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Human cytomegalovirus UL18 alleviated human NK-mediated swine endothelial cell lysis

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Abstract

Human cytomegalovirus UL18, a MHC class I homologue, is known to serve as a natural killer cell (NK) decoy and to ligate NK inhibitory receptors to prevent lysis of an infected target cell. To explore whether the cell surface expression of UL18 represents a potential immune suppressive approach to evade NK-mediated cytotoxicity in the prevention of xenograft rejection, we examined the effect of the UL18 expression in vitro upon human NK-mediated cytotoxicity against swine endothelial cells (SECs). UL18 expression on SECs by a retroviral vector (PLNCX2) significantly suppressed NK-mediated SEC lysis by approximately 25–100%. The protective effect of UL18 could be mediated through ILT-2 inhibitory receptor on NKs. Additionally, the interaction between UL18 and NKs resulted in the significant reduction of IFN-γ production. This study demonstrates that UL18 can serve as an effective tool for the evasion of NK-mediated cytotoxicity and for the inhibition of IFN-γ production during xenograft rejection.

Keywords: Human cytomegalovirus; UL18; Natural killer cell; IFN-γ; Xenograft rejection; Swine endothelial cell; ILT-2; YT cell line

Human cytomegalovirus (HCMV) has evolved a number of strategies to evade host immune response. These include the down-regulation of class I molecule on the surface of host cells to escape cytotoxic T cell recognition by utilizing the unique short region protein (US), US2, US3, US6, and US11 [1–6]. In theory, a lack of MHC-1 surface expression on the target cell might trigger focused lysis of that target cell and initiate the secretion of many inflammatory cytokines by NK cells. Strikingly, HCMV encodes a β2m-binding class I MHC heavy chain homologue gpUL18 [7], which it is believed acts as a decoy to block NK cell cytotoxicity in the absence of host class I molecules [8]. Moreover, it has been reported that the HLA-A, B, and C-deficient human B cell line, 721.221, expressing HCMV UL18 reduces susceptibility to various NK cell lines [9]. Increasing evidence on the possible role of UL18 indicates that UL18 binds CD85/LIR-1/ILT-2 molecule [10], an inhibitory receptor ubiquitously expressed on B lymphocytes, monocytes, dendritic cells, on a subset of NK cells, and on T lymphocytes [11]. Thus, the possibility exists that the ILT-2 receptor can provide a global inhibitory signal to multiple components of the host's immune system [12,13].

Swine has been considered as an ideal source of donor organs for xenotransplantation, primarily because of their size, their plentiful supply, and their physiological and anatomical comparabilities with human organs [14]. However, vigorous humoral and cellular immune responses to xenografts severely limit the clinical applications of xenotransplantion. Our

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understanding of the early rejection process, namely hyperacute and acute vascular rejection, which is elicited by xenoantibodies and subsequently activated complements, has led to significant progress in the prevention of humoral immune responses against porcine xenografts [15]. Of the available techniques, the depletion of xenoreactive natural antibodies (XNAs) by immunoadsorption, (over)expression of human complement-regulatory molecules (CD46, CD55, and CD59), and the elimination of the 1,3-galactosyltransferase gene by gene targeting of somatic cells or the nuclear transfer technique are probably the most effective and promising [16–19].

This advance has also increased interest in the nature of vigorous cellular immune response to xenoantigens. Previous studies have shown that large numbers of natural killer (NK) cells and macrophages infiltrate xenografts, and play important roles in the initial stage of human cellular immune response against porcine endothelial cells [20,21]. In addition, IFN-γ is mainly produced by NK cells, not by $CD4^+$ or $CD8^+$ T lymphocytes [22]. IFN-γ secreted by NK cells can also significantly influence specific xenoreactive T-cell immune response [22]. For this reason, it appears crucial that the xenografted endothelium be protected from NK-mediated damage.

Materials and methods

Cell lines and cell cultures. A swine endothelial cell (SEC) line (MYP30) and a retrovirus packaging cell line (PT67) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). An NK-like cell line (YT), which was kindly provided by Dr. Junji Yodoi (Kyoto University, Japan), was cultured in 80% Iscove's MDM supplemented with 20% FBS. Both media were supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, 100 μM non-essential amino acids (Gibco-BRL, Grand Island, USA), and 55 μM β-mercaptoethanol. Cultures were maintained in a 5% CO_2, 95% air atmosphere at 37 °C.

