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Human TIGIT on porcine aortic endothelial cells suppresses xenogeneic macrophage-mediated cytotoxicity

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ABSTRACT

Purpose: The delayed rejection caused by strong cell-mediated innate and adaptive xenogeneic immune responses continues to be a major obstacle.

Therefore, suppressing macrophage function could be effective in avoiding this type of rejection. In this study, the suppression of T-cell immunoglobulin and ITIM domain (TIGIT) function against macrophage-mediated xenogeneic rejection was investigated.

Material and methods: Naïve porcine aortic endothelial cell (PAEC) and PAEC transfectant with TIGIT (PAEC/TIGIT) were co-cultured with M1 macrophages, and the degree of cytotoxicity was determined by a counting beads assay. The anti/pro-inflammatory gene expression was determined by RT-PCR and the phosphorylated SHP-1 in the macrophages after co-culturing with PAEC or PAEC/TIGIT was evaluated by western blotting.

Results: CD155 was expressed at essentially equal levels on both M1 and M2 macrophages, whereas TIGIT was highly expressed on M2 macrophages but not in M1 macrophages. TIGIT on PAEC significantly reduced the cytotoxicity of M1 macrophages but no significant suppression of phagocytosis was detected. TIGIT also caused a decrease in the expression of pro-inflammatory cytokines, namely TNFα, IL-1β and IL-12 in M1 macrophages. Furthermore, PAEC/TIGIT caused a significant increase in phosphorylated SHP-1 in M1 macrophages compared to PAEC.

Conclusion: The findings of this study indicate that TIGIT suppresses xenogeneic M1 macrophage-induced cytotoxicity, probably at least in part, via the phosphorylation of SHP-1. In addition, the reduced expression of some pro-inflammatory cytokines, namely TNFα, IL-1β and IL-12, was observed in M1 macrophages that had been cultured with PAEC/TIGIT.

1. Introduction

Xenografts typically show a more severe immune reaction from the recipient than allografts because of species differences. However, with the advent of pigs lacking Gal α 1,3 Gal and overexpressing human complement regulatory proteins, the hurdle of hyperacute rejection has largely been overcome (Ekser et al., 2012; Klymiuk et al., 2009; Miyagawa et al., 2010, 2015). On the other hand, the delayed xenograft rejection caused by strong cell-mediated innate and adaptive xenogeneic immune responses continues to be a major obstacle (Scalea et al., 2012; Schneider and Seebach, 2008). Among such cells, the first focus is on NK cells because xenografts has been believed to be more susceptible to these cells due to the lack of inhibitory signals to human NK cells caused by MHC (SLA) class I incompatibility. It has, in fact, been shown that the ectopic expression of human MHC class I molecules on porcine cells reduces NK cell-induced xenocytotoxicity (Matsunami et al., 2002; Forte et al., 2005; Lilienfeld et al., 2007; Matsunami et al., 2008; Yung et al., 2018; Abicht et al., 2018). However, macrophages are thought to be much more important than NK cells because they are responsible, not only for being an important player in innate immunity and priming T cells as a bridge from innate to adaptive immunity, but also are the primary effector cells in xenograft

Abbreviations: cDNA, complementary deoxyribonucleic acid; PAEC, porcine aortic endothelial cell; GM-CSF, Granulocyte-macrophage colony-stimulating factor; MHC, major histocompatibility complex; SLA, Swine leukocyte antigen; RNA, ribonucleic acid; RT-PCR, reverse transcription - polymerase chain reaction

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rejection (Fox et al., 2001). In fact, some reports have shown that macrophages are dominantly infiltrating in rejected xenografts (Appels et al., 1989; Schwizer et al., 1984), suggesting that they are involved in the pathogenesis of delayed xenograft rejection. Therefore, the suppression of macrophage function would be predicted to be quite effective in terms of avoiding cell-mediated delayed rejection.

Both of these cell-mediated innate and adaptive immunity processes, several receptors related to the activation of these cells contain cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) and the signals induced by ITAM are regulated by other receptors with immunoreceptor tyrosine-based inhibitory motifs (ITIM) (Veillette et al., 2002). However, it would be impossible for wild porcine cells to interact with these inhibitory receptors on human immune cells because they do not produce the ligands that are needed. Consequently, the transgene of human ligands for these inhibitory receptors in pigs represents an excellent strategy to inhibit macrophage-mediated immune responses in pig-to-human xenotransplantation. ITIM-dependent inhibitory receptors and their respective ligands, such as SIRPα-CD47 or the Surfactant protein D (SP-D), actually have been demonstrated to play a role in controlling the phagocytosis that is mediated by macrophages (Barclay and Brown, 2006; Ide et al., 2007; Jiaravuthisan et al., 2018). However, CD47 has also been reported to inhibit eNOS and VEGF activity and to suppress the survival of endothelial cells from cellular stress via binding to TSP-1 (Schwartz et al., 1993; Ramanathan et al., 2011; Wang and Yang, 2012; Martinelli et al., 2013; Sharifi-Sanjani et al., 2014). A recent study by Chen et al. demonstrated that in an allogeneic cardiotransplant model, the elimination of CD47 from donor grafts induced long term survival (Chen et al., 2019). These findings suggest that the effect of CD47 in vascularized xenograft needs to be given more consideration. Furthermore, because other candidates such as HLA-E (Maeda et al., 2013) and G1 (Esquivel et al., 2015) also bind, not only to inhibitory receptors, but also activation receptors, in previous studies gene modification was required to induce a greater inhibitory function (Matsunami et al., 2002, 2006). These findings indicate that the appearance of a new candidate is an important subject in terms of suppressing macrophage-mediated xenogeneic rejection.

