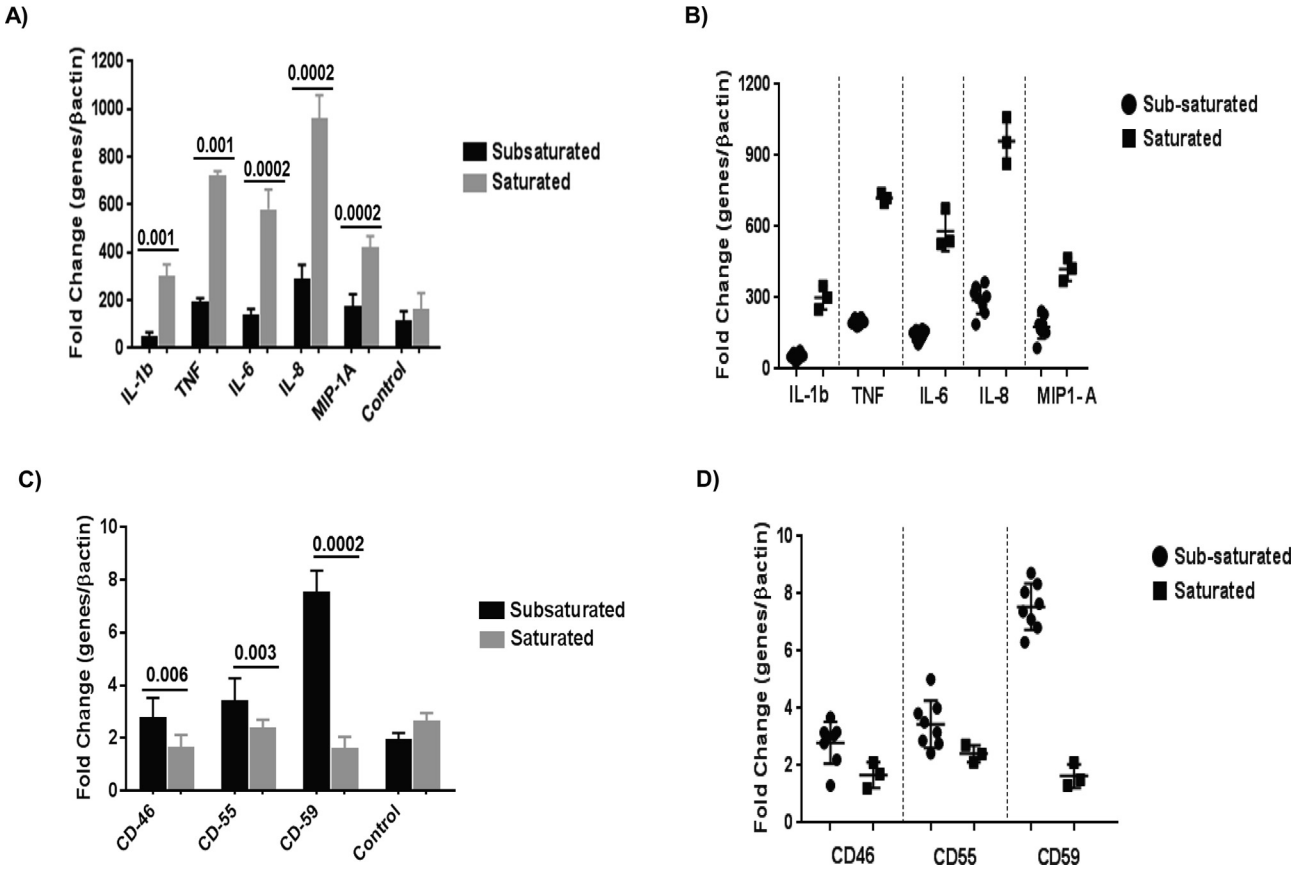

Fig. 4. Low levels of priming and challenge with high PRA (saturating concentration) downregulates inflammatory cytokines and upregulates complement regulatory proteins. A) Pre-exposure to sera (n = 8) downregulates expression of inflammatory cytokines. HAEC were exposed to sera for 3 days. EC were rested for 2 days in EC media and then exposed to high PRA sera for 24 h. Cell lysate was used to determine the concentration of cytokines (p value < 0.05 consider as significant). (C) Exposure of sera (low levels of priming) (n = 8) upregulates complement regulatory proteins. HAEC were exposed to sera for 3 days. EC were rested for 2 days in EC media and exposed to high PRA sera for 24 h. RNA from exposed EC was used for expression of CD46, CD55 and CD59 by qPCR, and changes were calculated in comparison to β-actin expression ($p < 0.002$). B and D are representing individual patient's data. All the experiments were repeated twice.

(Fig. 5D) in comparison of ECs expressing donor HLA (Fig. 5D lane 2 with pre-transplant sera (1:5), and lane 3 with w6/32 (1:100)).

3.4.4. EC incubated with low levels of priming followed by challenge with saturating concentration of anti-HLA induces cell survival signal Akt

Because Akt has been shown to promote cell survival [18,20], we analyzed Akt phosphorylation in EC incubated with pre-transplant sera for 3 days (priming). After 3 days incubation, the EC were washed and incubated in EC media for 2 days with high PRA sera (challenge). As shown in Fig. 6, significantly higher expression of phosphorylated Akt (Ser473) protein was noted in EC exposed to pre-transplant sera containing low levels of anti-HLA. These results demonstrate activation of the Akt pathway after exposure of EC to sub-saturating concentration of HLA Ab, i.e., priming event. On the other hand, no significant expression of phosphorylated Akt (Ser473) protein was noted in EC exposed only to saturating concentrations of anti-HLA.

4. Discussion

