

Soluble HLA-G induces NF- κ B activation in natural killer cells

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Abstract Human leukocyte antigen (HLA)-G is an immunomodulatory molecule discovered for the first time in the maternal–fetal interface. In cancer context, where high number of natural killer (NK) cells is described, the presence of HLA-G in its soluble form is thought to be essential for NK cells signaling. To evaluate intracellular signaling in NK cells upon HLA-G soluble forms stimulation, we investigate the role of soluble HLA-G (HLA-G5- and HLA-G1 shedding form) stimulation on classical nuclear factor (NF)- κ B pathway activation. We reported that these two forms of soluble HLA-G could activate NF- κ B in NK cells. NF- κ B activation in NK cells does implicate neither phosphatidylinositol 3-kinase (PI3K) nor MEK (MAP kinase kinase) as demonstrated after specific inhibition experiments. We demonstrated elsewhere that NF- κ B activation in NK cells is not implicated in cytotoxicity inhibition by HLA-G. Our findings may suggest the important role played by

NF- κ B activation after soluble HLA-G stimulation in other NK cells function.

Keywords HLA-G · NF- κ B · NK cells · Signal transduction

Introduction

HLA-G is a non-classical human leukocyte antigen (HLA) class I antigen playing an important role in immune tolerance [1]. For instance, HLA-G molecule could inhibit cytotoxicity of natural killer (NK) [20, 27, 29] and CD8⁺ T cells [15]. In addition, it could inhibit alloproliferation of T cells in mixed lymphocyte reaction [17, 18] and polarize cytokine secretion to the Th2 profile [11, 12]. Many studies have greatly advanced in the knowledge about HLA-G functions in immune responses regulation, thus underlining its crucial role in understanding pathologies. The expression of HLA-G molecule by grafts avoids their rejection [28]. By contrast, the expression of HLA-G molecule by tumoral cells allows their escape from immune system [30].

After alternative splicing of the HLA-G primary transcript, seven isoforms are, at least, produced. Four are membrane-bound forms (HLA-G1, HLA-G2, HLA-G3, and HLA-G4), and three are soluble forms (HLA-G5, HLA-G6, and HLA-G7). In addition, a shedding form of HLA-G1 was described. This later is produced by metalloproteinases proteolytic cleavage at the cellular membrane. The cleavage site is situated at ten or 20 amino acids apart from the distal

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of $\alpha 3$ domain. The produced shedding HLA-G1 form is smaller than HLA-G5 by about 1–2 kDa [22].

HLA-G receptors are divided into specific receptors (killer immunoglobulin-like receptor, KIR2DL4) and non-specific [immunoglobulin-like transcript receptor (ILT)-2, ILT-4] inhibitory receptors [3, 39]. However, questions remain regarding the signaling pathways driven by HLA-G in immune cells. In a single and recently published study, we demonstrated that HLA-G activates the classical NF- κ B pathway, with subsequent production of I κ B- α subunit after accumulation of NF- κ B in the nucleus. We showed that a membrane form of HLA-G (HLA-G1) rapidly activates NF- κ B in NK cells. Interestingly, we demonstrated that this activation occurs through the alpha1 domain of HLA-G1. Finally, we hypothesized that NF- κ B activation may result from a direct interaction of HLA-G1 with KIR2DL4 receptor [9].

NF- κ B is a ubiquitous heterodimeric transcription factor implicated in pro-inflammatory responses, auto-immune diseases, and cancer. The most known complex is constituted from p50 and p65 (relA) subunits. In its inactive form, NF- κ B is associated to the inhibitory protein I κ B- α . After activation of NF- κ B, I κ B- α will be degraded by the 26S proteasome, thus releasing the dimers and allowing their translocation to the nucleus.

Since the presence of high levels of HLA-G soluble forms in the serum of patients with melanoma [36] or lymphoproliferative disorders [31] and of high number of NK cells in tumor tissues associated to NF- κ B constitutive activation in numerous tumors [14], we investigated in this present work the classical NF- κ B signaling pathway activation mediated by soluble HLA-G in NK cells. We focused on the role of two soluble forms of HLA-G: HLA-G5 and the shedding form of HLA-G1, in NF- κ B activation in NK cells, which has not been addressed yet. In addition, we investigated whether NF- κ B activation by HLA-G could implicate PI3K/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways. We focused also on the implication of NF- κ B activation in NK cells cytotoxicity inhibition by HLA-G.

