Residues Met^{76} and Gln^{79} in HLA-G α1 domain involved in KIR2DL4 recognition

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ABSTRACT

Human leukocyte antigen-G (HLA-G) has long been speculated as a beneficial factor for a successful pregnancy for its restricted expression on fetal-maternal extravillous cytotrophoblasts and its capability of modulating uterine natural killer cell (uNK) function such as cytotoxicity and cytokine production through NK cell receptors. HLA class I α1 domain is an important killer cell Ig-like receptor (KIR) recognition site and the Met^{76} and Gln^{79} are unique to HLA-G in this region. NK cell receptor KIR2DL4 is a specific receptor for HLA-G, yet the recognition site on HLA-G remains unknown. In this study, retroviral transduction was applied to express the wild type HLA-G (HLA-wtG), mutant HLA-G (HLA-mG) on the chronic myelogenous leukemia cell line K562 cells and KIR2DL4 molecule on NK-92 cells, respectively. KIR2DL4-IgG Fc fusion protein was generated to determine the binding specificity between KIR2DL4 and HLA-G. Our results showed that residue Met^{76}, Gln^{79} mutated to Ala^{76,79} in the α1 domain of HLA-G protein could affect the binding affinity between KIR2DL4 and HLA-G, meanwhile, the KIR2DL4 transfected NK-92 cells (NK-92-2DL4) showed a considerably different cytolysis ability against the HLA-wtG and HLA-mG transfected K562 targets. Taken together, our data indicated that residue Met^{76} and Gln^{79} in HLA-G α1 domain plays a critical role in the recognition of KIR2DL4, which could be an explanation for the isoforms of HLA-G, all containing the α1 domain, with the potential to regulate NK functions.

Keywords: NK cell, KIR2DL4, HLA-G, receptor.

INTRODUCTION

The non-classical HLA class I antigen HLA-G is mainly expressed on extravillous cytotrophoblasts that invade deciduae in uterine pregnancy. Furthermore, HLA-G can modulate the function of most immune component cells such as NK cells, T cells and Dendritic cells, indicating that it may contribute to the maintaining of the maternal-fetal immunotolerance by protecting the allogenic fetus from maternal immune response and/or pathogen infections which threat a successful pregnancy [1, 2]. The primary transcript of HLA-G is alternatively spliced to produce four membrane-bound (HLA-G1, -G2, -G3,-G4) and three soluble (HLA-G5, -G6, -G7) isoforms [3]. It is worthy noted that HLA-G3, which only contains the α1 domain, has the ability to regulate NK functions through KIR2DL4, suggesting the α1 domain of HLA-G protein might be involved in KIR2DL4 recognition [4]. KIR2DL4, a member of KIR family, featured with both inhibitory and activating functional structure, has been described as the specific receptor for HLA-G [5]. However, the definite recognition sites between them yet to be elucidated.

Extensive progresses have been made in determining receptor-ligand binding specificity of KIRs. Generally, KIR3D recognize HLA-A and HLA-B, whereas KIR2D recognize HLA-C allelic products. Of particular interest is that KIR2DL1 and 2DS1 recognize the C2 epitope (Asn^{77}, Lys^{80}) in HLA-Cw2, 4, 5, 6, 17, 18, while KIR2DL2, 2DL3 and 2DS2 recognize the C1 epitope (Ser^{77}, Asn^{80}) in HLA-Cw1, 3, 7, 8, 13, 14 [6]. In addition, residues at position 77~83 in α1 domain also get involved in the interaction between KIR3D and HLA-B. Therefore, it appears that MHC Class I specific KIRs share a common feature of binding to their

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Abbreviations: HLA-wtG, wild type HLA-G; HLA-mG, mutant HLA-G; HLA, Human Leukocyte Antigen; KIR, killer cell Ig-like receptor; IFN-γ, interferon-γ.

corresponding ligands recognizing the residues77~83 in MHC Class I α1 domain [7]. Given that Met^{76} and Gln^{79} are unique to HLA-G molecule in this region, we hypothesized that these two sites might play an important role in the specific binding of KIR2DL4 and HLA-G [8].