Genes and construction of stable cell line. UL18 cDNA and human β2m cDNA were kindly provided by Dr. Kwangseog Ahn (Korea University, Korea). To establish stable cell lines expressing UL18, hβ2m or HLA-G1, we cloned UL18 cDNA into pLNCX2 (Clontech, CA, USA), a retroviral expression vector, hβ2m cDNA into pLHCX (Clontech), and HLA-G1 into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Recombinant UL18-pLNCX2 and hβ2m-pLHCX were transfected into PT67 cells by using the calcium phosphate precipitation method, to produce high titer retrovirus. Supernatants collected after 48 h of culture were used to infect SECs to produce a stable cell line expressing UL18 or hβ2m. G418 (650 μg/ml, Gibco-BRL) and hygromycin (125 μg/ml, Invitrogen) were used to select stable SEC transfectants.

Flow cytometry and antibodies. Flow cytometric analysis was carried out by standard protocols using a FACS Calibur running CELLQUEST software (Becton–Dickinson, Mountain View, CA, USA). Monoclonal antibody (mAb) 10C7 (American Type Culture Collection) was used as anti-UL18 monoclonal antibody. mAb N-19 (Santa Cruz Biotechnology, Santa Cruz, CA) is specific for human β-2 Microglobulin. mAb VMP55 (Santa Cruz Biotechnology) was used to detect the CD85(ILT-2) molecule on the YT cell line. Normal mouse IgG1 (κ; Sigma–Aldrich, St. Louis, MO, USA) was used as an isotype control. Transfected cells (1×10^6) were incubated with mAb 10C7, mAb N19 or mAb VMP55 for 30 min at 4 °C to check the expression of each molecule, and then with FITC-labeled anti-mouse IgG (Fab specific; Sigma) for mAb 10C7 and mAb VMP55, or with PE-labeled anti goat IgG (Santa Cruz Biotechnology) for mAb N19, for 45 min at 4 °C. The cells were then washed twice with FACS buffer (PBS containing 1% BSA and 0.01% sodium azide) and analyzed.

NK cytotoxicity assay. Cytotoxicity was measured by ^{51}Cr-release assay, as previously described [23]. Naive SECs or transfected SECs were cultured at 1×10^4 cells/well in a flat-bottomed, 1% gelatin-coated 96-well tray for about 15 h. These were then labeled with 100 μCi/ml $Na_2{}^{51}CrO_4$ (Perkin–Elmer Life Science, Boston, MA, USA), by incubating for 2 h at 37 °C.

During this incubation, fresh NK cells were prepared from the peripheral blood of a healthy donor using a RosetteSep NK Cell Enrichment Cocktail, according to the protocol provided by StemCell Technologies (Vancouver, Canada). Briefly, freshly prepared human blood was mixed with the RosetteSep antibody cocktail and incubated for 40 min. This sample was then diluted with an equal volume of PBS + 2% FBS and layered on top of Ficoll (1.077 ± 0.001 g/m, Sigma). After centrifugation at 2500 rpm (1370 g) for 40 min, enriched cells were removed from the Ficoll:plasma interface. These were then washed with PBS + 2% FBS twice and used as effector cells. In the cytotoxicity assay, peripheral blood NKs or YT cells were added at the required effector/target cell ratios (1:1, 2.5:1, 5:1, and 10:1) and then incubated for 4 h. The released ^{51}Cr was measured using a Packard Cobra Gamma counter (GMI, Albertville, MN, USA). To calculate the relative percentage of inhibition, NK-mediated lysis of SECs and those of each transfectant were compared at all four E:T ratios.

Cytokine assays. Naive SECs or transfected SECs were cultured at 1×10^4 cells/well, as described above. Freshly prepared peripheral blood NK cells were then added and incubated from 6 to 36 h. The supernatants were harvested at different times and assayed for cytokine concentration. The IFN-γ concentration in the culture supernatants was determined using an IFN-γ ELISA detection kit (Endogen, Woburn, USA). All assays were performed in triplicate and concentrations were determined from a standard curve freshly prepared for each assay.