Materials and methods

Antibodies and reagents For immunoblotting, we used specific antibodies raised against I κ B- κ anti-

I κ B- α (Santa Cruz, CA, USA) against the phosphorylated form of I κ B- α (Ser32/Ser36, anti-pI κ B- α Cell Signalling Technology, Danvers, USA) and against tubulin (Sigma, Saint-Quentin Fallavier, France). Recombinant human TNF- α purchased from Santa Cruz Biotechnology was dissolved in sterilized water. phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294.002 and MAP kinase kinase (MEK) inhibitor PD-98.059, purchased from Sigma, were dissolved, respectively, in ethanol and dimethyl sulfoxide (DMSO). The synthetic peptide corresponding to the alpha-1 domain of HLA-G was produced by Jerini (Berlin, Germany) and dissolved in 10% acetonitrile.

Cell culture and treatments M8 melanoma cells transfected with HLA-G1cDNA (M8-HLA-G1) or with empty vector pcDNA3.1/hygro(-) (M8-pcDNA) as previously described [23] were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 μ g/ml hygromycin B. Two NK cell lines were used: the NKL and YT2C2-PR. NKL cell line (kindly provided by E.H. Weiss, Munich, Germany) was cultured in RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, and 50 U/ml IL-2 (Sigma-Aldrich). NK-like YT2C2-PR cell line was maintained in RPMI medium supplemented with 10% FCS and 2 mM L-glutamine. All maintenance media contained 10 μ g/ml gentamicin and 0.25 μ g/ml fungizone (Invitrogen). NKL cells express both ILT2 and KIR2DL4 receptors whereas YT2C2-PR cells express only KIR2DL4 as previously described [4, 9, 26]. For co-culture experiments, we incubated non-adherent NK cells, as previously described [9], on a layer of M8-pcDNA or M8-HLA-G1 adherent cells. PI3K inhibitor LY-294.002 and MEK inhibitor PD-98.059 were used at 50 and 30 μ M, respectively, as previously described [5, 8].

Preparation of shedding HLA-G1 and HLA-G5 beads HLA-G1 shedding form was produced in the supernatant of M8-HLA-G1 cells treated with TNF- α at 50 ng/ml for 12 h as previously described [40]. Recombinant HLA-G5 protein was produced in SF9 insect cells transfected with both HLA-G5 baculovirus and human beta2-microglobulin (Appligene-Oncor, Illkirch, France). After purification of HLA-G5 protein from SF9 supernatant, it was captured onto magnetic microbeads (diameter 300 nm, Ademtech,

Pessac, France). HLA-G5-coated beads were used as a source of HLA-G5 at 4×10^3 beads/cell.

Immunoblot analysis The immunoblotting procedure used in the present study has already been described in our previous study [40]. Briefly, after cell stimulations proteins were extracted in a buffer containing 50 mM Tris-HCl, pH7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, and complete protease inhibitor (Roche, Basel, Switzerland) and quantified by the micro-BCA assay (Pierce, Rockford, USA). Protein extracts were mixed with an equal volume of Laemmli's buffer with 10% dithiothreitol followed by heating for 5 min at 95°C. Proteins were loaded on SDS-polyacrylamide gel, subjected to electrophoresis and finally transferred onto nitrocellulose membrane. Blots were either incubated with anti-I κ B- α or with anti-pI κ B- α . For the detection of the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated antibodies and treated with enhanced chemiluminescent substrate (SuperSignal West Pico, Pierce). Intensities of signals were analyzed using scanning densitometry AlphaEaseFC Software (Fluorchem 8800 imaging). Probed membranes with anti-I κ B- α or with anti-pI κ B- α were stripped of bound antibodies and reprobed with anti-tubulin used for normalization of densitometric measurements.

Cytotoxicity assay The implication of NF- κ B activation in the decreased cytotoxicity after HLA-G stimulation was determined by cytotoxicity assay that measure ^{51}Cr released from target cells (M8-pcDNA cells versus M8-HLA-G1). NKL effector cells were previously stimulated overnight with IL-2 (100 U/ml, Sigma) at 37°C. M8-pcDNA or M8-HLA-G1 target cells previously labeled for 1 h with 100 μCi ^{51}Cr were incubated for 4 h at 37°C with NKL cells. Supernatants (50 μl) were harvested for scintillation counting by Wallac 1450 Microbeta (EGG instruments, Every, France). The experiment was reproduced four times in triplicate. We calculated the percentage of specific lysis as previously described. Briefly, percentage of specific lysis equals $[(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$. Blockage experiments of NF- κ B with a specific inhibitor: BAY 11-7082 (BAY, Calbiochem, Darmstadt, Germany) [24] were carried out by stimulation of NKL cells with 5 μM of BAY for 1 h at 37°C under 5% CO_2 before cytotoxic assay.