In this study, our results indicated that residues Met^{76}, Gln^{79} in the α1 domain of HLA-G protein could affect KIR2DL4 binding specificity, which was confirmed by cytotoxicity assay using NK-92-2DL4 cells against HLA-wtG (Met^{76}, Gln^{79}) and HLA-mG ($Ala^{76,79}$) transfected K562 cells.

MATERIALS AND METHODS

Cell lines and antibodies

The erythroleukemia cell line K562 and HLA-wtG and HLA-mG K562 transfectants were maintained in complete RPMI 1640 media supplemented with 15% heat-inactivated FBS. Human leukemia cell line NK-92 (ATCC, MD) and its KIR2DL4 transfectants were cultured in Alpha Minimum Essential media (α-MEM) without ribonucleosides and deoxyribonucleosides (Gibco BRL, USA) and supplemented with 12.5% heat-inactivated FBS, 12.5% horse serum, 0.2 mM inositol, 0.1 mM 2-mercaptoethnol, 0.02 mM folic acid, and 200 U/ml of rIL-2 (Bioscience, NY). COS-7, PA317 and NIH3T3 cell lines were maintained in DMEM medium. All cells were cultured at 37 ºC in a humidified 5% CO_2 atmosphere.

The following mAbs are indicated: anti-KIR2DL4 mAb #33 (IgG1), anti-KIR2DL4 mAb #36 (IgM) were generously provided by E.O. Long (NIH, MD, USA); anti-pan-HLA I mAb W6/32 (IgG1) was kindly provided by D.E. Geraghty (Fred Huchinson Cancer Research Center, Seattle, USA).

KIR2DL4-Ig fusion protein expression and binding assay

KIR2DL4-Ig fusion protein was produced as previously described [9]. The extracellular region of KIR2DL4 gene was amplified from a cDNA from decidual tissue with the forward primer 5'-CAGAGTGTGCTAGCGCACGTGGGTGGTCAGGACAAGCC3' containing an *Nhe*I site, and the reverse primer 5'-GAGTACCTAGGATCCGCATGCAGGTGTCTGGCGATACC-3' containing a *Bam*HI site [5]. The amplified sequence was inserted into the fusion protein expression vector Cd5lneg1 (provided by B. Seed from Massachusetts General Hospital, Charlestown, MA and E.O. Long from NADID, NIH, MD). The reconstructed vector was transfected into the COS-7 cell using DEAE-Dextran according to the manufacturer's instruction. After transient expression, the transfectant cell culture supernatant was harvested, purified with Protein A Sepharose CL 4B (Pharmacia, USA) and concentrated with Centriprep-30 concentrators (Millpore, USA). Reducing condition SDS-PAGE and Western blot analysis were performed to identify the fusion protein. Direct binding assay was performed using mAb#33 and FITC-conjugated goat anti-human Fc by flow cytometry as described in previous studies [9].