Results

Cell surface expression of the recombinant UL18 and HLA-G1 gene

Using a retroviral packaging system, we harvested retroviral particles containing either recombinant UL18 or hβ2m genes. Recombinant UL18 retroviral particles were then infected into SECs with or without recombinant hβ2m retroviral particles. Cells stably expressing UL18 or hβ2m were selected using appropriate antibiotics (650 μg/ml of G418 for UL18 and 150 μg/ml of hygromycin for hβ2m). The expression levels of UL18 or hβ2m were assessed by flow cytometric analysis, using UL18-specific or hβ2m-specific monoclonal antibodies. Typical flow cytometric histograms for the selected stable cells are shown in Fig. 1. A low level of UL18 expression, as compared to that of HLA-G1, was observed in all analyzed transfectants. Furthermore, the additional transfer of hβ2m was not effective at increasing UL18 surface expression. For reasons that are not clearly understood, all transfectants selected gradually lost UL18 surface expression regardless of hβ2m

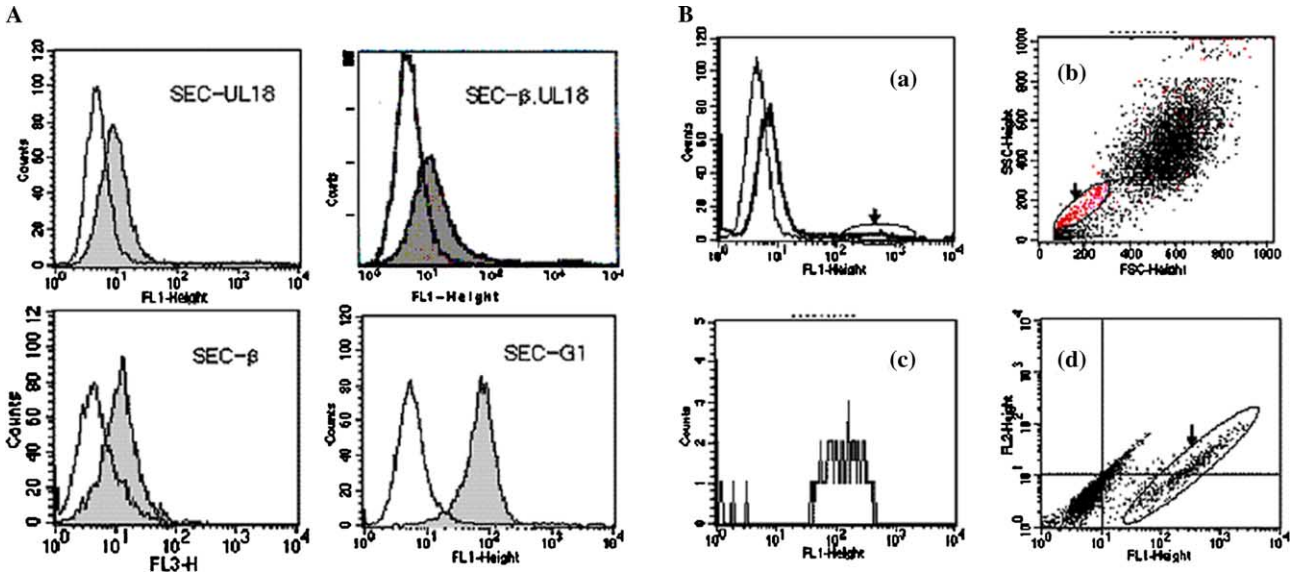

Fig. 1. Cell surface expression profiles of the transfectants with UL18, hβ2m, and HLA-G1. The expression of UL18 with or without hβ2m and of HLA-G1 on transfected swine endothelial cells (SECs) is shown in (A). Naive SECs and stable transfectants were stained with mAb 10C7 for SEC-UL18 and SEC-β.UL18; N-19 for SEC-β; MEM9/6 for SEC-G1. SEC-UL18, SEC expressing UL18; SEC-β.UL18, SEC expressing hβ2m and UL18; SEC-β, SEC expressing hβ2m; and SEC-G1, SEC expressing HLA-G1. Relatively high level expression of UL18 in dying cells (circle with arrow in (a), (b), and (d)), analyzed by flow cytometry are shown in (B). Single-parameter fluorescence histograms in (c) is obtained from gating the population of SEC-UL18 transfectant marked by arrow and circle in (b).