Statistical analysis Statistical differences between means in each experiment were performed using Student's *t* test and ANOVA test. The difference was considered statistically significant when $p < 0.05$ with 95% confidence interval.

Results

Soluble HLA-G5 activates NF- κ B in NK Cells HLA-G5 is the most important soluble form of HLA-G. It is implicated in various functions of HLA-G either in vitro or in vivo [16, 20]. NF- κ B activation by soluble HLA-G5 stimulation was determined by Western blotting using anti-phospho-I κ B- α . NKL cells ($5 \times$

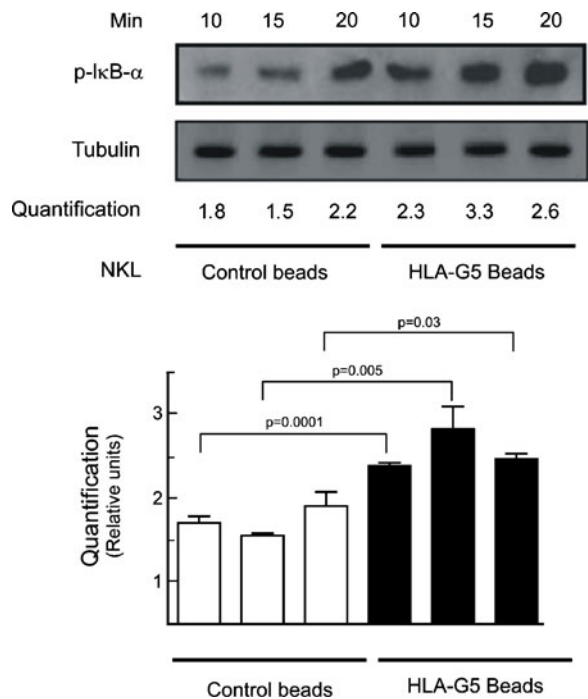


Fig. 1 Soluble HLA-G5 induces I κ B- α phosphorylation in NK cells. NKL cells were incubated either with control beads suspended in PBS 0.1% BSA or with beads coated with soluble HLA-G5 for various period of time: 10, 15, 20 min. After incubation, cells were lysed and subjected to immunoblotting assay with an antibody specific of I κ B- α phosphorylated form (anti-pI κ B- α). The same blot was probed with an antibody directed against tubulin to control quantities of loaded proteins. Bands were revealed using chemiluminescence and were quantified by scanning densitometry. The *upper panel* shows results from one representative experiment from four others. The *bottom panel* shows the quantification of I κ B- α phosphorylation. Values represents means \pm SEM ($n=4$). *p* values were measured by *t* test

10^5 cells/ml) were treated by HLA-G5-coated beads (at 4×10^3 beads/cell) at three different times: 10, 15, and 20 min. We observed, as shown in Fig. 1, that soluble HLA-G5 phosphorylates the I κ B- α subunit, in a time-dependent manner with a maximum at 15 min. Densitometric measurements show statistical significance of I κ B- α phosphorylation which reveals the activation of NF- κ B pathway in NKL cell line by soluble HLA-G5.

Shedding HLA-G1 activates NF- κ B in NK cells It was reported that the proteolytic cleavage of HLA-G1 by metalloproteinases produced another HLA-G soluble form commonly known as HLA-G1 shedding [22] which is thought to be abundant in cancer context [2]. To assess whether this soluble form (HLA-G1 shedding) was capable to activate the classical NF- κ B pathway, we performed experiments providing NF- κ B pathway activation through the I κ B- α subunit cleavage.

NKL cells (5×10^5 cells/ml) were treated with supernatants isolated from M8-HLA-G1 cells previously stimulated or not with 50 ng/ml of TNF- α for 12 h. These supernatants, respectively, contain or not, the shedding HLA-G1 form. As previously described [40], 50 ng/ml of TNF- α gives a higher amount of HLA-G1 shedding form reaching 115 ng/ml.