Full length KIR2DL4 and HLA-G cDNA constructs

Total RNA extracted from decidua was used as a RT-PCR template to generate full-length KIR2DL4 with following primers: forward primer containing a *Hind*III site (underlined), 5'-TCAAGCTT AGAAGCTGCACCATGTCCATGT-3', reverse primer containing a *Cla*I site, 5'-CCCGATCGATTGGAGACTCACGCCTTCAGA-3', PCR product was digested with *Hind*III and *Cla*I, subcolned into the vector pBlurescripts and sequenced, then the confirmed target sequence was ligated into the retrovirus expression vector pLNCX to generate recombinant retrovirus for generation of NK-92 cell line with stably integrated cDNA. To produce wild type HLA-G, primers were used as the following : sense (*Hind*III) 5'-TCAAGCTTGG TCCTGGTTCTAAAGTCCT-3', antisense (*Cla*I) 5'-CCG ATCGAT TGAGACAGAGAGACGGAG ACAT-3'. To mutate the Met^{76}, Gln^{79} residues in the HLA-Gα1 domain, the wild type HLA-G cDNA was used as PCR template. Site-directed mutagenesis was performed with the following sets of mutageneic primers to produce the HLA-mG (Met^{76}, Gln^{79}-$Ala^{76,79}$). Sense 5'-GACTGACAGAGCGAACCTGGCG ACCCTGCGCG-3', antisense 5'-CGCGCAGGGTCGCCAGGTT CGCTCTGTCAGTT-3'. The *Hind*III and *Cla*I digested fragment of HLA-G and HLA-mG were cloned into the retrovirus expression vector pLNCX respectively to produce the recombined vector. The nucleotide sequence of HLA-G and HLA-mG insert was sequenced to confirm that only the expected mutations were present (GenBank accession No. AY359817, AY359818).

Retroviral transfection into K562 or NK-92 cell lines

The packaging cell line PA317 was transfected with the recombined pLNCX vector containing HLA-wtG, HLA-mG and full-length KIR2DL4 cDNA respectively using Lipofectamine Plus reagent (Life Technologies). Two-day culture supernatant in serum-free DMEM (Gibco Life Technologies) medium was incubated with NK-92 or K562 cells for 8 h in the presence of Lipofectamine Plus reagent, and then the transfected NK-92 cells were maintained in the complete α-MEM (Gibco Life Technologies) containing rIL-2. The transfected K562 cells were cultured in the RPMI 1640 (Gibco Life Technologies) for 3 d before screened with G418. Cell surface protein expression on the transfectants was detected by flow cytometry. Stable expression of exogenous HLA-wtG, HLA-mG on K562 cells, as well as KIR2DL4 on NK-92 cells, was measured by flow cytometry. Uniform HLA-wtG, HLA-mG expression in K562 transfectants, and KIR2DL4 expression in tranfected NK-92 cells were confirmed by Western blot at the same time using mAb W6/32 and mAb#33 respectively.

Cytotoxicity assay

Cytotoxicity experiment was performed using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, USA). NK-92-2DL4 effector cells were maintained in complete α-MEM medium containing rIL-2 on the day before assay. During cytolysis assay, effector cells were mixed with 1×10^4 target cells at a E:T ratio of 10:1 in V-bottom 96-well plates (Costar, Cambridge, MA) as the manufacture's recommendation. Spontaneous release and maximal release of LDH in target cells and effector cells were measured by keeping these cells in medium alone. Each assay was performed in quadruplicate. Percentage of specific lysis was determined as follows:

(Experimental Release-Effector Spontaneous Release-Target Spontaneous Release) / (Target Maximum Release -Target Spontaneous Release)×100.

Antibody blocking assay

During cytotoxicity assay, anti-KIR2DL4 mAb#64 (IgM) was added to block the KIR2DL4 expression on the NK-92 transfectants. Effector cells were pre-incubated at room temperature in culture medium containing 200U/ml rIL-2 for 20 min and then incubated

for 15 min at room temperature with mAb#64 (mouse anti-human IgM) before the target cells were added. An isotype matched IgM ntibody was used in the experiment as a negative control.

Flow cytometry and immunofluoresence microscopy analysis

For cell surface staining, standard method was performed. Briefly, the parent NK-92, K562 cells and their transfectants were harvested, washed with cold 1% BSA-PBS three times, then resuspended to a concentration of 2×10^6 cells/ml. Then 50 μl of cell suspension was added into the 96-well round-bottom culture plate. Cells were incubated with primary mAb W6/32 or mAb#33 respectively at 4 °C for 1h, washed with cold 1% BSA-PBS and incubated with the FITC-labeled goat-anti-mouse IgG at 4 °C for 30 min. Isotype-matched antibody was used as the negative control. Finally, the cells were washed twice in cold PBS and fixed with 1% (w/v) paraformaldehyde/PBS solution, and analyzed with flow cytometry or subjected to fluorescent microscopy.

