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HLA-Class Ib Expression Suppresses Neutrophil Xenogeneic Immune Responses Against Pig Cells

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Abstract

Purpose: The expression of Human Leukocyte Antigen (HLA) class I molecules regulates innate immune responses of Natural Killer (NK) cells and macrophages. In this study, we investigated the regulatory responses of Human Leukocyte Antigen G1(HLA-G1) and Human Leukocyte Antigen E (HLA-E) on human neutrophils.

Material and methods: The expression of counter receptors on neutrophils was detected using flow cytometry. Complementary DNA (cDNA) of HLA-G1 and HLA-E were introduced into Swine Endothelial Cells (SECs) to establish SEC/HLA-G1 and SEC/HLA-E cell lines, respectively. These cell lines were then co-cultured with neutrophils to assess cytotoxicity. Subsequently, Reactive Oxygen Species (ROS) production levels were calculated using Cell Reactive Oxygen Green (CellROX Green). Neutrophil Extracellular Traps (NETosis) in neutrophils of SEC cells was measured using SYTOX Green.

Results: The expression of Natural Killer Group 2 member A(NKG2A), Immunoglobulin-Like Transcript 2 (ILT-2) and ILT-4 on neutrophils was <20%, approximately 30% and approximately 65%, respectively. No significant differences in cytotoxicity were observed in SEC/HLA-E and wild-type SEC cells, whereas SEC/HLA-G1 cells exhibited significant reduction of approximately 35% and 25% in the cytotoxicity after 4 h of incubation with Phorbol 12-Myristate 13-Acetate (PMA) and 24 h of incubation without PMA, respectively. Additionally, SEC/HLA-G1 cells significantly inhibited ROS production. HLA-G1 also suppresses NETosis induction in neutrophils.

Conclusion: These results indicate that HLA-G1 effectively regulates the xenogeneic immune responses of neutrophils against SECs in xenotransplantation.

Keywords: HLA-G1; Xenotransplantation; Neutrophil; Innate immunology; Internal ribosome entry site; Human beta-2 microglobulin (hβ2m)

Introduction

Several Genetically Edited (GE) pigs have been developed and used for xenotransplantation [1]. The six transgenes in “10GE-pigs” contain two complement regulatory proteins, two anti-coagulant factors, one anti-inflammatory molecule and Closs of Differentiation 47 (CD47), a factor that regulates monocytes and macrophages [2,3]. Another venture company developed pig lines expressing CD47 and Human Leukocyte Antigen E (HLA-E), but their expression levels were insufficient [4].

After years of basic research and preclinical experiments using non-human primates and brain-dead recipients, it has become evident that hyperacute rejection can be overcome using GE-pigs along with medication [5-10].

Despite this, gene editing in these pigs is insufficient and acute rejection follows. It is important to regulate innate immune cells, including Natural Killer (NK) cells, monocytes/macrophages and neutrophils, to suppress secondary rejection reactions [11-14].

Regarding the regulation of these innate immune cells in xenotransplantation research, the issue of graft damage caused by NK cells has become a key focus area from an early stage. The introduction of HLA class Ib into pig grafts has received attention as a potential solution to this issue.

The next challenge was to regulate monocytes and macrophages. In this regard, the expression of CD47 in grafts has been implemented and GE-pigs expressing CD47 have been developed. Issues associated with the expression of CD47 in GE-

pigs have been reported. A novel molecule, a hybrid of Collectin-P1 (CL-P1) and Surfactant Protein-D (SP-D) (CL/SP-D), can regulate the activity of macrophages better than CD47 in GE-pig cells [15,16]. Additionally, we previously reported that regulation of cells by recognizing HLA class Ib molecules such as HLA-G1 and HLA-E can be implemented for NK cells, monocytes/macrophages [17,18].

In this study, we focused on neutrophils and examined whether HLA class Ib molecules exhibit regulatory functions on neutrophils. The expression of HLA-G1 and HLA-E counter receptors was assessed in human neutrophils. In addition, xenogeneic rejection of Swine Endothelial Cells (SECs) was investigated in an *in vitro* system.

Materials and Methods

Cells

MYP30 lineage SEC were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS). Human peripheral blood neutrophils were isolated and kept in a humidified incubator at 37°C, 5% CO_2, in Roswell Park Memorial Institute Medium (RPMI)medium containing 10% FBS [19].