As shown in Fig. 2, we described a significant degradation of I κ B- α in column 5 corresponding to a co-incubation of NKL cells with culture supernatant of M8-HLA-G1 cells containing HLA-G1 shedding ($p < 0.005$ vs. column 4 corresponding to co-incubated NKL cells with culture supernatant of M8-HLA-G1 cells without HLA-G1 shedding molecule and $p < 0.05$ vs. column 1 corresponding to NKL cells alone control). We also described an I κ B- α degradation when we co-incubate NKL cells with culture supernatant of M8-pcDNA cells without HLA-G1 shedding form. This later degradation, statistically not significant (Fig. 2), could be attributed to an induced cleavage due to the excess of TNF- α in the supernatant. Taken together, these data suggest that HLA-G1 soluble form is implicated in I κ B- α degradation.

NF- κ B pathway activation by HLA-G1 in NK cells does not implicate PI3K/Akt pathway PI3K/Akt pathway is one of the ubiquitous signaling pathways leading to NF- κ B activation [34]. In order to study the involvement of this pathway in NF- κ B activation

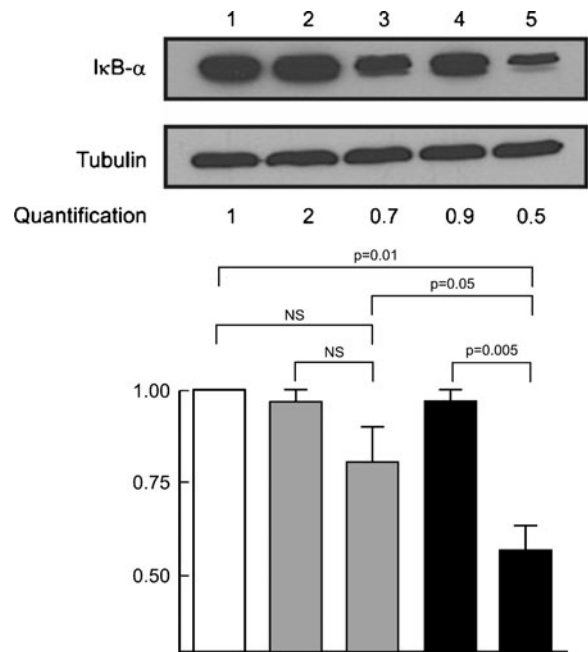


Fig. 2 HLA-G1 shedding form induces I κ B- α degradation in NK cells. NKL cell lines were incubated for 1 h at 37°C with supernatants of M8-HLA-G1 cells (versus M8-pcDNA), treated or not with TNF- α for 12 h. These supernatants, respectively, contain or not the HLA-G1 shedding molecules. After incubation, cells were lysed and subjected to SDS/PAGE and Western blotting assay with the specific antibody of I κ B- α subunit (anti-I κ B- α). Bands were revealed using chemiluminescence and were quantified by densitometry. Level 1 was assigned to the non-treated NKL cell lines. The upper panel shows results from one experiment representative of two others. The bottom panel shows densitometric measurements of I κ B- α phosphorylation after tubulin normalization. Values represent means \pm SEM. p values were measured by t test or ANOVA test (column 1 vs. column 3 or vs. column 5). Abbreviations: NS not significant p value. 1 NKL, 2 NKL+culture supernatant of M8-pcDNA (untreated cells), 3 NKL+culture supernatant of M8-pcDNA (cells treated during 12 h with TNF- α), 4 NKL+culture supernatant of M8-HLA-G1 (untreated cells), 5 NKL+culture supernatant of M8-HLA-G1 (cells treated during 12 h with TNF- α)

after HLA-G1 stimulation, cells were pre-incubated with 50 μ M of LY-294.002, a specific inhibitor of PI3K for 1 h before incubation on a layer of M8-HLA-G1 cells for 15 and 30 min. The representative experiment shown in Fig. 3 demonstrates that there are no significant differences in I κ B- α phosphorylation between treated and non-treated cells with the PI3K inhibitor in respective times. Statistical tests performed on densitometric measurements confirmed this observation. I κ B- α phosphorylation was not blocked after inhibitor use, proving that NF- κ B