RESULTS

HLA-wtG and HLA-mG expression on K562 cells

As previous mentioned, the primary transcript of HLA-G can be alternatively spliced to give rise to four membrane-bound proteins (HLA-G1 to HLA-G4) and three soluble isoforms (HLA-G5 to HLA-G7). All of these isoforms contain the α1 domain that can be recognized by KIR2DL4. *In vivo* experiments indicated that the interaction between HLA-G and NK cell receptors could affect cytotoxicity activity of both the decidual and peripheral blood NK cells, as well as allogenic proliferative responses induced by antigen specific CTL or CD4+ T cells. Residues 77~80 in the α1 domain of HLA class I molecule has been considered as an important KIR recognition site. In this region, Met^{76} and Gln^{79} are unique to HLA-G, suggesting these two residues might be involved in KIR2DL4 recognition.

To examine the potential role of these two residues to KIR2DL4 recognition, we generated a mutant HLA-G (Met^{76}, Gln^{79}-$Ala^{76,79}$, HLA-mG) which allowed us to directly compare the binding ability of HLA-wtG or HLA-mG to KIR2DL4-IgFc fusion protein. HLA-wtG and HLA-mG expression on K562 cells was measured by flow cytometry. We found that HLA-wtG and HLA-mG on cell surface were expressed at similar levels in retrovirus-transfected K562 cells (Fig. 1). The expression of HLA-wtG and HLA-mG was further confirmed by immunofluorescent staining (Fig. 2).

Direct binding of soluble KIR2DL4-Ig fusion proteins to K562 cell and its transfectants

To test whether Met^{76}, Gln^{79} in HLA-G α1 domain get involved in KIR2DL4 recognition, soluble receptors were produced in the form of KIR2DL4-IgG Fc fusion protein. A band of expected molecular weigh (70 kD for KIR2DL4-IgGFc) was detected, then the protein was assessed by Coomassie blue and confirmed by Western blot with the

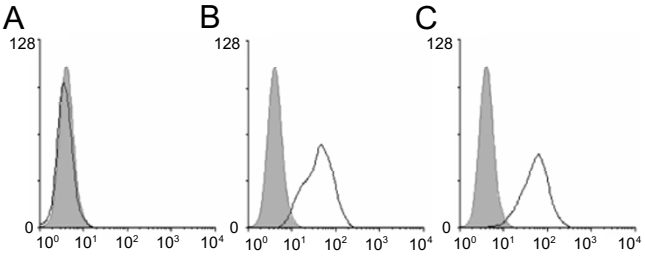

Fig. 1 Expression of HLA on K562 cells detected by flow cytometry analysis. (**A**) Vac-transfected K562 cells. (**B**) K562 cells transfected with HLA-wtG. (**C**) K562 cells transfected with HLA-mG.

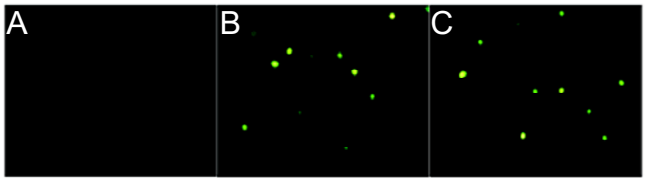

Fig. 2 Immunofluorescence staining of HLA-wtG and HLA-mG expression in transfected K562 cells. (**A**) Un-transfected K562 cells. (**B**) HLA-wtG transfected K562 cells; (**C**) HLA-mG transfected K562 cells.

mAb#33 (Fig. 3). Direct binding results showed that KIR2DL4-IgG Fc fusion protein bound strongly to K562-wtG cells, while a much weaker binding was detected in the K562-mG cells (Fig. 4, $P < 0.01$), suggesting residues Met^{76} or Gln^{79} or both in HLA-G α1 domain 1 could be the recognition site(s) responsible for HLA-G's interaction with KIR2DL4. Titrations of the fusion protein between 20 μg/ml and 40 μg/ml revealed a dose-dependent manner when binding to K562 transfectants.