expression after about five generations of passages in culture in the presence or absence of the appropriate antibiotics. It was also observed by FACS staining that relatively high levels of UL18 were expressed in dying cells during culture as shown in Fig. 1B. In contrast to UL18 expression, HLA-G1 was readily expressed on SECs and its expression level was maintained throughout the experiments. Because of the low level of expression and the instability of UL18 on SECs, the UL18 cell surface expression was checked by flow cytometry on the day of the experiment and only samples showing substantial expression were used for further experiments.

UL18 gene expression protected swine endothelial cells from lysis by peripheral blood human NKs

To study whether UL18 expressed on SECs helps protect them from human NKs, we assayed 4 h ^{51}Cr release at effecter/target ratios (1:1, 2.5:1, 5:1, and 10:1), using NK cells freshly prepared from the peripheral blood of a healthy donor. Naive swine endothelial cells were susceptible to the cytolytic activity of human NKs. However, despite its poor surface expression, UL18-transfectants showed a significant ability to resist NK cell lysis. The relative protection defined as (% cytolysis of SECs control − % cytolysis of transfectants)/(% cytolysis of SECs control) against NK-mediated cytotoxicity ranged from 25% to 100% (Fig. 2). It is noteworthy that complete protection was observed in cells expressing UL18 with or without hβ2m at a NK/SEC ratio of 1:1. Furthermore, transfectants without UL18 surface expression were not protected from NK-mediated cytotoxicity (data not shown).

The NK-like cell line, YT, does not express the ILT-2 inhibitory receptor, and the NK inhibitory signal in YT cells involved HLA-G1 not UL18

In previous reports, it is suggested that UL18 interacts predominantly with ILT-2 inhibitory receptor to induce NK inhibition [24,25], and that HLA-G1 exerts a direct inhibitory function by interacting with at least three inhibitory receptors, i.e., ILT-2 [11,24], ILT-4, and KIR2DL4/p49 [26,27]. Interestingly, we found that YT cells (a human T/NK leukemia cell-line) did not express ILT-2 receptor, as shown in Fig. 3. Therefore, we evaluated the cytotoxic effect of YT cells against stable transfectant cells expressing UL18 or HLA-G1 and assessed the potential contribution of the ILT-2 receptor in the mediation of NK inhibition. As shown in Fig. 3, UL18 was not able to inhibit the lytic activity of YT cells. However, HLA-G1 showed an inhibitory function, strongly indicating that HLA-G1 mediates inhibitory signals through other than ILT-2 inhibitory receptors, supposedly via ILT-4 or KIR2DL4/p49.

Both UL18 and HLA-G1 significantly suppress IFN-γ secretion in peripheral blood NK cells

In general, the important NK effector functions are its cytotoxicity and its ability to induce cytokine