The T-cell immunoglobulin and ITIM domain, TIGIT, was first reported to inhibit the activation of NK cell and T cell as an immune checkpoint (Stanietsky et al., 2009; Yu et al., 2009). TIGIT consists of four domains, as follows: an IgV domain, a transmembrane domain, an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an Ig tail-tyrosine (ITT)-like motif (Levin et al., 2011; Yu et al., 2009), and has a competitive relationship with the immune activation receptor CD226 or the DNAX accessory molecule-1 (DNAM-1) for CD155 (PVR or the poliovirus receptor) (Zhang et al., 2016). Trans interactions of TIGIT with CD155 induce the suppressive downstream signaling of both TIGIT and CD155 (Stengel et al., 2012). TIGIT can compete CD226 for binding to CD155 (Fig. 1), thus TIGIT is allowed to induce an inhibitory signal in TIGIT-expressing cells by disrupting CD226 co-stimulation, while also triggering another inhibitory signal in opponent cells via CD155, which also has an ITIM domain in itself (Dougall et al., 2017). Chauvin et al. reported that the expression of TIGIT in tumor antigen-specific $CD8^+$ T cells is upregulated and tumor infiltrating $CD8^+$ T cells ($CD8^+$TILs) that are involved in adaptive immune tumor surveillance (Chauvin et al., 2015). In addition, TIGIT has been reported to express on a subset of $Foxp3^+$Treg cells in human lymphocytes (Yu et al., 2009) and TIGIT$^+$Tregs co-express other immunosuppressive genes, including CTLA-4 and PD-1(Bottino et al., 2003). All the facts mentioned above indicate that TIGIT should be able to serve as a break for the immune system.

Given the fact that TIGIT suppresses activated acquired immunity and also influences innate immunity including NK cells (Stanietsky et al., 2009), it is possible that activated macrophages might be suppressed as well. However, another new strong candidate with the ability to suppress not only T cells and NK cells, but also macrophages have not been identified. This motivated us to investigate whether the suppressive function of TIGIT exerts a similar immunomodulatory function on macrophages in an *in vitro* pig-to-human xenograft model.

2. Materials & methods

2.1. Cells

A porcine aortic endothelial cell (PAEC) line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

2.2. Plasmid construction

cDNA for human TIGIT without its intracellular inhibitory motif (Gene Bank No. EU675310.1) was synthesized by Japan Integrated DNA Technologies (Tokyo, Japan). The construct was cloned into the pCXN2L expression vector, followed by checking by an ABI 310 sequencer (Applied Biosystems, Waltham, MA, USA). The plasmid was amplified and isolated using standard techniques after transformation into *Escherichia coli*Dh5α.

2.3. Preparation of PAEC transfectant by lipofection

Five microgram plasmid was mixed with 40 μl Lipofectamine 2000™ reagent (Thermo Fisher Scientific, Tokyo, Japan) and introduced into PAEC. The PAEC transfectants were cultured in DMEM complete medium for 24 h in 5% CO_2 at 37 °C. The cells were selected by culturing in complete medium with 800 μg/mL G418 (Thermo Fisher Scientific, Tokyo, Japan). The PAEC expressing TIGIT (PAEC/TIGIT) used in this study was isolated by magnetic beads sorting after G418 selection.

2.4. Generation of M1 and M2 macrophages

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors using lymphocyte isolation solution (Nakarai Tesque, Kyoto, Japan). Human monocytes were isolated by a plastic adherence method. To generate M1 macrophages, human blood monocytes were cultured in the presence of 100 ng/ml rGM-CSF (Peprotech, Rocky Hill, NJ, USA). Five days after, cells were harvested and purified with negative selection using Pan Monocyte Isolation Kit, human (Miltenyi Biotec Inc., Bergisch Gladbach, Germany). The negative cells were used as M1 macrophages. M2 macrophages were developed by culturing in the presence of 100 ng/ml rM-CSF (R&D system, Minneapolis, MN) for 5 days. To confirm the differentiation of macrophages, the fluorometric signals were detected by flow cytometry after staining of macrophages with APC-labeled anti-human CD14 (BioLegend, San Diego, CA) and PE-labeled anti-human HLA-DR antibodies (BioLegend, San Diego, CA).

2.5. Assessment of cell proliferation by a WST-8 assay

PAEC and PAEC transfectants (PAEC/TIGIT) were plated at a concentration of 1.0×10^4/well in a 96-well plate. After 48 h, 10 μl WST- 8 reagent solution (Nakalai Tesque, Kyoto, Japan) was added to each well, and absorbance was measured at 450 nm using a microplate reader (Thermo Fischer Scientific, Tokyo, Japan).

2.6. Counting beads assay/phagocytosis assay and anti-CD155 blocking assay

To confirm the expression of transfected molecules, PAEC transfectants were stained with PerCP/Cyanine5.5 labeled anti-human TIGIT (clone: VSTM3) antibody (BioLegend, San Diego, CA) for 30 min. at 4 °C. The expression of human TIGIT and of CD155 (PE labeled anti-CD155 antibody (Thermo Fisher Scientific, Tokyo, Japan)) on human