Establishment of NK-92 cells expressing functional KIR2DL4

KIR2DL4 (CD158d) displays unusual characters among KIR family members for carrying both a single ITIM in its cytoplasmic tail and a charged Arg residue in its transmembrane region, suggesting a possible function of activating and inhibitory of KIR2DL4 [11]. Long *et al* confirmed both the inhibitory and activating functions of KIR2DL4 in terms of the different stages of NK cells [12].

NK-92 cell line is an IL-2-dependent NK-like cell line. NK-92 severs as an excellent model system to study NK cell biology and KIR functions for its strong target cell cytotoxicity and well-defined cell surface markers [13]. We choose NK-92 cell line in this experiment, because it has been used successfully for retrovirus mediated expression, despite its low endogenous KIR2DL4 expres-

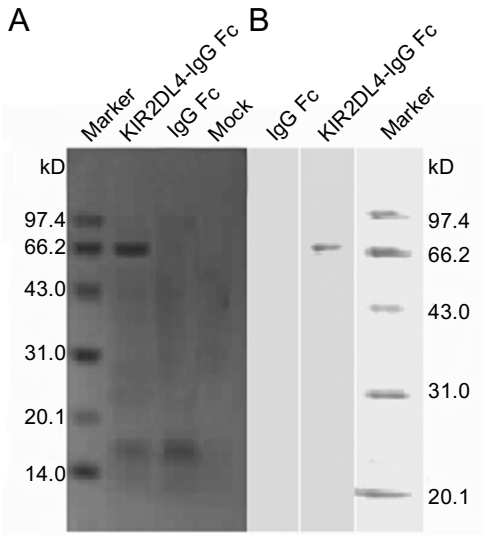

Fig. 3 Generation of soluble KIR2DL4-IgG Fc fusion proteins. (**A**) SDS-PAGE analysis of purified soluble KIR2DL4-IgG Fc fusion protein from supernatants of transfected COS-7 cells. (**B**) Purified soluble KIR2DL4-IgG Fc fusion protein from supernatants of transfected COS-7 cells were detected by Western blot.

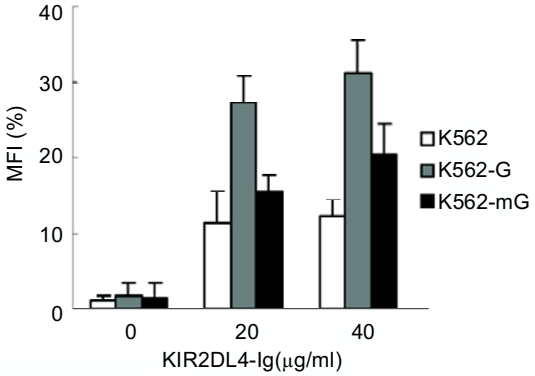

Fig. 4 Direct binding assay of soluble KIR2DL4-IgG Fc fusion protein to K562 cells. Parent K562 cells and K562 transfectants expressing HLA-wtG or HLA-mG were incubated without the fusion protein, or with 20 μg/ml and 40 μg/ml KIR2DL4-IgGFc fusion protein. The binding index was expressed as median fluorescence intensity (MFI) by flow cytometry.

sion and lacking of FcγR III (CD16) expression, which benefits applying intact Abs instead of $F(ab')_2$ to manipulate surface receptors [14].