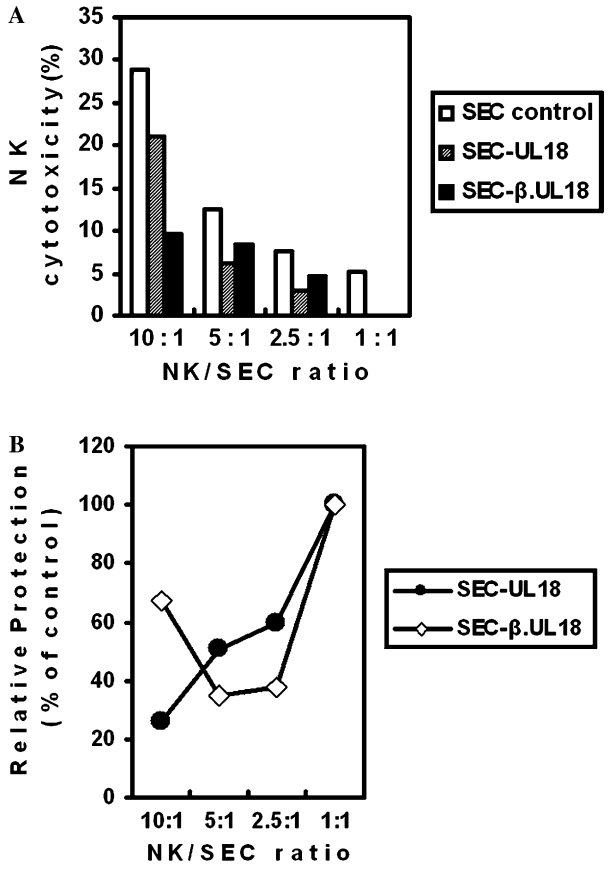

Fig. 2. Protective effect of the UL18 gene product against human NK cell-mediated cytotoxicity. Peripheral blood human NK cells were prepared by using a Rosettesep NK cell enrichment cocktail. Cytotoxicity was measured by a ^{51}Cr-release assay after 4 h incubation at 37 °C in 5% CO_2. Results were expressed as the percentage of lysed cells versus naïve SECs as a control in (A). The relative protection defined as (% cytolysis of SECs control − % cytolysis of transfectants)/(% cytolysis of SECs control) against NK-mediated cytotoxicity is shown in (B).

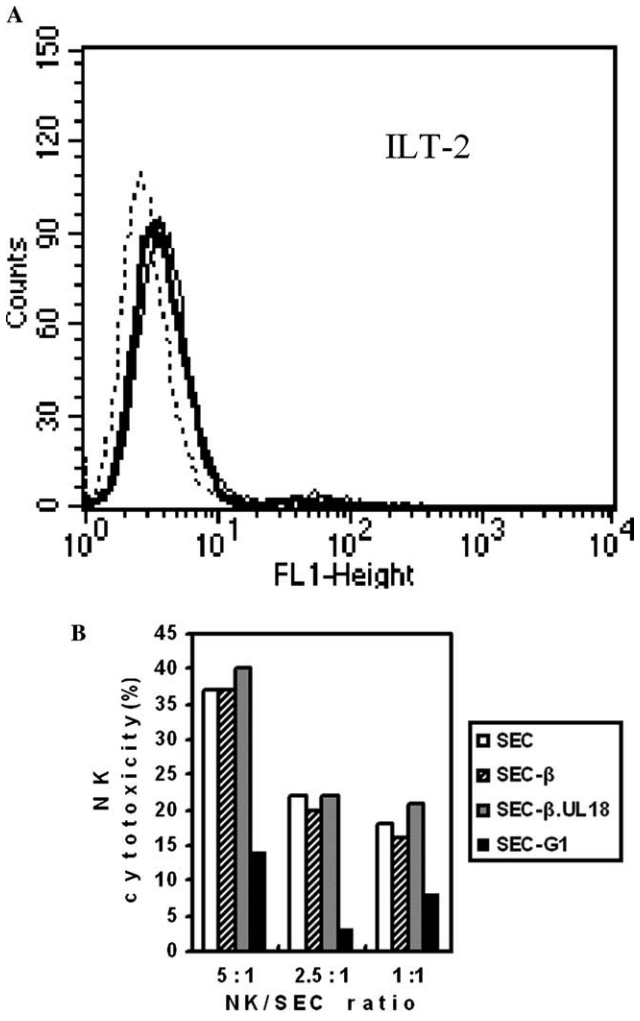

Fig. 3. ILT-2 expression on YT cells (a NK-like cell line) (A) and their cytolytic activity against either UL18 or HLA-G1 transfectants (B). YT cells were labeled by indirect immunofluorescence with murine anti-ILT-2 (VMP55) antibody. Mouse IgG1 (κ) was used as an isotype control. 4 h ^{51}Cr release assay was performed in triplicate and results are expressed as the percentage of lysed cells versus naïve SECs as a control.

secretion, especially IFN-γ secretion. Therefore, we investigated the effects of UL18 and HLA-G1 on the down-regulation of IFN-γ secretion. Supernatants were collected from the culture plates in which NK cells were co-cultured with naive SECs or with each stable transfectant expressing UL18 or HLA-G1. As shown in Fig. 4, both UL18 and HLA-G1 expression on SECs significantly reduce the amount of IFN-γ cytokine secreted by freshly prepared peripheral blood NKs from 1.14 to 0.67 μg/ml in UL18 and to 0.72 μg/ml in HLA-G1. Thus, the relative suppression on cytokine production was 40% for UL18 and 37% for HLA-G1 respectively, after 36 h of incubation.

