Depletion of Cytoplasmic Tail of UL18 Enhances and Stabilizes the Surface Expression of UL18

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Background: Human cytomegalovirus UL18, a MHC class I homologue, has been considered a natural killer (NK) cell decoy. It ligates LIR-1/ILT2 (CD85j), an NK inhibitory receptor, to prevent lysis of infected target cells. However, precise role of UL18 to NK cell cytotoxicity is yet elusive. Difficulty in clarifying the function of UL18 lies in complication in detecting UL18 mainly due to low level expression of UL18 on the surface and gradual loss of its expression. **Methods:** To overcome this hurdle, cDNA of cytoplasmic tail-less UL18 was constructed and expressed in swine endothelial cell (SEC). The expression level and its stability in the cell surface were monitored with FACS analysis. **Results:** Surface expression of UL18 is up-regulated by removing cytoplasmic tail portion from UL18F (a full sequence of UL18). SECs transfected with a cDNA of UL18CY (a cytoplasmic tail-less UL18) stably expressed UL18 molecule on the surface without gradual loss of its expression during 6 week continuous cultures. In the NK cytotoxicity assay, UL18 functions either inhibiting or activating NK cell cytotoxicity according to the source of NK cells. We found that there is individual susceptibility in determining whether the engagement of NK cell and UL18 results in overall inhibiting or activating NK cell cytotoxicity. **Conclusion:** In this study, we found that cytoplasmic tail is closely related to the regulatory function for controlling surface expression of UL18. Furthermore, by constructing stable cell line in which UL18 expression is up-regulated and stable, we provided a useful tool to clarify exact functions of UL18 on various immune cells having ILT2 receptor.

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INTRODUCTION

Human cytomegalovirus (HCMV) has evolved various strategies to evade host immune system. Down regulation of class I molecule on the surface of host cell is one of the mechanisms to escape cytotoxic T cell recognition. However, the lack of MHC class I expression on the infected cell is vulnerable to the focused attack of NK cells. Strikingly, HCMV utilizes many regulatory genes including UL16, UL18, UL40, UL141, UL142 and a microRNA (miR-UL112) to systematically suppress NK cell recognition (1).

UL18, a 348-residue type-I membrane glycoprotein, is associated with β_2-microglobulin (β_2m) and a mixture of endogenous peptides similar to elutes from MHC class I molecules (2). The NK cell inhibitory receptor ILR1/ILT2 was reported to bind soluble gpUL18 with 1000-fold higher affinity than HLA-I molecules (3). Due to its remarkable structural similarity and affinity to inhibitory receptor ILT2, UL18 has been recognized as a NK decoy which could compensate reduced surface expression of host MHC class I to avoid NK cell cytotoxicity. However, precise role of UL18 to the NK cell function has been elusive. UL18 expressed in endothelial cells and macrophage showed no inhibitory effect on NK cells and sometimes increased NK-mediated cytotoxicity (4). On the other hand, HLA-deficient human B cell line expressing UL18 reduces susceptibility to various NK cell lines (5). UL18-expressing swine endothelial cells were also reported to be protected from the cytotoxicity of NK cells (6). Difficulties in clarifying *in vitro* / *in vivo* functions of UL18 lie in the complica-

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tion on the detection of UL18 expression mainly due to a low level of expression and confounding antibody interaction with virus-encoded Fc receptors (7,8). In addition, stable cell lines expressing UL18 have been difficult to construct due to their gradual loss of UL18 surface expression regardless of β_2m co-expression (4,6).

Several attempts proved to be unsuccessful in enhancing and stabilizing the UL18 expression on the cell surface (5). They include addition of an RNA stabilizing sequence to the original UL18 sequence and using an episomal vector. On the other hand, cytoplasmic tail of Membrane Cofactor Protein (MCP, CD46) was reported to directly related to the intracellular transport of its protein and tail-less mutant showed the longest half life on the cell surface (9). Possible role of cytoplasmic tail of UL18 in delivering putative toxic signals or in enhancing metabolic turnover was postulated in our previous reports (6). Nucleotide sequence analysis revealed that the cytoplasmic region of UL18 contained two tyrosine residues on position 332 and 342 (10). This implies that functional engagement of two tyrosine residues might be possible by phosphorylation and following signal cascades.