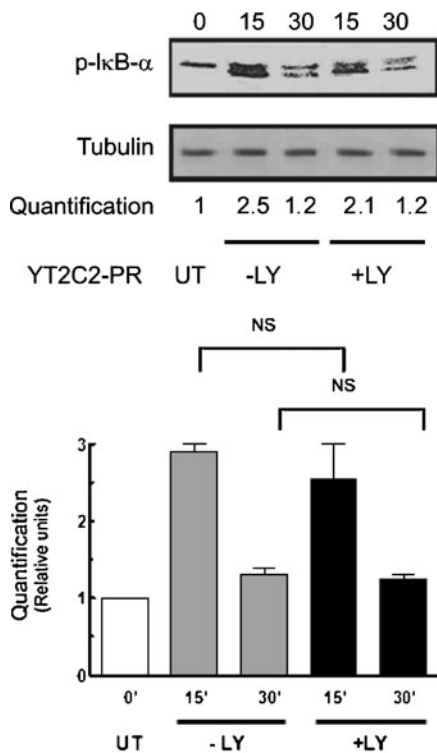


Fig. 3 PI3K is not implicated in NF- κ B activation by HLA-G1. YT2C2-PR were pre-treated with 50 μ M PI3K inhibitor LY-294.002 ($n=2$) for 1 h at 37°C and then incubated on a layer of M8-HLA-G1 cells for 15 and 30 min. After extraction, proteins were analyzed for the phosphorylation of I κ B- α (pI κ B- α). The nitrocellulose membrane was stripped and reprobed with anti-tubulin antibody to confirm equal loading. In quantification by scanning densitometry, we assigned the level 1 to the control lane corresponding to cells treated with ethanol. The *upper panel* shows a representative experiment, and the *bottom panel* shows the mean \pm SEM. p values were measured by t test. Abbreviations: LY LY-294.002, NS not significant p value, UT untreated cells, *minus sign* absence; *plus sign* presence

activation by HLA-G1 in NK cells does not implicate PI3K in this experimental condition.

To ascertain the obtained results, we studied the effect of a synthetic HLA-G peptide corresponding to the α 1 domain (90 first amino acids of HLA-G exon 2). This later domain is, in one hand, shared by all HLA-G forms, and in the other hand, implicated in the inhibition of NK-cytotoxicity [29]. The performed experiments with the synthetic peptide gives the same result as presented in Fig. 3 (data not shown).

NF- κ B pathway activation by HLA-G1 in NK cells does not implicate MAPK/ERK pathways To determine whether NF- κ B activation involves other path-

ways like MAPK/ERK pathway, we employed a specific inhibitor of MEK which is PD-98.059. Pre-treatment of NK cells with 30 μ M of PD-98.059 for 1 h before incubation on a layer of M8-HLA-G1 cells for 15 and 30 min did not change the extent of I κ B- α phosphorylation. This result suggests that MEK is not implicated in activation of NF- κ B by HLA-G1 in NK cells (Fig. 4). We found the same results with the synthetic HLA-G peptide corresponding to the α 1 domain (data not shown).

NF- κ B pathway activation by HLA-G1 in NK cells is not implicated in cytotoxicity inhibition To evaluate the implication of NF- κ B activation by HLA-G1 molecule, we performed a cytotoxicity assay employing NKL cells as effectors cells and M8-pcDNA or M8-

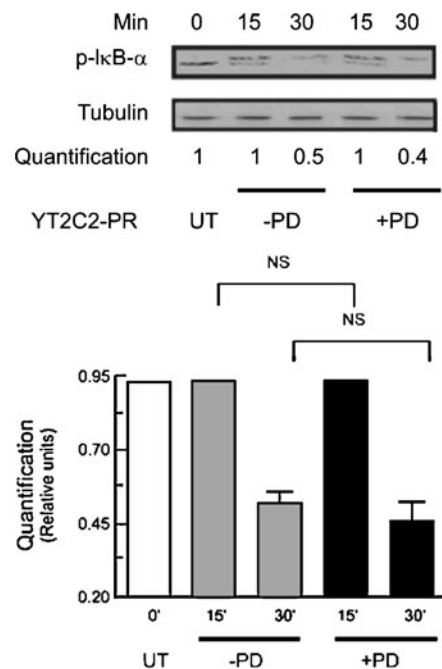


Fig. 4 MEK is not implicated in NF- κ B activation by HLA-G1. YT2C2-PR were pre-treated with 30 μ M MEK inhibitor PD-98.059 ($n=2$) for 1 h at 37°C and then incubated for 15 or 30 min on a layer of M8-HLA-G1 cells. After protein extraction, samples were subjected to SDS/PAGE and Western blotting with anti-phosphorylated I κ B- α (pI κ B- α). The nitrocellulose membrane was stripped and reprobed with anti-tubulin antibody to confirm equal loading. In quantification by scanning densitometry, we assigned the level 1 to the control lane corresponding to cells treated with DMSO. The *upper panel* shows a representative experiment, and the *bottom panel* shows the mean \pm SEM. p values were measured by ANOVA test. Abbreviations: NS not significant p value, PD PD-98.059, UT untreated cells, *minus sign* absence, *plus sign* presence