Pre-existing Abs to mismatched donor HLA in patients awaiting cardiac transplantation is generally considered a contraindication for transplantation, especially when DSA are associated with positive donor-specific cytotoxicity crossmatch. These patients have been considered at high risk for AMR and poor allograft survival. In pediatric patients waiting for cardiac transplantation there is often sensitization to HLA as they may have undergone surgery and transfusions [1–3]. These candidates often result in positive cross matches and, therefore, do not receive transplants in a timely manner [21]. Intraoperative plasma exchange at transplantation (to remove Ab), followed by post-transplant plasmapheresis, and augmented immunosuppression, often with the addition of post-transplant intravenous immunoglobulin therapy, has been used by several centers to minimize risk of wait list mortality for highly sensitized children awaiting heart transplantation [5–7]. Initial reduction/depletion in Ab is often associated with persistence of DSA post-transplant, yet with continued long-term graft function [5–7]. The resistance of Ab mediated damage resulting in rejection of transplanted organ despite the presence of Ab and complement deposition has been termed accommodation [22].

Graft accommodation has been described primarily in xeno-transplantation models [22,23]. EC of accommodated xenografts has increased expression of the anti-apoptotic genes Bcl2 and HO-1 [24]. Narayanan et al. also demonstrated that human allele-specific anti-HLA and monoclonal anti-HLA in sub-saturating concentrations can result in similar changes in EC *in vitro* [15,16]. Dorling et al. demonstrated that low levels of anti-HLA binding can promote expression of protective genes leading to accommodation of renal allografts [25]. Over expression of the anti-apoptotic genes A20, BAX and Bcl2 (which can inhibit activation of transcription factor NF-κB and suppression of pro-inflammatory cytokines and apoptosis) has also been reported in the EC of transplanted xenografts [26–28]. Increased expression of Bcl2 and HO-1 in grafts exposed to natural xenoreactive Abs and complement can confer protection against hyperacute, AMR [29,30].