In this study, we revealed that cytoplasmic tail is responsible for enhancing surface expression and stability of UL18. In addition, we found that UL18 could either activate or inhibit NK cytototoxicty according to the source of NK cells.

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). Media was supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin, 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 β_2m cDNA were kindly provided by Dr. Kwangseog Ahn (Korea University, Korea). To establish stable cell lines expressing UL18, hβ_2m or HLA-G1, we cloned UL18 cDNA into pLNCX2 (Clontech, CA, USA), a retroviral expression vector, hβ_2m cDNA into pLHCX (Clontech), and HLA-G1 into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Recombinant UL18-pLNCX2 and hβ_2m-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 hβ_2m. 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 FACScalibur 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. Normal mouse IgG1 (kappa; 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, 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 [34]. Naive SECs or transfected SECs were cultured at 1×10^4 cells per well in a flat-bottomed, 1% gelatin-coated 96-well tray for about 15 h. These were then labeled with 100 μCi/ml $Na_2{}^{51}CrO_4$ (PerkinElmer 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 Inc. (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 2,500 rpm (1,370 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 was 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 Inc., Albertville, MN, USA). To calculate the relative percentage of inhibition, NK-mediated lysis of SECs and that of each transfectant were compared at all four E:T ratios.

Construction of cytoplasmic tail-less UL18 and glycosylphosphatidy linositol (GPI)-anchored UL18

Tail-less mutant of UL18, whose cytoplasmic tail (331-348 amino acid residues) was deleted for blocking the possible cascades of signal transduction, was amplified by standard PCR method and subcloned into pLNCX2. The primers used for this were as followed: 5'primer (5'-CTAAGCTTACCA CCAT GATGACAATGTG-3'), 3'primer (5'-GCCAGGTTACG ATC GATTCATTTCAAG-3'). Glycosylphosphatidylinositol (GPI)-anchored UL18 recombinant DNA was constructed by overlapping PCR techniques, which is designed to replace transmembrane and cytoplasmic portion (302～348 amino acids) in UL18 full sequence by GPI anchor cleavage/attachment signal sequence. GPI signal sequence was obtained by amplifying a single GPI cleavage/attachment signal sequence (315～347 amino acid residues) in human decay-accelerating factor (hDAF) gene. To amplify the fragment for GPI signal, we used overlapping forward primer 1-F (5'-ATCACCCGACA GCTAACAAAACCAC TTCAGGTACTAC-3'), which is designed to contain both 18 nucleotide sequences from the COOH terminal of UL18 (1～301 amino acid) and 18 nucleotide sequences from the NH_2 terminal of GPI signal sequence (315～347 amino acid residues) and reverse primer 1-R (5'-CGCATCGATCTAAGTCAGCAAGCCCAT-3'). UL18-GPI recombinant DNA was generated by PCR amplification with PCR amplified-GPI signal fragment (A) and UL18 DNA (B) as a template and forward primer 2-F (5'-CTAAGCTTACCACCT GATGACAATGTG-3') and reverse primer 1-R. Because the gene fragment A and B have the junction region, the fragments would anneal together to form UL18-GPI under specific temperature conditions (35 cycles at 94°C for 30 s, at 58°C for 30 s and at 72°C for 1 min), thus triggering the amplification cascade. Amplification was performed by using Pyrobest DNA polymerase (Takara Biomedical, Japan), which has an inherent 3'→5' exonuclease proofreading activity. To determine the nucleotide sequence, each DNA insert was ini-