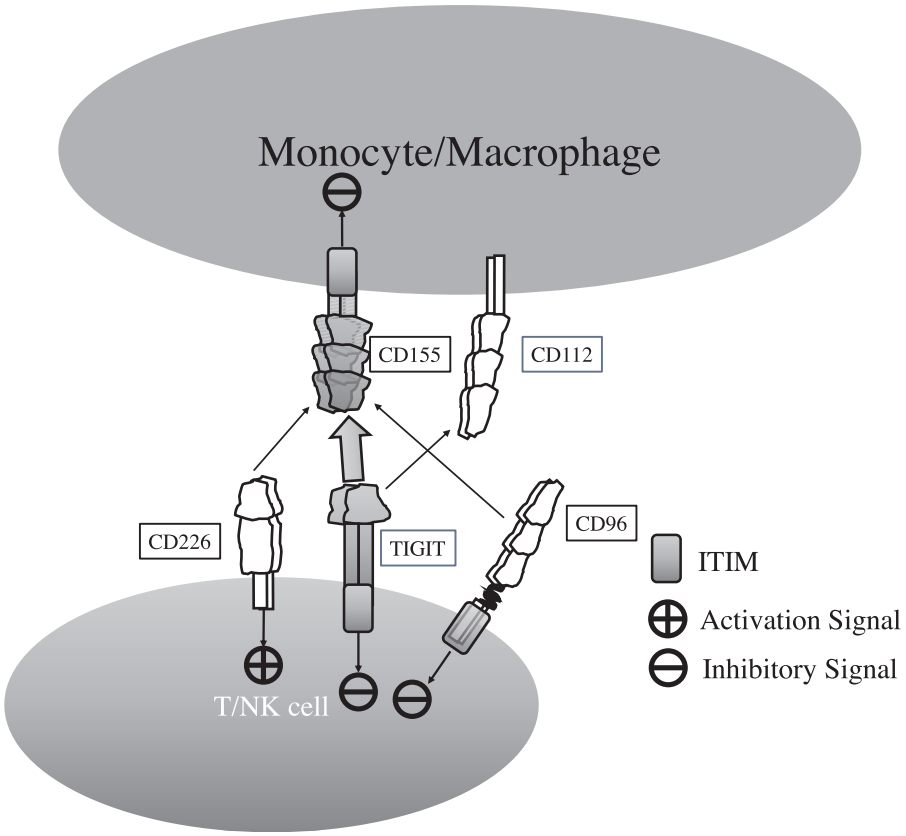


Fig. 1. TIGIT-CD155 inhibitory signal between T/NK cell and monocyte/macrophage. TIGIT on activated T cells and NK cells induces an inhibitory signal via binding to CD155 on monocytes and macrophages. CD226 and CD96 compete with TIGIT for the binding to CD155 and CD112 competes with CD155 for the binding to TIGIT. CD155-CD226 signaling induces the activation signal on NK/T cell. TIGIT-CD155 signaling and CD96-CD155 signaling induce the inhibitory signal both on NK/T cell and monocyte/macrophage. TIGIT has a significantly higher affinity for CD155 than CD226 and CD96. CD155 also has a significantly higher affinity for TIGIT than CD112.

macrophages were evaluated in the same manner.

For the counting beads assay, PAEC and PAEC transfectants were labeled with calcein AM (Nakalai Tesque, Kyoto, Japan) for 10 min. at 37 °C. Forty thousand cells/well of calcein AM-stained PAEC or PAEC transfectants were seeded in 24-well plates. After 24 h, 1.6×10^6 macrophages were added and co-cultured for 48 h. The cells were harvested and stained with APC labeled anti-human CD14 (BioLegend, San Diego, CA) and PerCP/Cy5.5 labeled anti-human TIGIT antibodies. The counting beads assay was conducted after 25 μL of counting beads (Thermo Fisher Scientific, Tokyo, Japan) were added to each sample. The relative cell numbers of PAEC or PAEC transfectants and of macrophages corresponding to 1500 beads were measured by flowcytometery. % Phagocytosis was calculated as ($CD14^+$Calcein AM^+cells)/($CD14^+$cells) × 100 (%).

For the blocking assay, macrophages were processed with an anti-CD155 antibody (BioLegend, San Diego, CA) or isotype IgG (normal mouse IgG2a) (SANTA CRUZ Biotechnology, Dallas, Texas, USA) before being co-cultured with PAEC or PAEC transfectants. They were then co-cultured and their cytotoxicity was assessed by an assay based on counting the number of beads, as described.

A FACS CantoII(BD Biosciences, San Diego, CA, USA) was used to achieve the experiments.

2.7. Quantitative PCR

Pan-monocytes were isolated by magnetic beads sorting using negative selection for pan-monocytes explained above after co-culture with PAEC or PAEC transfectants. After extraction of total RNA using TRIZOL reagent (Thermo Fisher Scientific, Tokyo, Japan), the expression of specific mRNA was amplified with an One-Step SYBR®Prime SCript®RT-PCR kit (Takara Bio Inc., Otsu, Japan) and quantified using a Smart CyclerII(Takara Bio Inc.). The primers were purchased from Thermo Fisher Scientific. The sequences of the primers were shown in Table 1.

The mRNA expression levels of specific genes are expressed as [the specific mRNA expression in macrophages co-cultured with PAEC/TIGIT]/[the specific mRNA expression in macrophages co-cultured with PAEC].

2.8. Western blotting

Two hundred thousand cells/well of calcein AM-labeled PAEC or PAEC/TIGIT were seeded in 6-well plates. After 24 h, 2.0×10^6 macrophages were co-cultured with PAEC or PAEC/TIGIT for 24 h. The resulting cells were harvested, suspended in 100 μL RIPA buffer, homogenized by sonication with a handy ultrasonic disruptor UR- 20 P (TOMY SEIKO Co.,LTD, Tokyo, Japan) and incubated on ice for 30 min. for western blotting. The protein samples were heated at 95 °C for 5 min for denaturing and loaded in 10% SDS-PAGE. After loading, the gel was transferred to a PVDF membrane (Immobilon® P; Millipore, Billerica, MA, USA) and incubated in TBS/0.05% Tween 20 (TBST) with 3% bovine serum albmin (BSA) for 1 h at room temperature. After washing with TBST, the membrane was stained with an anti- phosphorylated Src homology 2 (SH-2)-domain-containing phosphatase 1 (SHP- 1) rabbit mAb (clone D11G5; Cell Signaling Technology Japan, Tokyo, Japan) for 1 h at room temperature. The blot was stained with HRP-labeled anti-rabbit IgG after washing with TBST. The signal development was conducted with an ImmunoStar® Zeta (WAKO, Osaka, Japan) and the signal was detected by LAS4000 mini (GE Healthcare, Little Chalfont, GB). Western blotting with anti-GAPDH (Proteintech, Tokyo, Japan) was used as the reference. Signal intensity of all specific bands was quantified using Image *J*.