Based on these findings, we hypothesized that exposure of EC *in vitro* to low concentration of Abs to HLA present in pre-transplant sera

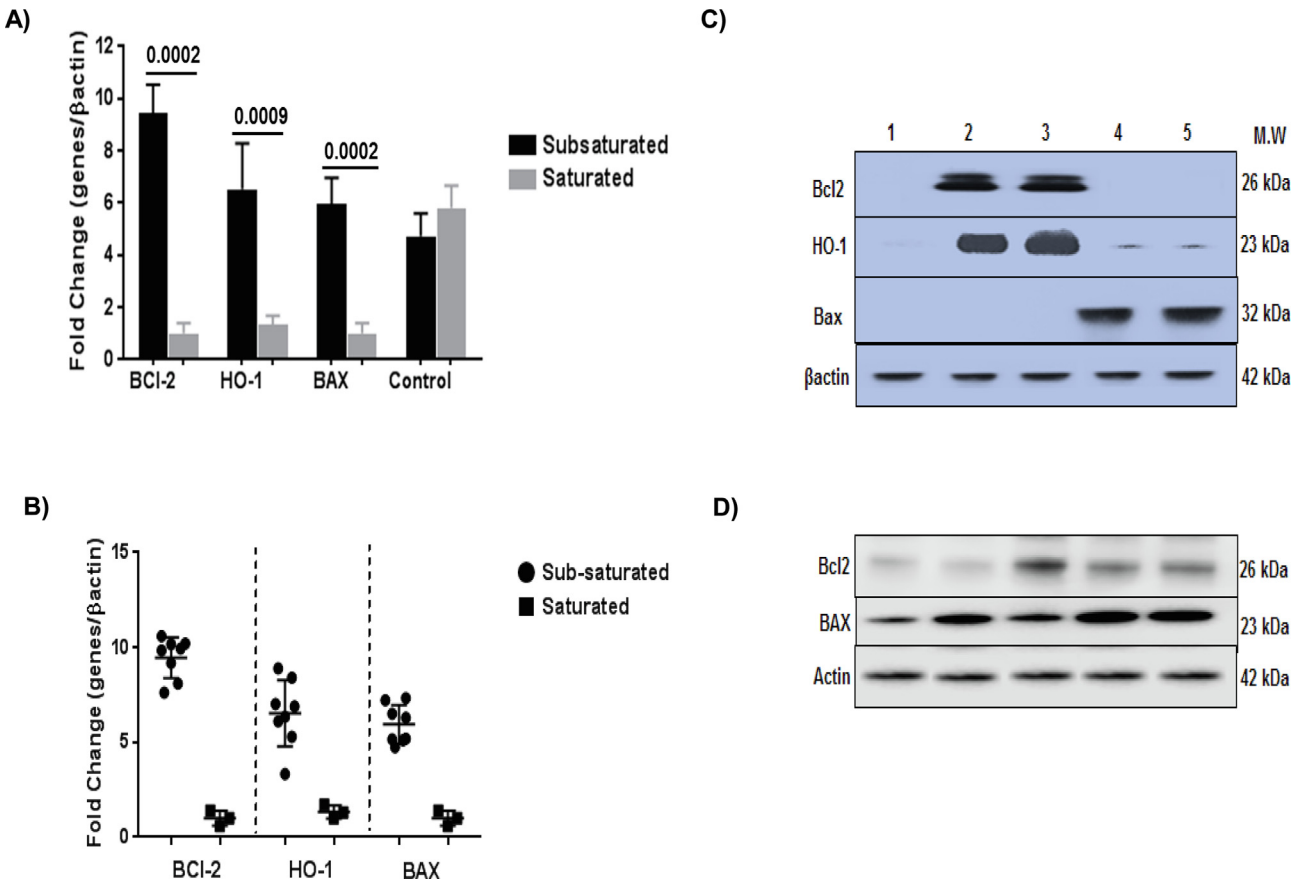

Fig. 5. Increased expression of anti-apoptotic genes after exposure of EC to sera (priming) and challenge with saturating concentration of HLA Abs. HAEC were exposed to pediatric patients (n = 8)' pre-cardiac transplant sera (1:5 dilution) for 3 days. Cells were washed with EC media and rested in EC media for 2 days, then exposed to high PRA sera for 24 h. Expression of anti-apoptotic genes was the analyzed. (A) qPCR analysis: RNA was isolated from treated EC and used for qPCR analysis; changes in gene expression were calculated using β-actin as a housekeeping gene. Figure A represents sum of all 8 patient's data and B is Individual patient results. Experiment is repeated twice. All the genes p value < 0.05 consider as significant. (C) and (D) Western blot analysis: The samples were analyzed for anti-apoptotic gene expression by western blot analysis using anti Bcl-2, anti Bax, anti hemeoxygenase 1 Abs. βactin was used as loading control. Lane 1: control; lane 2: Pre-transplant serum (1:5); lane 3: Sub saturated (1:100) w6/32; Lane 4: Saturated (1:5) w6/32; Lane 5: High PRA serum (1:5). EC which did not express donor HLA was analyzed by western blot as negative control. lane1: control; lane 2: High PRA sera at saturating levels (1:5); lane 3: High PRA sera at sub saturating concentration (1:100); lane 4: Pre-transplant serum patient 9 (1:5); lane 5: Pre-transplant serum patient 10 (1:5).

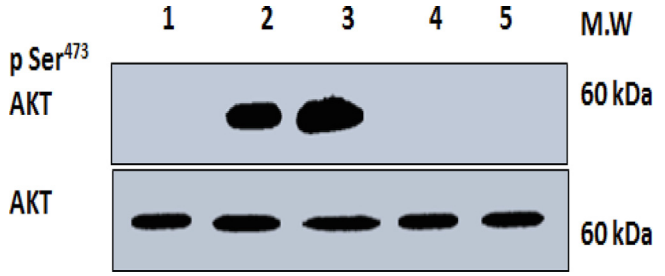