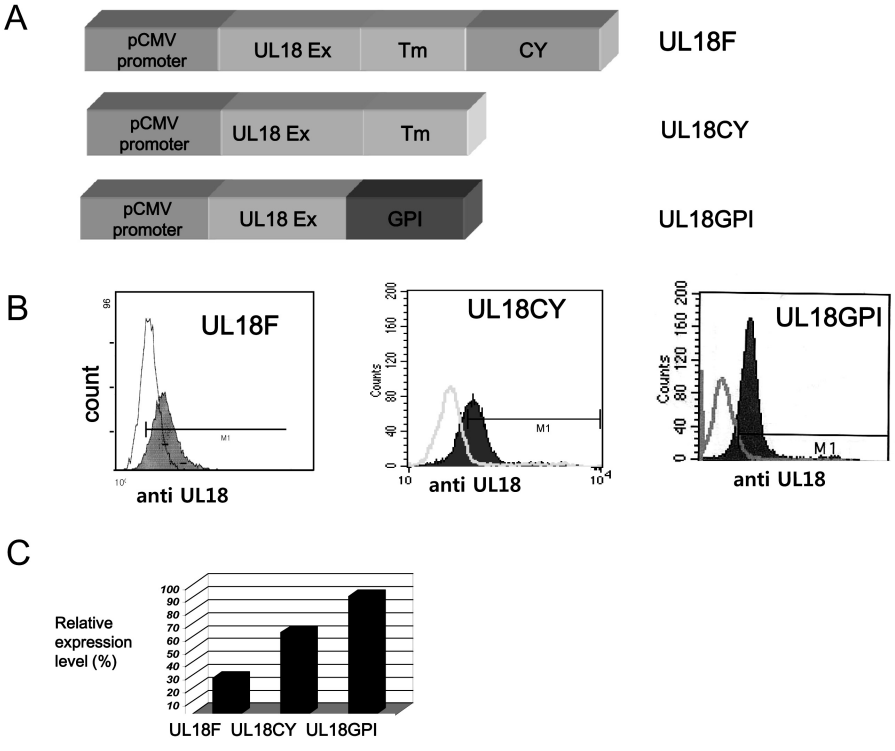

Figure 1. Increased expression level of cytoplasmic tail-less UL18 and GPI -anchored UL18 in SECs. (A) Schematic diagrams of UL18 recombinant cDNA constructs. The wild type UL18F consisted of CMV promoter (pCMV) region, extracellular domain region (UL18 Ex), transmembrane domain region (Tm), and cytoplasmic domain region (CY). UL18CY cDNA was constructed by removal CY portion from UL18F cDNA. In the case of UL18GPI, the Tm and CY were substituted with glycosylphosphatidylinositol (GPI) attachment/cleavage signal sequence which originated from human DAF cDNA. Either UL18F, UL18CY or UL18GPI recombinant cDNA were introduced into SECs. The stable cells expressing UL18 were selected under the maintenance in the 650 ug/ml concentration of G418. (B) Flow cytometric analysis of expression level of UL18F, UL18CY, and UL18GPI molecules on the surface of SECs. (C) Relative expression level of UL18F, ULCY, and UL18GPI was defined as % of the frequency of positive cell out of the frequency of 30,000 gated transfectants.

tially sequenced by using pLNCX sequence/primers (5 primer, 5'-AGCGCGTTTAGTGAACCGTCAGATC-3', and 3' primer, 5'-ACGTACAGGTGGGGTCTTTCATTCC-3'). Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems, USA).

RESULTS

Cytoplasmic tail of UL18 is closely related to the control of its surface expression

Surface expression of UL18 was reported to be very low and inconstant in several reports. Only marginal levels of UL18 were expressed in our previous study as well. Another study reported that the significant amount of mature UL18 protein is accumulated in the compartments of ER and the cis-Golgi (11). By the analysis of structure-based functional domain of UL18 protein, we found that UL18 contained two possible phosphorylation sites that might initiate following signal cascades. Therefore, it is postulated that cytoplasmic tail of UL18 might be responsible for controlling the surface expression of UL18 molecule. To prove this, cDNAs of UL18CY, cytoplasmic tail-less mutant of UL18, and UL18GPI, a substitute of transmembrane and cytoplasm portion with GPI anchor, were constructed by overlapping PCR technique. Each cDNA was then transfected into the SEC line, and its expression level was compared day by day by cell surface immunostaining. As shown in Fig. 1, as we expected, both deletion of cytoplasmic tail and replacement of cytoplasmic tail and transmembrance portion as a GPI anchor from mature UL18 increased the surface expression level of UL18 to 63% (UL18CY) or to 91% (UL18GPI), respectively, whereas UL18F only reaches 28% of surface expression. This strongly indicated that cytoplasmic tail region exerts an unknown regulatory function for expressing UL18 protein from the cytoplasmic compartment to the cell surface.