2.9. Statistical analysis

The data described above were compared with student's *t*-test as continuous values. The threshold for significance was $p < 0.05$. For statistical analysis, R (The R Foundation for Statistical Computing,

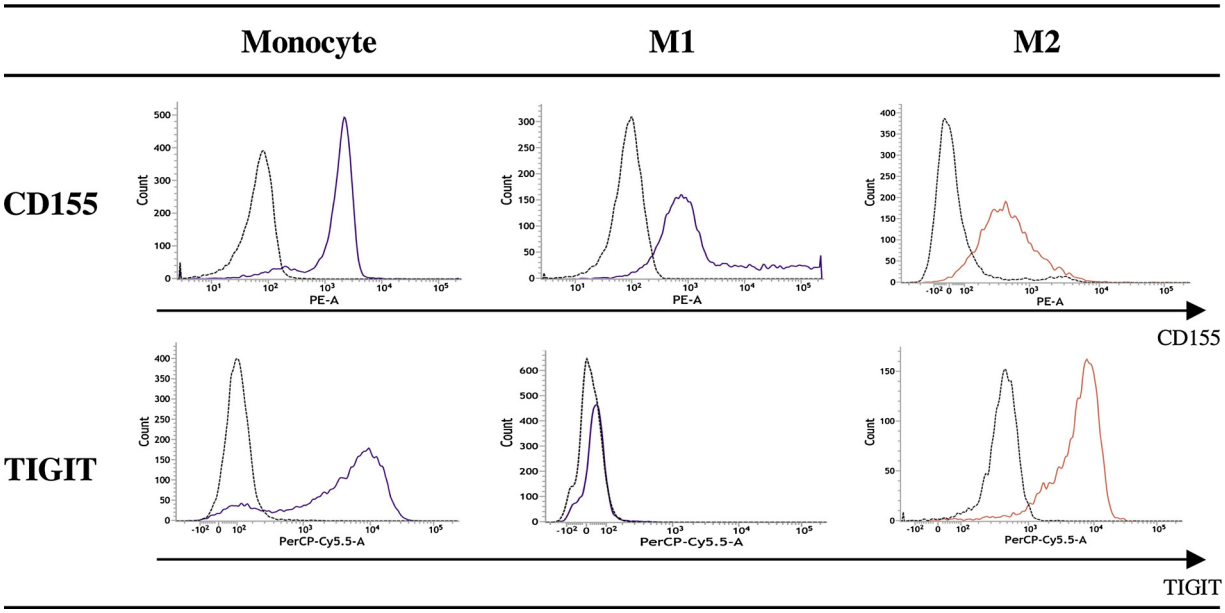


Fig. 2. Expression of an inhibitory receptor for TIGIT, CD155 on monocytes, M1 and M2 macrophages. Histograms show the expression of CD155 and TIGIT on monocytes, M1 and M2 macrophages. M1 and M2 macrophages were generated from peripheral blood monocytes by culturing with 100 ng/ml GM-CSF or 100 ng/ml M-CSF, respectively.

Vienna, Austria) was used.

3. Results

3.1. The expression of CD155 & TIGIT on macrophages

We first investigated the expression of CD155 and TIGIT on undifferentiated monocytes, M1 and M2 macrophages. While a significant expression of CD155 and TIGIT was detected on these monocytes, CD155 was expressed on both M1 and M2 macrophages equally, whereas TIGIT was expressed more highly on M2 macrophages compared to M1 macrophages (Fig. 2). This suggests that TIGIT was able to suppress the function of M1 macrophages via interacting with CD155 on them.

3.2. Human TIGIT on PAEC suppressed human macrophage-mediated xenocytotoxicity, whereas the phagocytosis of macrophages was not suppressed

We next introduced human TIGIT molecules into PAEC cells by a lipofection method (Fig. 3(a)). In order to evaluate the cytotoxicity of macrophages against PAEC cells, M1 macrophages were used as effector cells (Fig. 3(b)). After co-culturing PAEC cells (PAEC) or TIGIT-expressing PAEC cells (PAEC/TIGIT) with the effector cells, we evaluated the cytotoxicity of the effector cells by a counting beads assay. Before evaluating the results, we first confirmed that the ability to proliferate was equal between PAEC and PAEC/TIGIT (Fig. 3(c)). As a result of the counting beads assay, we found that PAEC/TIGIT was significantly more alive than PAEC(55.1% v.s. 17.1%; $p < 0.005$ ($n = 7$)) (Fig. 3**(d) and (e)**). However, no significant suppression in phagocytosis by macrophages was observed in the case of PAEC/TIGIT (Fig 3 (d) and (f)).

We next investigated the effect of CD155 on these cytotoxicities by blocking them with an anti-CD155 antibody. Non-processed macrophages and macrophages processed with isotype IgG showed a reduced cytotoxicity against PAEC/TIGIT (23.2% and 31.6%, respectively; not significant ($n = 5$)), whereas macrophages processed with the anti-CD155 antibody showed no inhibitory effects on PAEC/TIGIT (71.2% v.s. 23.2% (non-processed macrophages) and 31.6% (processed with isotype IgG); $p < 0.05$ ($n = 5$)) (Fig. 4(a) and (b)) and showed the same or a substantially greater cytotoxicity than those of non-processed macrophages against PAEC (Fig. 3(d) and (e)).