Construction of the plasmids

cDNA was prepared for HLA-G1 alone and subcloned into the pCXN2L (β-actin promoter) expression vector. cDNAs for the HLA-Ev (147) (Human Leukocyte Antigen E Variant) and human β2-microglobulin(hβ_2m) were also prepared [20]. Subsequently, the hybrid gene, HLA-Ev (147) +IRES+hβ_2m, was constructed and cloned into the pCXN2L expression vector. All sequences were verified by means of an ABI 310 autosequencer (Perkin-Elmer Corporation, Norwalk, CT).

Establishment of the transfected cells

Plasmids encoding HLA-G1 and HLA-E were transfected into SECs by electroporation using the neon® transfection system (Thermo-Fisher Scientific, Tokyo, Japan). The transfected cells were then subjected to drug selection by adding G418 (NacalaiTesque, Kyoto, Japan) to the medium and clonal individuals of SEC/HLA-G1 and SEC/HLA-E were established by limiting dilution.

Flow cytometry

The SEC cells (1 x 10^6) were stained with a mouse monoclonal Antibody (mAb) against HLA-class I, B9.12.1 (CosmoBio, Tokyo, Japan) for 30 min at 4°C, followed by a secondary antibody, Fluorescein Isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (Cappel ICN) for 30 min at 4°C.

The expression of counter inhibitory receptors on neutrophil was evaluated using flow cytometry. The expression levels of Immunoglobulin-Like Transcript（ILT-2, ILT-4) and NKG2A were assessed using Alexa Fluor 647 anti-human CD85j mAb (GHI/75) (Biolegend, San Diego, CA), Phycoerythrin (PE)-labeled anti-human CD85d mAb (42D1) (Biolegend) and PE-labeled anti-human NKG2A mAb (R and D Systems, Minesota, MN), respectively. The stained cells were analyzed using a FACS (Fluorescence-Activated Cell Sorting) Calibur flow cytometer (BD Biosciences, San Diego, CA).

Neutrophil isolation

Human peripheral blood mononuclear cells were isolated from healthy volunteer donors, followed by differential density gradient separation with Polymorphprep® (Alere Technologies AS, Oslo, Norway). The remaining erythrocytes were dissolved with a lysis buffer (0.83% NH4Cl, 10 mmol/L Hepes-NaOH, pH 7.4) and incubated at 37°C for 3 min. The isolated cells were labeled using PE-labeled anti-CD66b mAb (Bio Legend) and a purity of approximately 95% was confirmed by flow cytometry [21].

Cytotoxicity assay

Naive SEC, SEC/HLA-G1 and SEC/HLA-E were plated on day 0 at 37°C in flat-bottom gelatin-coated 96-well plates at a concentration of 1.0 x 10^4 cells/well. Neutrophils (1.0 x 10^5 cells/well) were added to each well with 50 nmol/L Phorbol 12-Myristate 13-Acetate (PMA) on day 1 of experiment 1. Then, 10 µl of the Water-Soluble Tetrazolium-8 (WST-8) solution (NacalaiTesque) was added to each well and the absorbance was measured using a microplate reader (Thermo-Fischer Scientific). The %cytotoxicity was calculated as follows:

%cytotoxicity= (OD (co-culture)-OD (neutrophils or dHL-60))/(OD(SEC)-OD (medium)) x 100 (%) [21].

In experiment 2, the same WST-8 assay was performed after 24 h of coculture without PMA.

Detection of Reactive Oxygen Species (ROS) in neutrophil

ROS levels were quantified after staining with CellROX™ Green Reagent (Thermo-Fischer Scientific). CellROX™ reagent was added to cells at a final concentration of 5 µM, followed by incubation in the dark at 37˚C for 30 min. Subsequently, the stained cells were resuspended in Phosphate-Buffered Saline (PBS) and fluorescence images were evaluated using a FACSVerse™ flow cytometer (BD Biosciences) [16].

NETosis assay by SYTOX green

The SECs were plated in 24 well dishes at a concentration of 4 × 10^4 cells/well. After 24 h of incubation, 4 × 10^5 neutrophils were mixed into individual wells; after 2.5 h of incubation in the presence of 50 nmol/L PMA, the cells were collected by gentle pipetting. The wells were stained with 30 nmol/L SYTOX Green (Thermo-Fischer Scientific) and PE-labeled anti-human CD66 antibodies for 15 min at room temperature. The percent value of NETosis was calculated as (the number of CD66b+SYTOX Green+ cells/the number of CD66b+ neutrophils) x 100 (%), using a FACSVerse™flow cytometer [16].