The expression of KIR2DL4 on NK-92 transfectants was measured with mAb#33 (IgG) at a recommended concentration using flow cytometry. NK-92 cells express a low level of endogenous KIR2DL4 (4.2%) as revealed by flow cytomerty. While on the transfected NK-92 cells, ex-

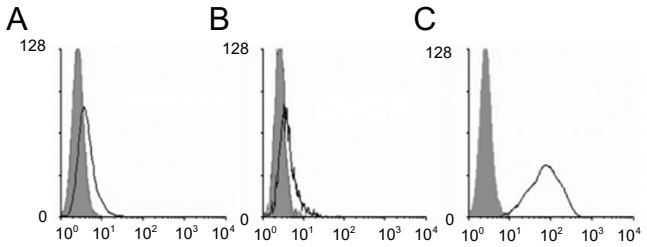

Fig. 5 Expression of KIR2DL4 on NK-92 cells detected by flow cytometry analysis. (**A**) Non-transfected NK-92 cells. (**B**) Vac-NK-92 cells. (**C**) KIR2DL4 transfected NK-92 cells.

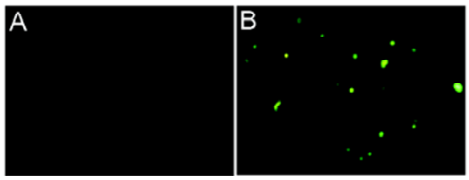

Fig. 6 Immunofluorescence staining of KIR2DL4 expression on NK-92 cells. (**A**) Un-transfected NK-92cells; (**B**) KIR2DL4 transfected NK-92 cells.

pression of KIR2DL4 is relatively high, with the mean percentage of 87.52%. These results demonstrated that the recombined construct was successfully set up and KIR2DL4 was expressed stably on NK-92 cells (Fig. 5). Immunofluorescence analysis revealed that KIR2DL4 was densely epressed on the KIR2DL4 transfected NK-92 cell surface (Fig. 6).

HLA-G induced NK-92 cytolytic activity though KIR2DL4

Previous studies report that KIR2DL4 is an activating receptor leading to IFN-γ production in resting NK cells. Increasd IFN-γ and cytotoxicity in activated NK cells suggested that NK cell stage might be one of critical factors affecting the signal transducted by KIR2DL4 [12]. To determine what kind of signal would be transducted by KIR2DL4 and whether it was modulated by HLA-mG with respect to NK-92-2DL4 cells in current experiments, cytotoxicity assay was performed applying NK-92-2DL4 as the effector cells against the K562, K562-wtG and K562-mG targets. We observed that lysis of K562-wtG target cells by NK-92- 2DL4 cells were significantly increased compared to that of the K562-vac cells. Surprisingly, cytolysis of HLA-mG transfectants was considerable lower when comparing with that of NK-92-2DL4 against K562 cells, and the lysis of NK-92-2DL4 to K562-mG was similar to that of the K562-vac. The cytotoxicity of NK-92-2DL4 also displayed a phase-dependent manner that

compared to the 8 h co-culture killing system. Increased specific lysis could be observed in 12 h co-culture of the effectors with target cells. It should be noted that blocking of KIR2DL4 expression on NK-92 cells with mAb#64 (IgM) could dramatically decrease the lytic ability of KIR2DL4-NK-92 against K562-wtG target cells (from 98.237% to 56.124%; $P < 0.01$), while no significant changes were observed with the lysis of NK-92-2DL4 against the K562-mG or K562-vac targets (Fig. 7; $P > 0.05$). Taken together, these results showed that KIR2DL4 is an activating receptor by interacting with HLA-G to induce the cytotoxicity of NK-92 cells, strongly supporting that the Met^{76}, Gln^{79}-$Ala^{76,79}$ mutation could modulate the recognition of KIR2DL4 to HLA-G protein.