3.3. Human TIGIT on PAEC increased the expression of anti-inflammatory cytokines and decreased those of pro-inflammatory cytokines

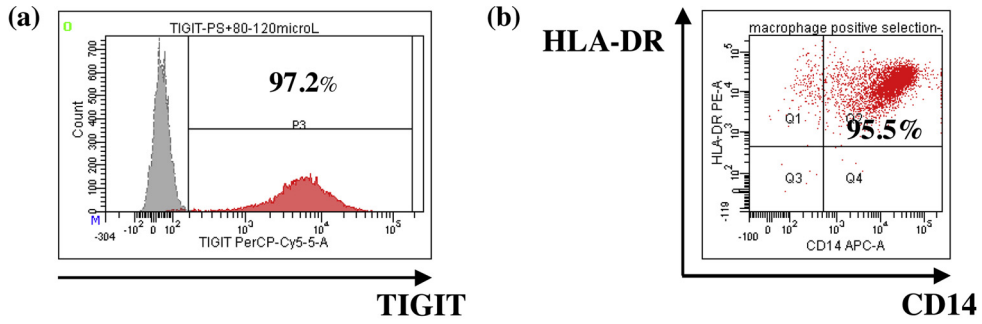
We then evaluated the expression of several genes related to the regulation of inflammation, such as ARG-1, IL-10 and TGFβ for anti-inflammatory cytokines, iNOS, TNFα, IL-1β and IL-12 for pro-inflammatory cytokines. A significant suppression of pro-inflammatory cytokines, such as TNFα, IL-1β and IL-12 was observed in macrophages cultured with PAEC/TIGIT (Figure 5).

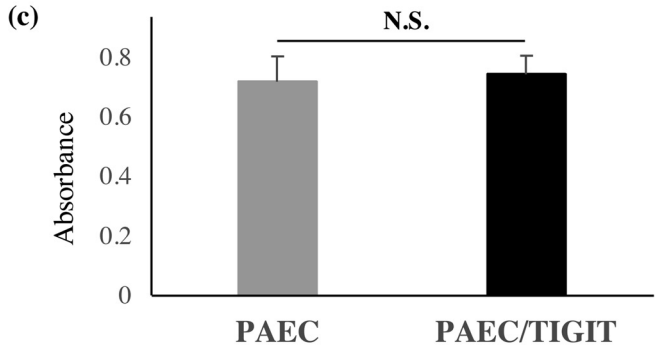
3.4. Human TIGIT on PAEC caused the phosphorylation of SHP-1 in human macrophages to be enhanced as an inhibition pathway

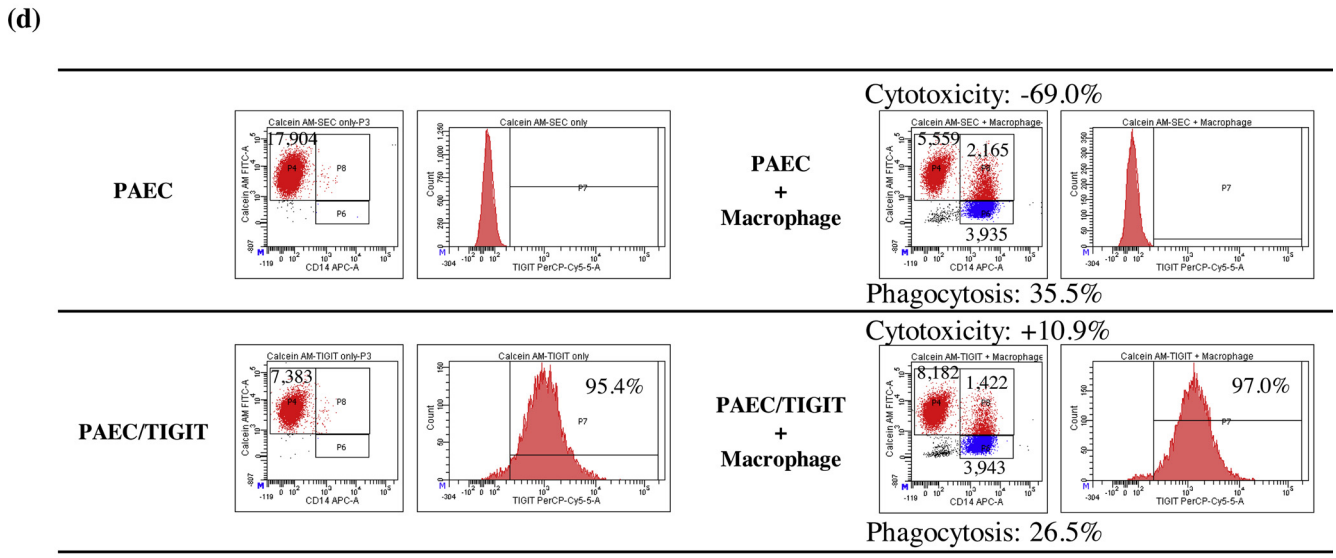
Finally, to investigate whether human TIGIT on SEC induce the inhibitory signal in macrophages, we determined the extent of phosphorylation of SHP-1 because it has been reported that the phosphorylation of ITIM in ITIM-containing receptors triggers the recruitment of SHP-1 to the phosphorylated tyrosine within the motif (Coggeshall et al., 2002) . We found that M1 macrophages cultured with PAEC/TIGIT increased the degree of phosphorylated SHP-1 compared to those cultured with PAEC (Fig. 6(a)). On the other hand, TIGIT failed to induce the phosphorylation of SHP-1 in macrophages cultured in a transwell plate, suggesting that the induction of SHP-1 phosphorylation is dependent on the TIGIT on PAEC and CD155 on macrophages coming into contact. Furthermore, quantitative analyses indicated that, TIGIT-stimulated macrophages had approximately a 5-fold increase in phosphorylated SHP-1 compared to the PAEC-stimulated ones (Fig. 6(b)).