Fig. 6. Induction of AKT in EC exposed to sera (priming) and challenge with saturating concentration of HLA Abs. HAEC were exposed to pediatric patients (n = 8)' pre-cardiac transplant sera (1:5dilution) for 3 days. After exposure, EC were incubated with EC media for 2 days, and then incubated with high PRA sera for 24 h. The cells were lysed using radio-immunoprecipitation assay buffer. Protein from lysed EC was electrophoresed in 10% SDS-PADE. Proteins were then transferred to a polyvinylidene difluoride membrane. The samples were detected by specific Abs to phospho-Akt and Akt. Lane 1: control; Lane 2: pre-cardiac transplant serum (1:5); Lane 3: sub-saturated W6/32 (1:100); Lane 4: saturated W6/32 (1:5) serum; Lane 5: high PRA sera (1:5).

of pediatric cardiac transplant patients may facilitate graft accommodation. We employed an *in vitro* model using cultured primary EC expressing a specific HLA mismatch for patients' Abs and incubated with sub-saturating concentration of either patient's pre-transplant sera containing anti-HLA or purified monoclonal Abs to HLA detecting framework determinants (W6/32). This *in vitro* model allowed us to demonstrate that exposure to sub-saturating levels of HLA Abs over a 3-day period initiated the cellular and molecular changes, priming, responsible for graft accommodation even when subsequently exposed to high levels of Abs to HLA. Accommodated EC exhibited a marked increase in the Bcl2, HO-1 mediated by BMP4/WNT activity and significant decrease in expression of adhesion molecules (ICAM-1, VCAM-1, and PECAM-1 molecule) following challenge with saturating concentrations of anti-HLA (Fig. 4). Therefore, sub-saturating levels of allo-Abs to HLA results in priming of EC, induces changes in EC and subsequent binding to saturating concentrations of Abs (challenge) will not result in damage to the EC. This is consistent with findings in xeno-transplantation wherein modification in EC by low levels of natural Abs can protect EC from further damage even when higher levels of Abs appear and is associated with increased expression of anti-apoptotic genes BCl-2 and Bcl-xl [31,32].