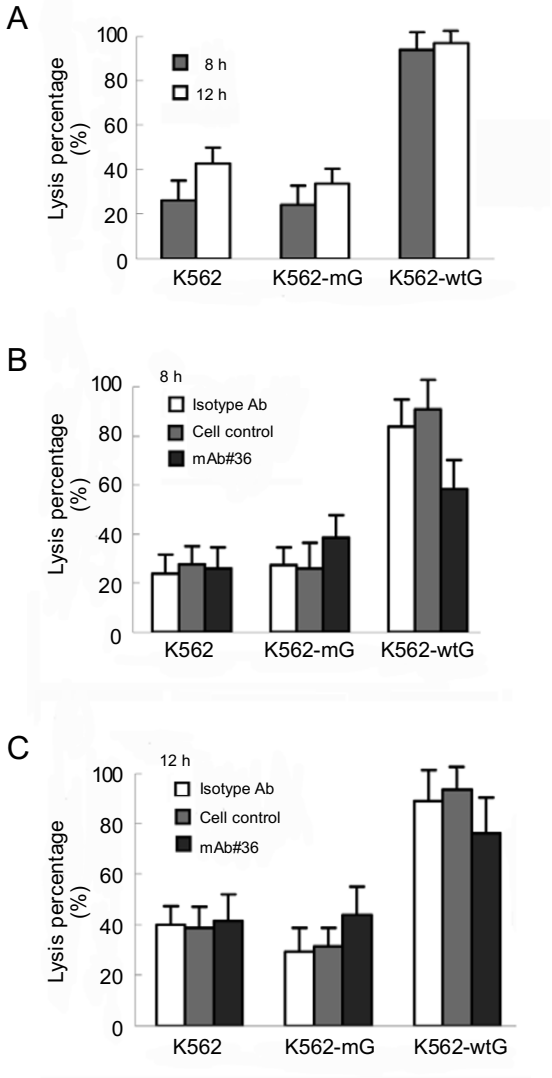

Fig. 7 HLA-wtG or HLA-mG transfected K562 cells regulate the cytolysis function of NK-92-2DL4 cells. K562-vac, K562-wtG and K562-mG were used as targets toward NK-92-2DL4 in LDH release assay. (**A**) Comparison of the mean cytolytic percentage of NK-92-2DL4 among different targets in a 8 h and 12 h co-culture at a 10:1 (E:T) ratio; (**B**,**C**) Ab#36 blocking assay was performed at a 10:1 (E:T) ratio in the presence of KIR2DL4 specific Ab#36 (IgM) in a 8 h and 12 h co-culture.

DISCUSSION

Reproduction, a successful allotransplantation, is an important biological phenomenon posing an immunological paradox because the semiallogeneic fetus survives by evading the maternal immune recognition [15]. The detailed mechanisms behind this maternal-fetal immunotolerance remain to be elusive. HLA-G has long been speculated as a major factor in maintaining the fetal-maternal immunotolerance for its restricted expression on extravillous cytotrophoblasts and potential ability in the regulation of uterine NK cell functions such as cytotoxicity and cytokine production by interacting with various NK cell receptors during pregnancy [16]. The special functions of HLA-G are contributed to its structures. HLA-G is highly homologus to classical HLA class I molecules, in spite of which it exhibits specific structural characteristics with a shortened cytoplasmic tail which stabilizes its expression on cell surface [17]. Furthermore, alternative splicing of its mRNA gives rise to seven isoforms including four membrane bound (HLA-G1, -G2, -G3, -G4) and three soluble (HLA-G5, -G6, -G7) proteins containing α1 domain. The third, the promoter region of HLA-G significantly differs from that of other HLA Class I genes, with most of the conserved regulatory boxes being deleted, altered or absent, including the interferon (IFN) regulatory sequence [1].

Extensive experiments have been done at both structural and functional levels on the full length of HLA-G1 since its discovery, demonstrating that receipts of HLA-G1 targets such as LCL721.221and K562 could resist NK cell cytolysis [18-20]. However, biological functions of other isoforms have been poorly defined till recently. Riteau *et al* [4] pointed out that HLA-G2, -G3 and -G4 truncated isoforms were expressed as non-mature cell-surface glycoproteins and all of them could inhibit both innate (NK cells) and acquired (CTL) effectors through KIR2DL4, suggesting the α1 domain of HLA-G that contains the functional region, since both α2 and α3 domains are not presented in HLA-G3.