Statistically analysis

The results of each assay are presented as means and standard errors. Statistical significance was determined using a 2-tailed t test. Statistical significance was set at $p<0.05$.

Results

Expression levels of Immunoglobulin-Like Transcript 2 (ILT-2), ILT-4 and NKG2A on neutrophils

Human peripheral neutrophils were isolated and the expression levels of NKG2A/CD94, ILT-2 and ILT-4 were analysed by flow cytometry. The expression levels of NKG2A, ILT-2 and ILT-4 on neutrophils was 19.6% ± 9.9%, 33.2% ± 21.8% and 65.9% ± 11.6%, respectively **(Figure1).**

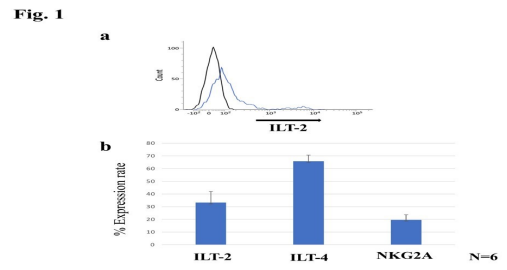

Figure1: Expression of each molecule on neutrophil. Representative FACS data for the expression of ILT-2 on peripheral blood neutrophils is shown; a). FACS data results for ILT-2, ILT-4 and NKG2A on peripheral blood neutrophils are shown in a histogram; b) (n=6).

Expression levels of HLA-G1 and HLA-E in the transfected SECs

The expression levels of HLA class Ib in SECs were verified using flow cytometry. Developed SEC/HLA-G1 and SEC/HLA-E clones exhibited expression levels of 97% and 99% of HLA-G1 and HLA-E, respectively **(Figure 2).**

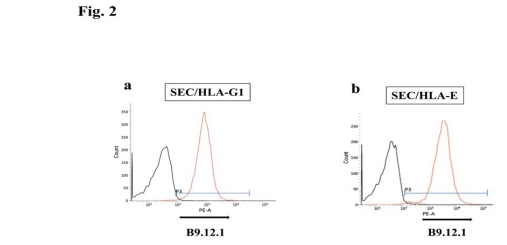

Figure 2: Expression of each molecule on SEC transfectants. Expression of HLA-G1; a) and HLA-E; b) in each SEC line was measured by flow cytometry using antibody B912.1 against HLA class I. Representative FACS data for SEC/HLA-G1 and SEC/HLA-E are shown.

Cytotoxicity assay using peripheral blood neutrophils

Peripheral blood neutrophils were used as effector cells to assess their cytotoxicity against SECs. Transfection with HLA-G1 significantly suppressed cytotoxicity in both experiments, whereas the suppression of cytotoxic activity by HLA-E was not significant, corresponding to the expression rate of NKG2A **(Figure 3).**

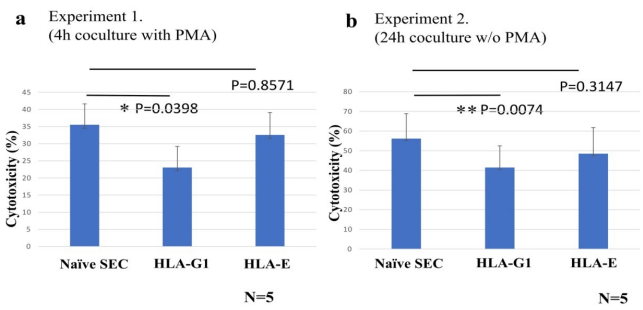

Figure 3: Cytotoxicity of each SEC by peripheral blood neutrophils. Suppression of neutrophil-mediated cytotoxicity by HLA-G1 and HLA-E was calculated in two ways, the 4 h incubation with PMA (experiment 1); a) and the 24 h incubation without PMA (experiment 2); b) by the WST-8 assay. Transfection of HLA-G1 significantly suppressed cytotoxicity in both experiments. (n=5, *: $p<0.05$, **: $p<0.01$).