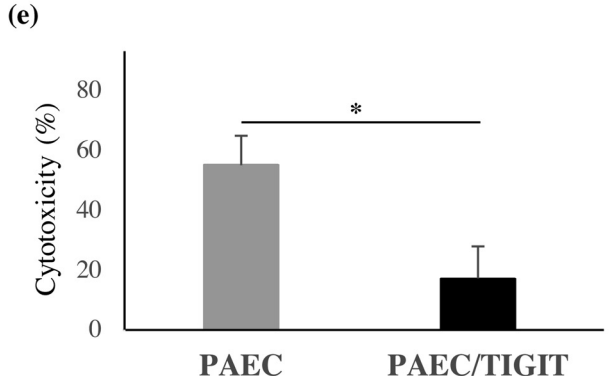
4. Discussion

In xenogeneic conditions, macrophages are activated in both an antibody and a complement dependent and independent manner (Ide et al., 2007; Yang and Sykes, 2007). Because we did not use human serum in culturing the macrophage and PAEC co-culture, it was possible to assess antibody independent cellular cytotoxicity in this study.









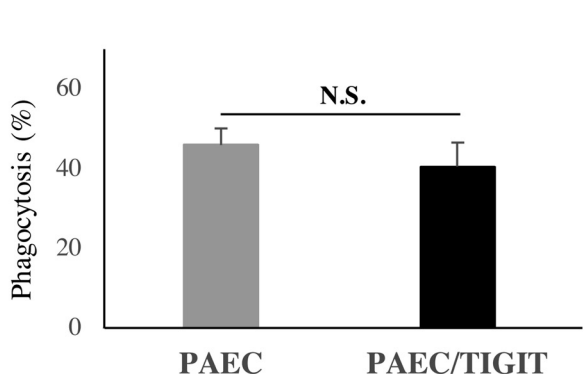


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Fig. 3. Evaluation of human macrophage-mediated cytotoxicity by a counting beads assay. In the counting beads assay, 4.0×10^4 cells/well of calcein AM-stained PAEC or PAEC/TIGIT were co-cultured with 1.6×10^5 cells/well of M1 macrophages at 37 °C for 48 h. Whole cells were stained with PerCP/Cyanine5.5 labeled anti-human TIGIT and APC-labeled anti-CD14 antibodies, and analyzed by flow cytometry. (a) The expression of human TIGIT in PAEC transfectants (PAEC/TIGIT) was evaluated by flow cytometry. Data are representative of more than five different experiments. (b) Dot plots shows the successful magnetic separation of $CD14^+HLA\text{-}DR^+$ macrophages. Data are representative of more than five different experiments. (c) comparison of cell proliferation between PAEC and PAEC/TIGIT cells after 48 h assessed by WST-8 assay (n = 4). (d) The cytotoxicity and phagocytosis for PAEC were calculated using the counting beads assay, Data are representative of seven different experiments. (e, f) Cytotoxicity and phagocytosis assessed by a counting beads assay are represented by histogram. Data represent mean ± SEM (n = 7; * $p < 0.005$).

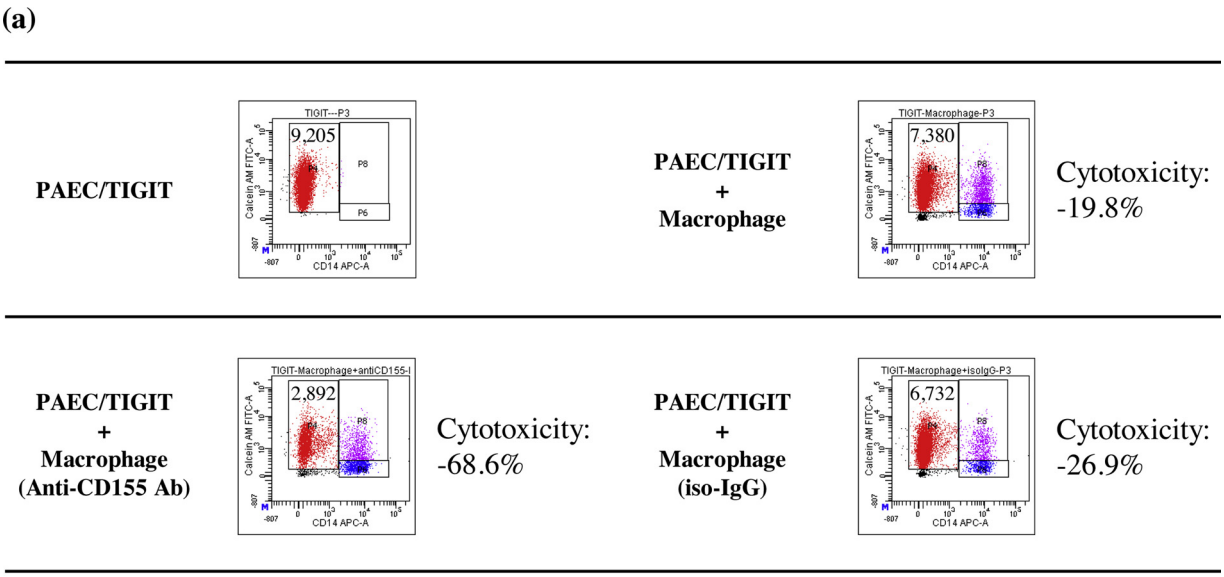

Fig. 4. Blocking assay with anti-CD155 antibody in PAEC/TIGIT.
In this blocking assay, 1.6×10^5 cells/well of M1 macrophages were processed with an anti-CD155 antibody or isotype IgG at room temperature for 1 h. These processed macrophages (4.0×10^4 cells/well_ were then cultured with calcein AM-stained PAEC or PAEC/TIGIT at 37 °C for 48 h. Whole cells were stained with PerCP/Cyanine5.5 labeled anti-human TIGIT and APC-labeled anti-CD14 antibodies, and analyzed by flow cytometry. (a) The cytotoxicity for PAEC were calculated using the counting beads assay, Data are representative of five different experiments. (b) Cytotoxicity assessed by a counting beads assay are represented by histogram. Data represent mean ± SEM (n = 5; * $p < 0.05$).