Residues from 77~83 in classical HLA class I α1 domain have been proposed as crucial sites for KIR recognition. In this region, Met^{76} or Gln^{79} are unique to HLA-G among all alleles discovered to date. Therefore, these two sites were speculated to be crucial for KIR2DL4 recognition and this hypothesis was confirmed by our results.

Our data showed that KIR2DL4-IgG Fc fusion protein

bound strongly to HLA-wtG, while much weakerly to the K562-mG cells. Cytotoxicity assay showed that KIR2DL4 could induce high cytotoxicity against K562-wtG cells as compared to the K562 and K562-mG cells. Treatment with anti-KIR2DL4 mAb#64 could dramatically block the lysis of NK-92-2DL4 against K562-wtG cells, while no obvious effects was found to the lysis of K562 and K562-mG with mAb#64 treatment. A discrepancy between previous reports and our results is that whether HLA-G was an immunotolerant molecule which could inhibit the cytolysis of NK cells to various HLA-G expressing targets. In these studies, polyclonal NK cells from peripheral blood were used, which express various NK cell receptors. Whatever interacting with known or unknown receptors, HLA-G renders its bearing targets resist the NK cells lyses [21-23]. Meanwhile, different stages of NK cells and NK cells from different individuals may also contribute to the complicated result interpretation [12]. Kikuchi-Maki A *et al* [24] recently provided strong evidences that the KIR2DL4 containing ITIM does not influence its activating function, which is an additional support to our results. Interestingly, genotype of KIR2DL4 may also influence its function in the view of what was reported by Goodridge *et al* [26], which described that KIR2DL4 is an activating receptor for NK cells from the individuals with at least one 10A allele. Cytotoxicity results in the present study further strengthen that residues Met^{76} or Gln^{79} or both in HLA-G α1 domain could be the recognition site(s) involved in the interaction with KIR2DL4. However, so far we could not exclude the possibility that the weak binding affinity was due to the altering or disrupting of other potential residues specific for KIR2DL4 recognition, for the mutation might change possible binding moiety conformation between HLA-G and KIR2DL4. The present study didn't examine whether the single residue Met^{76} or Gln^{79} mutation contribute to the change of the binding affinity, but this is clearly of interest to further understand the roles of these two residues in the interaction between KIR2DL4 and HLA-G molecules. To address this question, site-directed mutagenesis of the single amino acid residue of the Met^{76} and Gln^{79} is under investigation.

These findings provide insights into the KIR2DL4 recognition of HLA-G protein, and could offer an explanation to such questions as why homozygous HLA-G*0105N individuals didn't show any immunodeficiency, infections or tumors and no record of pregnancy and delivery pathology [27, 28]. Cytosine in exon 3 of HLA-G*0105N, a null HLA-G allele, is deleted to result in a frameshift mutation leading to a stop codon at the beginning of exon 4. Therefore, homozygous HLA-G*0105N individuals can't generate HLA-G1 protein. However, homozygous HLA-G*0105N individual could produce at least HLA-G2, -G3, -G6, and -G7 isoforms [3]. This was supported by Ober *et al* [25] that soluble HLA-G isoforms could be detected in placenta of homozygous HLA-G*0105N individual and Fuzzi *et al* [28] indicated that sHLA-G in early embryos is a fundamental prerequisite for the development of successful pregnancy.

In conclusion, our results not only demonstrate that KIR2DL4 can directly bind to HLA-G, but more importantly, residues Met^{76} and Gln^{79} in HLA-G α1 domain play a crucial role in KIR2DL4 and HLA-G interaction. Ponte *et al* [29] have reported expression of KIR2DL4 only on decidual-associated NK cells, thus raise the possibility that the signaling through KIR2DL4 might be of particular relevance to the maternal-fetal immunotolerance due to the high abundance of uNK cells in the deciduas in the first and second trimesters of pregnancy and their association with trophoblast cells [30].

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