Stable expression of UL18 could be maintained on the cell surface without gradual cell loss by the removal of cytoplasmic tail of UL18

In addition to gradual loss of UL18 expression in the SECs transfected with UL18 full sequence, it was observed that cells highly expressing UL18 proteins on their surface were likely to die within five generation of continuous culture, resulting in selective survival of SECs expressing relatively low level of UL18. To identify whether stable expression of UL18 was maintained without causing cell death by the removal of cyto-

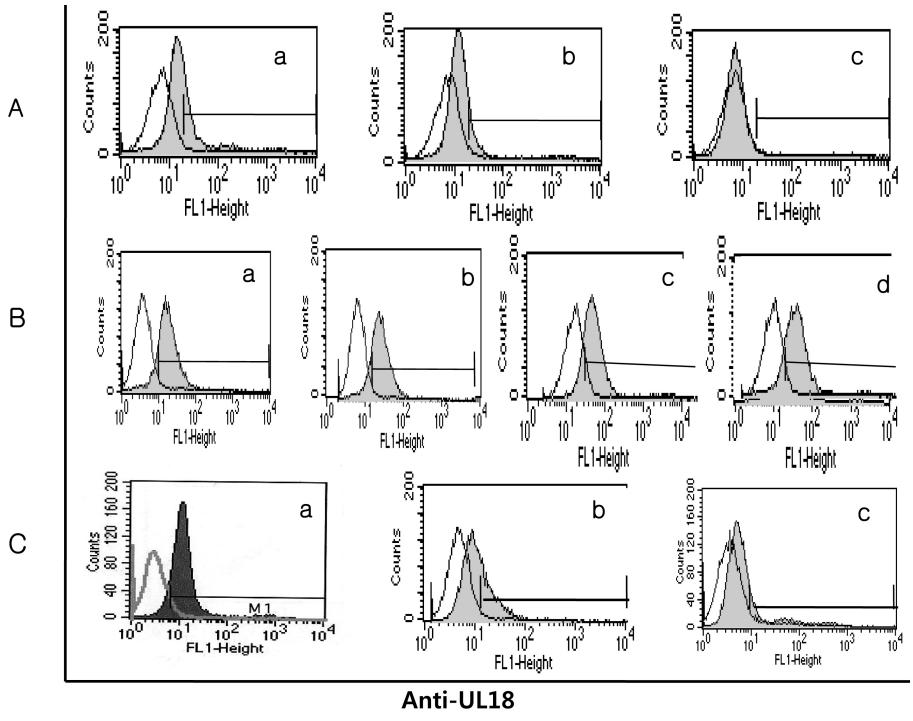

Figure 2. Stable expression of cytoplasmic tail-less mutant UL18CY without the loss of its expression on the cell surface. Cells were continuously cultured for more than 1 month, and examined expression level of UL18 at the designated time. Individual expression levels of UL18F (A), UL18CY (B) or UL18GPI (C) were examined by FACS analysis. Each generation obtained from continuous culture of cells was designated as the symbol, #(generation number). UL18F: a; #(1), b; #(3), c; #(5). UL18CY: a; #(2), b; #(5), c; #(7), d; #(9). UL18GPI: a; #(1), b; #(2), c; #(3).

plasmic tail of UL18, expression levels are monitored during several generations of continuous culture. As shown in Fig. 2, the expression level of UL18CY was maintained stably during the 6 week continuous cultures of transfectants, On the contrary, UL18GPI did not maintain UL18 expression in spite of cytoplasmic tail removal. Therefore, it suggested that removal of cytoplasmic tail in UL18 protein without any modification is important to maintain stable expression of UL18 on the surface.