Therefore, macrophages in our system were thought to be mainly activated by DAMPs (Hu et al., 2011) and alpha1,3Gal-Galectin-3 signal (Peterson et al., 2005; Jin et al., 2006). Numerous studies reported that Human CD47 suppresses not only in vitro but also in vivo and ex vivo macrophage-mediated xenogeneic rejection (Barclay and Brown, 2006; Ide et al., 2007; Teraoka et al., 2013; Navarro-Alvarez and Yang, 2014; Tena et al., 2014). While CD47 induces many beneficial effects in macrophages, CD47 has been reported to be harmful in endothelial cells. In the future, a careful examination of the effect of CD47 in vascularized transplantation should be done. In this study, we found that TIGIT is expressed only on M2 macrophages, while CD155 is expressed on both M1 as well as M2 macrophages after differentiation. Based on previous studies reporting that TIGIT expression is observed on the surfaces of activated T cells and on the surfaces of $Foxp3^+$Tregs (Bottino et al., 2003; Chauvin et al., 2015; Yu et al., 2009), it is reasonable to conclude that M2 macrophages, which are known to preferably have an inhibitory function among pan-monocytes, express more TIGIT than M1 macrophages, which would serve to function as

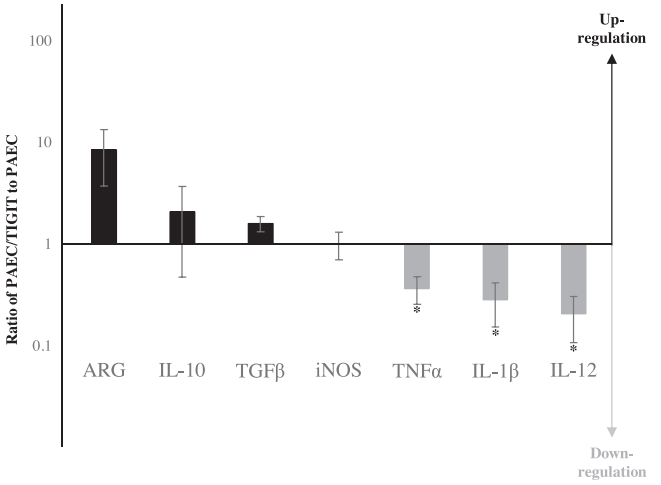

Fig. 5. Induction of gene expressions by TIGIT in macrophages.
Forty thousand PAECs or PAEC/TIGITs were co-cultured with 1.6×10^5 M1 macrophages for 24 h. The resulting cells were harvested by pipetting, processed as described in the Materials and Methods section to obtain the mRNA of target genes and analyzed. The histogram shows the ratio of gene expressions (ARG-1, IL-10, TGF-β, iNOS, TNFα, IL-1β and IL-12) in PAEC/TIGIT to those in PAEC (n = 7–8, * $p < 0.001$).

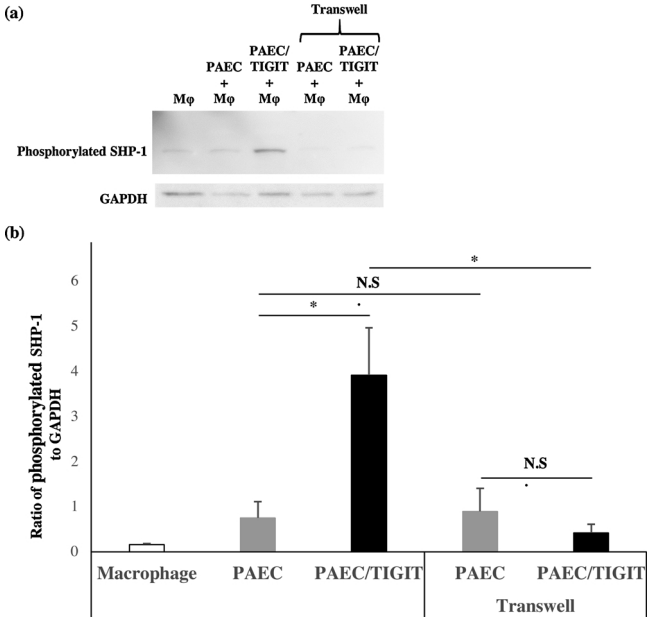

Fig. 6. Evaluation of the phosphorylation of SHP-1 in human macrophages by western blotting.
For western blotting, 2.0×10^5 PAEC or PAEC/TIGIT were cocultured with 2.0×10^6 macrophages in 24 well plates or 24 trans well plates. After 24 h of co-culturing, the resulting cells were harvested by pipetting, processed as described in materials and methods to obtain target proteins and analyzed. (a) The phosphorylation of SHP-1 in macrophages after co-culturing with PAEC or PAEC/TIGIT was evaluated by western blotting. Data are representative of five different experiments. (b) The ratio of phosphorylated SHP-1 to GAPDH is represented in a histogram. Data represent the mean ± SEM (n = 4–5; * $p < 0.05$).

inhibitors to prevent activated immune cells from overactivation via the ligation to CD155 like Foxp3$^+$Tregs. Indeed, it has been reported that TIGIT has ITIM and Immunoreceptor Tyrosine Tail (ITT)-like motifs within its sequence and that TIGIT stimulation induces inhibitory signals via these motifs in TIGIT-expressing cells (Levin et al., 2011; Stengel et al., 2012; Yu et al., 2009). However, in this study we introduced TIGIT without these inhibitory motifs, such as ITIM and ITT-like motifs, into PAEC. Therefore, it was not necessary to take into consideration the potential inhibitory signals toward TIGIT-introduced PAEC. On the other hand, in M1 macrophages no autologous binding of TIGIT to CD155 was induced, as evidenced by the fact that no TIGIT expression was detected (Fig. 2). Thereafter, TIGIT-expressing PAEC (PAEC/TIGIT) would suppress M1 macrophages via interactions between the two molecules.