To define the signaling mechanisms involved in allograft survival, we determined the activation of cell survival kinase pathways. There are reports demonstrating that MAPK/ERK is associated with anti-apoptotic molecule which facilitates cell survival and growth in various cells [33]. In agreement with this, we also observed the induction of MAPK/ERK pathway in cells treated with low levels of anti HLA Ab. It is known that many growth factors activate Akt in a PI3K-dependent

manner. Akt is activated when phosphoinositides bind to it, and this activation has a major effect on cell survival and apoptosis [34,35]. We evaluated the role of the Akt pathway in rescuing EC exposed to the low levels of anti-HLA that is present in patients' sera. Our results demonstrated upregulation of Akt, a well-known cell survival signal. Activation of the Akt pathway by phosphorylation results in increased expression and activation of protective genes, especially Bcl2 and HO-1. These results are in agreement with the reported findings by Jin et al., who demonstrated that exposure of EC with low concentrations of the HLA Ab W6/32 activated the PI3K/Akt pathway and increased expression of Bcl2 and BAX [32]. Further, we demonstrated that EC which did not express donor HLA showed downregulation of Bcl2 and upregulation of BAX genes which support our conclusion that low concertation of HLA Abs specific to EC express mismatch HLA may induce graft accommodation.

Regulation of tissue injury mediated by complement deposition is controlled by proteins belonging to the multigene family of regulators of complement activation [35] which includes the membrane proteins C3b receptor (CR1), C3d receptor (CR2), decay accelerating factor (CD55) and membrane cofactor protein (CD46) [36]. CD46 and CD55 are widely distributed in hematopoietic cells, EC, and epithelial cells. Increased expression of CD46 or CD55 following transplantation has been shown to result in decreased complement activation and tissue damage [36]. EC expression of CD55 and CD59 has been associated with improved graft function [19]. Our results demonstrate that priming with sub-saturating levels of anti-HLA can indeed activate various complement regulatory proteins on EC suggesting that this can contribute to continued survival of EC even in the presence of Ab and complement.

As this was a retrospective study using patient sera from pediatric cardiac transplant recipients, there are some limitations. Our report analyzed sera from a small group of 8 patients for whom we had appropriate HLA phenotyped cultured primary EC. However, results obtained were consistent with priming induced by low levels of anti-HLA resulting in molecular changes consistent with accommodation. We currently have only short term follow-up of these subjects, and long-term follow-up of the CTOTC-04 cohort is ongoing and includes assessment of persistence pre-transplant DSA (and *de novo* DSA development), acute rejection events (Ab and cellular), chronic graft function, and development of graft coronary vasculopathy [3].

Previous paper from our lab showed exposure of sub saturating concentration of HLA class1 Ab results in induction of signaling protein which confers resistance to EC against Ab- complement mediated cell death [16]. For that study we used adult sera and obtained similar results. Based on this, we hypothesized that low levels of HLA Abs in sera from pediatric patients will also induce resistance to EC. Since our results using pediatric sera paralleled with adult sera, we feel that Abs present in the pediatric patients behave similar to adult patients.

In summary, our study demonstrated that low levels of anti-HLA, when bound to EC, lead to priming of the EC which resulted in activation of survival signals and decreased expression of adhesion molecules. Subsequent challenge with saturating concentration of anti-HLA resulted in the downregulation of inflammatory signals, increased expression of complement inhibitory proteins, and upregulation of anti-apoptotic genes. Increased expression of Bcl2 and HO-1 was evident in resistant EC. Furthermore, we have demonstrated that activation of the PI3K/Akt pathway mediated by BMP4/WNT led to activation of phosphorylation of AKT. Taken together, our results demonstrate that sub-saturating concentrations of HLA Abs present in the pre-transplant sera of pediatric cardiac transplant patients activated MAPK/ERK pathway in EC, leading to changes consistent with the phenomenon of accommodation which results in continued allograft function.

Disclosure statement

The authors declare that they have no conflict of interest. All authors have approved the final article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.01.008.

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