Discussion

This study demonstrates the inhibitory role of UL18 in NK cytotoxicity against swine endothelial cells in vitro, and suggests the possible use of UL18 as an immune modulator in xenotransplantation. As is shown by the results obtained, UL18 expression on swine endothelial cells was lower than that of HLA-G1 and disappeared gradually during passage irrespective of the presence of appropriate antibiotics. But, it is noteworthy that even this low level of UL18 expression delivered a significant inhibitory signal to peripheral blood NKs and conferred protection on swine endothelial cells from NK-mediated cytotoxicity.

Therefore, it is expected that the up-regulated expression of UL18 on SECs might deliver a stronger inhibitory signal to NK cells, and that this would result in a more efficient down-regulation of human NK-mediated swine endothelial cell lysis. The underlying mechanisms of low level expression of UL18 might be

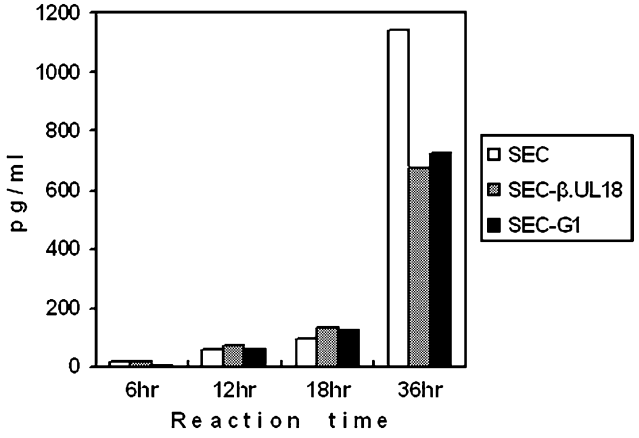

Fig. 4. Cell surface expression of UL18 or HLA-G1 significantly reduced IFN-γ secretion in peripheral blood human NK cells. The effector-to-target (E:T) ratio used was 10:1. 2×10^6 peripheral blood NK cells were co-cultured with either naïve SECs or UL18 or HLA-G1 transfectants. Supernatants were collected from each culture and tested to determine IFN-γ concentrations.

considered due to the segregation of the plasmid vector, the weakness of the promoter used, the mRNA instability, and/or rapid intracellular trafficking of protein expressed on the cell surface. Several attempts have been made to enhance the expression level of UL18 on SECs by us and others. To prevent the segregation of expression vectors in transfectants, the cells were kept in the presence of appropriate antibiotics after selection. For the alternative expression vector, pCDNA3.1 was tried other than a retroviral gene transfer system. To increase the stability of surface expression, we attempted to co-express hβ2m which has been previously reported to induce more efficient HLA-G1 expression [28]. Unfortunately, all of these efforts were not successful. Reyburn et al. [9] tried unsuccessfully to enhance the expression level of UL18 by modifying a gene to increase transcription and translation, by including an RNA stabilizing sequence, and by using an episomal vector. Similar efforts have been made by others to enhance the surface expression of HLA-E on SECs. The substitution of the HLA-G1 or HLA-A2 leader sequences for HLA-E successfully up-regulated HLA-E, otherwise not expressed on SECs [23]. Recently, an interesting report showed that an HLA-E single chain trimer, which encodes five peptides including the leader peptide of hβ2m, a 15 amino acid linker, mature hβ2m, a 20 amino acid linker, and a mature HLA-E heavy chain, was highly expressed on porcine cell surfaces [29]. The merits of the strategy of changing or substituting the leader sequence in terms of promoting UL18 expression are currently under investigation.