ROS production in peripheral blood neutrophils

ROS production was measured to examine the inhibitory effects of HLA-G1 and HLA-E on the innate immune responses of neutrophils. Although SEC/HLA-E cells showed a declining trend of ROS production in co-cultured neutrophils, a significant reduction in ROS production was induced by SEC/HLA-G1, indicating that HLA-G1 can inhibit ROS production in neutrophils **(Figure 4).**

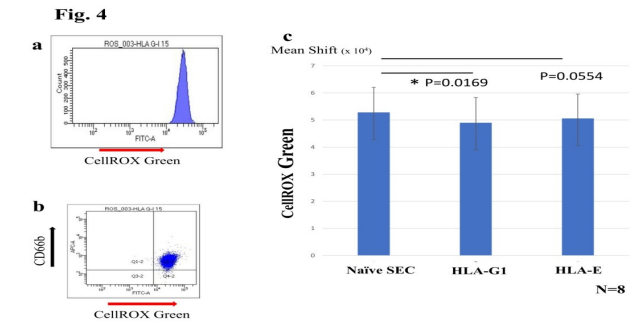

Figure 4: ROS of peripheral blood neutrophils after co-culture. Production of ROS in neutrophil under xenogeneic conditions was measured. Human peripheral blood neutrophils were co-cultured with SEC, SEC/HLA-G1 or SEC/HLA-E for 24 h and stained with CellROX green; a) and PE-labeled anti-CD66a mAb, then displayed in 2D; b). Histogram shows the results of FACS data for CellROX green; c) (n=6, *: $p<0.05$).

NETosis of peripheral blood neutrophils

Peripheral blood neutrophils were stained with SYTOX Green after incubation of 2.5 h with SEC, SEC/HLA-G1, or SEC/HLA-E to assess the influence of HLA-G1 and HLA-E on NETosis. Both HLA-G1 and HLA-E significantly inhibited NETosis **(Figure 5).**

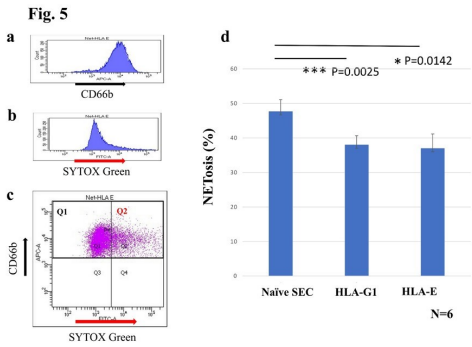

Figure 5: Suppression of NETosis by HLA-G1 and HLA-E. SEC, SEC/HLA-G1, or SEC/HLA-E was cocultured with peripheral blood neutrophils for 2.5 h in the presence of PMA. Cells were stained with PE-labeled anti-CD66b mAb; a) and SYTOX green; b). The percentage of SYTOX Green-positive dead cells among CD66b+ neutrophils is indicated, i.e., the percent of the cells in the region Q2 of the (Q1 plus Q2) region; c). Histogram shows the results of data for NETosis; d) (n=5, *: p<0.05, ***: p<0.005).

Discussion

Neutrophils are characterized by their fast response and extreme phagocytic ability compared with macrophages. In addition, neutrophils release various granules to attack the host, but it is very short-lived and usually replaced every 3-7 days.

Primarily, Fc and complement receptors trigger these reactions in neutrophils. However, the role of neutrophils in xenograft rejection has not been well studied. We previously reported for the first time that neutrophils can recognize and damage SECs, with CD31 and CD177 playing a regulatory role in this response. In addition, we investigated the regulating mechanism of neutrophils [21,22].

Xenotransplantation research began with a discovery of the species specificity of complement and Complement Regulatory Proteins (CRPs) [10]. Next, the focus shifted towards regulating xenogeneic carbohydrate antigens such as α-Gal, which were also implicated in the regulation of NK cells [23].

In the 1980s, the mechanism by which NK cells recognize themselves via HLA molecules was revealed, known as the "missing-self theory" [24]. Subsequently, inhibitory receptors that recognize HLA class I antigens and inhibit their harmful effects were discovered. NK receptors, including Killer Cell Immunoglobulin-Like Receptors (KIRs), Immunoglobulin-Like Receptors (LILRs) and members of the CD94/NKG2 family, Major Histocompatibility Complex (MHC) molecules and help regulate cytotoxic activities of NK cells. In this study, we investigated whether neutrophils have a self-recognition system similar to that of NK cells, focusing on the self-recognition of xenogeneic cells using HLA class Ib and its counter receptor.