In addition to CD155, TIGIT has also been reported to bind to another molecule, i.e., CD112 (Fig. 1). However, the affinity of TIGIT for CD155 is considerably higher than the affinity for CD112 (Yu et al., 2009; Zhu et al., 2016). In addition, CD155 can also interact with another molecule, CD96, except for CD226 and TIGIT (Fig. 1), but TIGIT has a far higher affinity for CD155 than CD96 or CD226 as well (Chan et al., 2014). Thus, TIGIT is thought to be able to dominantly interact with CD155 and induce negative signaling in CD155-expressing cells. To confirm the interaction between TIGIT and CD155 in this study, we investigated the TIGIT-induced phosphorylation of SHP-1 in macrophages. CD155 has an ITIM motif within the cytoplasmic tail (Dougall et al., 2017) and it is known that ITIM interacts with SHP-1 and phosphorylates it when stimulated (Coggeshall et al., 2002). Therefore, TIGIT suppresses xenogeneic M1 macrophage-induced cytotoxicity, probably at least in part via the phosphorylation of SHP-1. In our study, the phosphorylation of SHP-1 was significantly enhanced in macrophages by co-culturing them with PAEC/TIGIT, whereas phosphorylation was not observed in the absence of contact between both cells, indicating that TIGIT directly interacts with CD155 molecules on macrophages and induces an inhibitory signal via the phosphorylation ITIM. In addition to this, we also investigated what would happen when CD155 was blocked with a blocking antibody. The results indicated that CD155-blocked macrophages were not suppressed by the expression of TIGIT on PAEC, indicating that TIGIT-CD155 binding is required for a TIGIT-induced inhibitory effect on macrophages.

We also examined the issue of how TIGIT suppresses macrophage-mediated cytotoxicity using PCR. The results showed that TIGIT decreased the expression of pro-inflammatory genes, such as TNFα, IL-1β and IL-12. TIGIT also had a tendency to increase the expression of anti-inflammatory genes, although we were not able to show a significant difference in this study. The suppression of IL-1β expression was similar to our previous report regarding the effect of SP-D on macrophages (Jiaravuthisan et al., 2018).

In conclusion, the findings reported herein indicate that TIGIT suppresses macrophage-mediated cytotoxicity, probably at least in part via the phosphorylation of SHP-1 (Fig. 7) and that when macrophages interact with TIGIT the expression of some pro-inflammatory genes, such as TNFα, IL-1β and IL-12 is reduced. Furthermore, detailed investigations will need to be conducted, including studies of the molecular mechanism in the future.

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Authorship

YN and AM: Research design, Implementation of the research, Data analysis, and Manuscript writing.
PL, CT and TH: Implementation of the research, Data analysis.
TK, TY, CT and YT: Data collection and analysis.
HO and SM: Critical editing and Approval of the manuscript.

Declaration of Competing Interest

The authors have nothing to disclose.

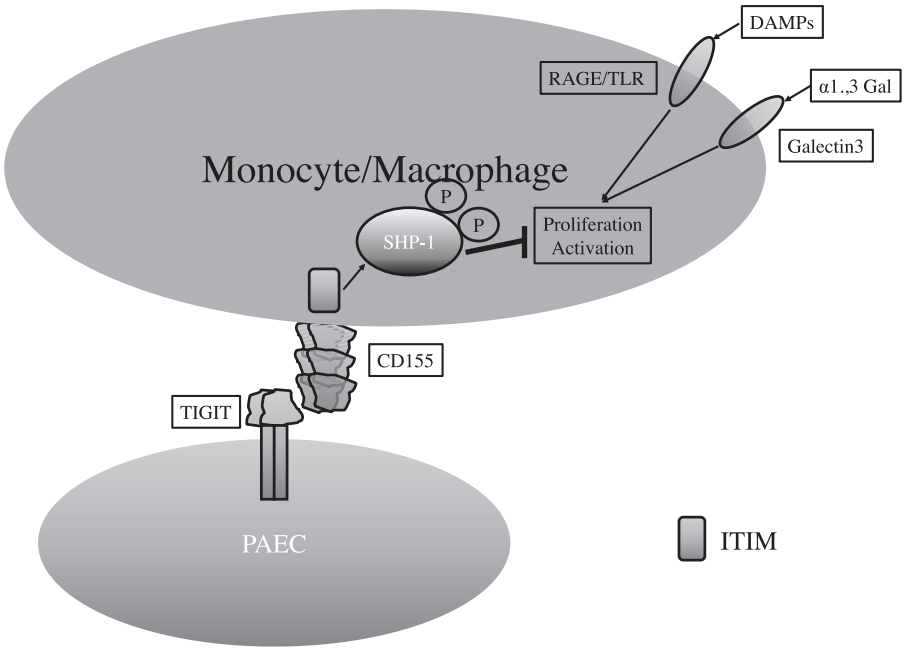

Fig. 7. The suppression of M1 macrophages by human TIGIT on PAEC.
Human TIGIT on PAEC induces ITIM phosphorylation in the cytoplasmic domain of CD155, followed by the SHP-1 phosphorylation. The phosphorylated SHP-1 suppresses the cell proliferation and activation of macrophages which were activated in xenogeneic conditions.

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