The finding that UL18 binds several endogenous peptides derived from cytoplasmic proteins [30] might lead to the belief that UL18, like class I molecules, might load the peptides during assembly in the endoplasmic reticulum, and that UL18 cell surface expression might be affected by binding specific peptides deficient in swine cells. As empty class I heterodimers are less stable than their peptide-filled counterparts, and rapidly disappear unless stabilized by the addition of exogenous peptides [31], the instability of UL18 expressed on SECs in our studies could be closely related to empty UL18 expression produced by the lack of suitable peptides in swine cells.

The gradual loss of UL18 expression on surface and the high level of its expression on dying cells prompted us to consider the possible role of cytoplasmic region of UL18 in delivering putative toxic signals or in enhancing metabolic turnover. Nucleotide sequence analysis revealed the cytoplasmic region of UL18 had two tyrosine residues on position 332 and 342 [32]. The possible phosphorylation of two tyrosine residues and functional engagement remains to be investigated. The cytoplasmic tail of membrane protein has been known to play an important role in metabolic turnover or recycling of the surface protein. Liszewski et al. [33] reported that cytoplasmic tail of membrane cofactor protein (MCP, CD46) was directly related to the intracellular transport of individual isoforms and the tail-less mutant showed the longest half life on the cell surface. We subcloned tail-less mutant of UL18 which lacked 331–348 amino acid residues of carboxyl terminal end into pLNCX. The expression level and stability of the mutant are under investigation.

We found that the protective effect of UL18 against NK cell cytotoxicity potentially occurs via a CD85/LIR-1/ILT-2 NK cell inhibitory receptor dependent pathway. It is an interesting observation that the YT cell line, a widely used NK-like cell line, does not express ILT-2 inhibitory receptor, and therefore, that the cytolytic activity of YT cell line is not suppressed by UL18. Similarly, it is possible that previously reported enhanced NK cytolysis against Human foreskin fibroblast (HFF) cells, where UL18 is transiently expressed [34], is due to the absence of ILT-2 inhibitory receptor on the surface of the NK clone.

Given the facts that to date, UL18 has only been shown to bind ILT-2 receptor [24,25], and that the affinity of UL18 for the ILT-2 receptor is approximately 1000-fold higher than for class I MHC [35], it appears that UL18 might affect the biological functions of components of immunity, including B cells, monocytes, dendritic cells, a subset of NK cells, and the majority of T cells, all of which express ILT-2 inhibitory receptor on their surfaces [11–13]. There is now increasing evidence that ILT-2 inhibitory receptor downregulates the Ag-specific cytolytic activity of $CD8^+$ T cells [36] and the proliferative response of $CD4^+$ T cells [37]. These findings are interestingly in accord with those of studies which showed that the various membrane-bound and soluble HLA-G1 isoforms are capable of inhibiting NK

cytolytic function [38], dendritic maturation [39], and the allo-proliferation of $CD4^+$ T cells and antigen-specific lysis [40], possibly by inhibiting receptors, including ILT-2, ILT-4, and KIR2DL4. Therefore, as UL18 binds to the ubiquitous ILT-2 inhibitory receptor like HLA-G1 and because its binding affinity is much higher than that of a class I molecule, it is possible that UL18 delivers an inhibitory signal to global cell subsets expressing surface ILT-2, i.e., NK cells, $CD4^+$, and $CD8^+$ T lymphocytes, macrophages, and dendritic cells, although this remains to be tested.

Finally, our cytokine data suggest that both UL18 and HLA-G1 suppress IFN-γ (Th1 cytokine) secretions. NK cells produce a range of cytokines, which drive the subsequent xenograft immune response. Of these, IFN-γ plays a crucial role in triggering xenograft rejection, by activating macrophages [41], promoting the recruitment of other effector cells, and augmenting T cell responses against porcine xenografts [22]. Therefore, the blockade of this important cytokine by UL18 could have much clinical benefit, although the role of the IFN-γ still remains controversial [42].

In summary, our findings demonstrate that UL18 can suppress the cytolytic activity of NK cells and their production of the important inflammatory cytokine IFN-γ, possibly through ILT-2 inhibitory receptor. Hence, the expression of UL18 on xenografts might serve as a new form of immune-modulation to protect xenografts from NK cell-mediated damage. Thus, now we are only beginning the processes of enhancing the stable surface expression of UL18 and of unraveling its immune regulatory functions.

Acknowledgments

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