The LILR gene family has been studied extensively. ILT-2 and ILT-4 molecules recognize a wide range of HLA class Ia and Ib molecules. Respective ILT-2 and ILT-4 have four and three Immunoreceptor Tyrosine-Based Inhibitory Motifs (ITIM), respectively, in the cytoplasm and transmit inhibitory signals. Many immune cells, including monocytes, macrophages, dendritic cells, B cells, T cells and NK cells, express ILT-2. Monocytes, macrophages and dendritic cells express ILT-4. In addition, the NKG2 family of C-type lectin-like receptors and CD94 form a heterodimer as an NKG2/CD94 molecule that is expressed on NK cells and a subset of T cells [25]. The expression of these molecules on neutrophils has not yet been thoroughly investigated; however, several reports have shown that ILT-2 is expressed at relatively lower and variable levels on neutrophils. In contrast, ILT-4 is highly expressed and localized in the rafts of neutrophils together with CD32a and has been reported to inhibit ROS production and phagocytic functions of neutrophils *via* stimulation of HLA-G1. These factors are associated with the neutrophil status during inflammation [26,27].

We investigated NKG2A, ILT-2 and ILT-4 expression levels on human neutrophils. ILT-2 and NKG2A were expressed at lower levels, but ILT-4 was relatively well expressed. Characteristically, ILT-2 and ILT-4 bind to the α3 and β2-microglobulin (β_2m) domains, which are low polymorphic regions of HLA. In this study, HLA-G1 was transfected into SECs to establish SEC/HLA-G1 cell lines without a hβ_2m domain. ILT-4 binds to the α3 domain side of HLA-G1 rather than hβ_2m. Therefore, it can bind to HLA-G1 even in the absence of hβ_2m. In contrast, ILT-2 is less likely to bind to HLA-G1 in the absence of hβ_2m [28-30]. These findings are consistent with those of this study. These findings correlated with expression levels of receptors on neutrophils. Although SEC/HLA-E cells showed a tendency to control regulation, which was not significant, the cytotoxicity of SEC/HLA-G1 cells was significantly inhibited. Furthermore, the ROS production measurement test results indicated that HLA-G1 significantly reduced ROS production levels. This is believed to be due to the properties of ILT-4. We investigated the suppression of NETosis by HLA-G1 and HLA-E. Results indicated that HLA-G1 exhibits significant inhibitory effects. Furthermore, significant results were obtained for the suppression of NETosis by HLA-E *via* the low expression of NKG2A.

Our results suggest that although the suppression of HLA-E in SECs is modest, the expression of HLA-G1 can suppress neutrophil-mediated xenograft immune responses. Additionally, simultaneous transfection of HLA-G1 and hβ_2m may enhance the expression level of HLA-G1, making the suppressive effect on neutrophils more effective. We have previously reported this in a study involving NK cells [31]. When developing GE-pigs, we believe that introducing the hβ_2m gene simultaneously with HLA-G1, rather than HLA-G1 alone, increases HLA-G1 expression. Furthermore, considering that hβ_2m can also serve as a ligand site for ILT2/4, it is believed that it would be better to introduce hβ_2m simultaneously with HLA-G1.

In conclusion, the expression levels of HLA-G1 in the graft are important, considering the simultaneous regulation of all

innate immune cells, neutrophils, macrophages and NK cells in xenografts [32].

While transplants of GE pig hearts and kidneys have begun, pancreatic islet transplantation remains the most common xenotransplantation to date. In pig pancreatic islet transplants, a phenomenon called the Instant Blood-Mediated Inflammatory Reaction (IBMIR) occurs [33,34]. This reaction involves antibodies, a complement system and coagulation factors, followed by the involvement of macrophages and neutrophils. Additionally, pig pancreatic islet transplants are tissue transplants. Rejection reactions in pancreatic islet transplants must occur similarly to those in the heart and kidney transplants. On the other hand, in red blood cell transplantation, is a cellular transplantation and even in this case, not only macrophages but neutrophils as well may also be involved [35-37].

Conclusion

In all clinical trials to date, neutrophil attacks on grafts that do not express HLA-G1/E have not been discussed. We think that there are many factors behind this. This is because xenoantigens such as such as α-Gal and H-D are knocked out and expressed CRPs are suppressing complement deposition. Furthermore, the expression of CD47 controls not only macrophages but neutrophils as well. In addition, large amounts of immune-suppressants, such as metabolic antagonists, calcineurin antagonists and anticomplement agents, are believed to suppress neutrophils. There are various theories regarding the effect of steroids on neutrophils and it is still unknown at present. In the future, we intend to examine the effects of each immune-suppressant on neutrophils both *in vitro* and *in vivo*.

These results indicate that HLA-G1 effectively regulates the xenogeneic immune responses of neutrophils against SECs in xenotransplantation.

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Conflicts of Interests

The authors declare that they have no competing financial interests.

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