NK cytotoxicity against SECs expressing up-regulated UL18 showed individual variance

Our previous study proposed that even low level expression of UL18 on the surface of SECs could confer resistance to NK-mediated cell cytotoxicity. Therefore we postulated that up-regulated UL18CY could more effectively suppress NK cytotoxicty by enhanced delivery of inhibitory signals by possible engagement with ILT2 receptor. To test this, we performed cytotoxicity assay with NK cells purified from diverse blood samples. Unexpectedly, individual NK cells were differently affected to the up-regulated UL18 protein. As shown in Fig. 3A, the up-regulated UL18 on the surface of SECs could inhibit the cytotoxicity of NK cells obtained from one donor. On the contrary, up-regulated UL18 could not inhibit the cytotoxicity of blood NK cells from the others or even activated NK-mediated cell lysis. Therefore, it strongly suggests that there are individual variances in the degree of inhibiting or activating NK cell cytotoxicty. Namely, it implies that individual NK cells determine their fate if they would be activated or inhibited by the engagement of UL18 by unknown mechanisms.

DISCUSSION

HCMV in the infected cells frequently makes complex interconnection with the receptor on host immune cells, resulting in remarkable resistance to the attack of T and NK cells. UL18 has been one of the molecules for down-regulating NK cell cytotocixity. However, low level expression of UL18 after HCMV infection and gradual loss of its expression in cell lines expressing UL18 have made it difficult to elucidate precise influence of UL18 on the function of NK cells. In this study, we demonstrated that surface expression of viral protein UL18 was regulated by the portion of cytoplasmic tail in UL18. In addition, we found that UL18-mediated NK cell suppression exhibited individual variance according to the source of NK cells.

The receptor for UL18 is a CD85j/LIR-1/ILT2 (CD85j), a molecule of the Ig superfamily (12). The UL18-LIR-1 interaction certainly plays a pivotal role in the immune response to HCMV infection. Binding of LIR-1 to UL18 requires surface expression of its viral protein. However, it was reported that UL18 accumulates in the cytoplasm and was hard to detect surface expression in HCMV-infected fibroblast (13). Surface expression of UL18 was also very low and hard to be maintained in the swine endothelial transfected with UL18-carring retrovirus in our previous study. Accordingly, it was requisite to construct stable cell line in which UL18 expression is up-regulated and stable without rapid turnover in the surface membrane. Biochemical analysis conducted by Griffin *et al.* also revealed that UL18 protein was significantly accumulated in the compartments of ER and the cis-Golgi but was undetectable on the cell surface (11). Our previous study also delivered a hypothesis that poor surface expression would be related to the blocking of UL18 molecules to reach the cell

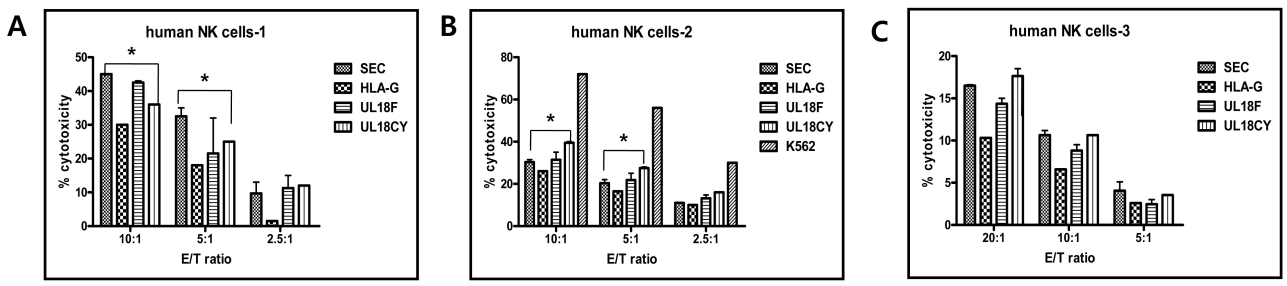

Figure 3. Different effects of up-regulated UL18 on the NK-mediated cell cytotocixity. Peripheral blood human NK cells were prepared by using RosettesepTM NK cell enrichment cocktail. ^{51}Cr-release assay was performed by 4 hr incubation at the designated E:T (effector cells: target cells) ratio. UL18CY suppressed (A), enhanced (B), or unaffected the cytotocixity of NK cells purified from individual NK cell donor blood. *represents statistical p value < 0.05.

membrance or the rapid internalization. Close examination of full length UL18 domains showed possible phosphorylation sites which could initiate unknown signal cascade in the cytoplasmic tail. In this study, removal of the cytoplasmic portion in the full length UL18 gene was sufficient to increase its surface expression and maintain stability without loss of expression level. Substitution of both the cytoplasm and transmemrance portion of UL18 with GPI up-regulated its expression as well, but UL18 expression was not maintained during the culture. Collectively, this strongly implies that cytoplasmic tail has an unknown regulatory function in controlling surface expression of UL18. Another interesting observation is that the cells expressing high level of UL18 are more likely to be placed in cell death, resulting in selective survival of the cells expressing the relatively low level of UL18. Cell death was only observed in the cells transfected with full length UL18 or UL18GPI gene but was not detected in the case of the cells expressing UL18CY gene. Therefore, there might be another regulatory mechanism, possibly by delivering putative toxic signals, to control protein expression on the surface.

UL18 initially was considered to be a decoy molecule for NK cells. However, the results for UL18-mediated inhibition of NK cells are still controversial. Leong *et al.* proposed that endothelial cell infected with AD169 or dUL18 did not show an inhibitory effect on NK cell cytotocicity (4). On the other hand, Reyburn *et al.* and our previous studies reported that UL18 could suppress NK cell lysis. HLA-deficient human B cell line expressing UL18 reduced susceptibility to various NK cell lines (5). SEC transfected with UL18 was protected from the cytotoxicity of NK cells (6). In this study, by using SEC expressing the high level of UL18CY, we demonstrated that the effect of UL18 on the NK cytotoxicity exhibits individual variance. The reason why UL18 affects differently on the individual NK cell is elusive. Possible explanation might be in the proportion of $ILT2^+$NK cells in the overall NK cell population. Recently, the effect of UL18 on NK cell lysis has been explained in the context of LIR-1 expression. Prod'homme *et al.* demonstrated that UL18 inhibited $LIR\text{-}1^+$ NK cells but activated LIR^-NK cells by unknown mechanisms. They proposed that the outcome of UL18/NK interaction might depend on the prevalence of $LIR\text{-}1^+$NK cells in the whole NK cells (8). Namely, in a polyclonal setting in which blood NK cell consists of LIR^-NK cells and LIR^+NK cells, UL18 encounter might lead to activation or inhibition according to the prevalence of either subpopulation. Alternatively, when inhibitory signals by $LIR\text{-}1^+$NK cells are equaled by activating stimuli by LIR^-NK cells, UL18 encounter might result in no overall effect at al. Therefore, Individual susceptibility on the NK cytotoxicity shown in this study might be related to the different frequency of $LIR\text{-}1^+$NK cells in blood. It is now undergoing works to examine the variance of LIR^+NK cell proportion among the NK cells purified from individual human blood. The frequency of LIR^+NK cells and its relation to NK cytotoxicity should be investigated with SECs expressing high level UL18 in our further study.

Given the fact that UL18 has only been shown to bind ILT2 receptor and that the affinity of UL18 for the ILT2 receptor is approximately 1,000-fold higher than for class I MHC (3). 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 ILT2 inhibitory receptor on their surface (14-16). There is now increasing evidence that ILT2 inhibitory receptor downregulates the Ag-specific cytolytic activity of $CD8^+$T cells and the proliferative response of $CD4^+$ T cells (17). 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 (18), dendritic maturation (19), and the allo-proliferation of $CD4^+$ T cells and antigen-specific lysis (20), possibly by inhibiting receptors, including ILT2, ILT4, and KIR2DL4. Therefore, as UL18 binds to the ubiquitous ILT2 inhibitory receptor like HLA-G1 and as 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 ILT2, i.e. NK cells, $CD4^+$ and $CD8^+$ T lymphocytes, macrophages, and dendritic cells, although this remains to be tested. Therefore, UL18CY-expressing cell in which UL18 expression was up-regulated and stable could be a useful tool to clarify exact UL18 functions on various immune cells having ILT2 receptor.

In summary, we found that removal of UL18 cytoplasmic tail could up-regulate and stabilize the surface expression UL18. By using SEC which UL18 expression was up-regulated, we also demonstrated there is individual susceptibility in determining whether the engagement of NK cell and UL18 results in overall inhibiting or activating NK cell cytotoxicity. Finally by constructing cell line which stably expresses UL18 molecule on its surface, we approached new step to clarify precise effects of UL18 on the function of various immune cells.

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