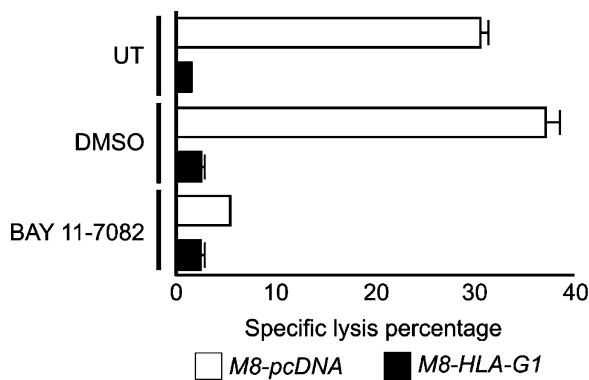


Fig. 5 NF- κ B is not implicated in NK cytotoxicity inhibition after HLA-G1 stimulation. The figure shows an experiment of ^{51}Cr release assay. NKL effectors were pre-treated or not with either 5 μM NF- κ B inhibitor BAY 11-7082 ($n=4$) or its control DMSO, for 1 h at 37°C, and then incubated for 4 h with chromium-labeled target cells (M8-pcDNA or M8-HLA-G1 cells). Each treatment cell condition was performed in triplicate. Values represent means \pm SEM. Abbreviations: *UT* untreated NKL cells

HLA-G1 as target cells. The assay confirmed the decrease in lysis capacity of NKL cells against M8-HLA-G1 both with untreated NKL cells and the DMSO-treated one. Moreover, NKL treatment with BAY 11-7082, a specific inhibitor of NF- κ B pathway, has shown that NKL cell cytotoxicity inhibition by HLA-G1 is independent of NF- κ B pathway. In fact, specific lysis percentage was not reversed after the use of M8-HLA-G1 cells (Fig. 5). Cytotoxicity assay analysis after NF- κ B inhibition revealed also the same significant decrease with M8-pcDNA cells. These results obtained with NF- κ B inhibitor demonstrated the implication of NF- κ B pathway in NKL cytotoxicity.

Discussion

NK cells are known as potent immune cells implicated in innate responses as well as in adaptative responses after interaction, like with dendritic cells [6]. In the privileged cancer context, NK cells were largely represented [21, 35] as well as soluble HLA-G molecules [31, 32] suggesting a potential role of HLA-G molecules in NK cells signaling and probably in the modulation of their function. Membranous HLA-G1 molecules have been described as activators of NF- κ B pathway toward NK cell lines [9]. However, the implication of soluble HLA-G forms in classical NF- κ B pathway activation has not been

investigated. To provide more insights into this activation, we used HLA-G5 and HLA-G1 shedding soluble molecules. Our choice was guided by the fact that, in one hand, HLA-G5 molecule, generated by alternative splicing, is the most described in cancer [31] and that, in the other hand, shedding HLA-G1 form, generated by proteolytic cleavage, is proposed to be enhanced in stress conditions associated to cancer [2].

In the current study, we reported for the first time that NK cell stimulation by soluble HLA-G5 activates NF- κ B in a time-dependent manner (Fig. 1). The basal observed activation obtained with control beads could be explained by their composition and/or their elevated concentration of 4×10^3 beads/cell.

Moreover, we demonstrated an expressed I κ B- α degradation, showing NF- κ B activation upon stimulation by HLA-G1 shedding form (115 ng/ml; Fig. 2). In the same figure, we observed a significant decrease between control (column 1) compared with column 3 probably due to the excess of TNF- α needed for the cleavage of membranous HLA-G1 present in the surface of M8-HLA-G1 cells, contained in used culture medium. This observed decrease is enhanced after exposure to shedding HLA-G1 (column 3 vs. 5 in Fig. 2) showing that NF- κ B activation, in NK cells, is dependent from shedding HLA-G1. These findings are in line with our previous study which has yielded that NF- κ B was activated by membranous HLA-G1 form in NK cells after their co-culture with transfected melanoma cell line M8-HLA-G1 [9].

The demonstrated activation of NF- κ B pathway either by soluble HLA-G5 or by shedding HLA-G1 form may occur probably through KIR2DL4 receptor. In fact, NF- κ B activation was shown in YT2C2-PR cell line which expresses only KIR2DL4 receptor. However, we have not confirmed directly KIR2DL4's implication in NF- κ B activation by soluble forms of HLA-G because of the absence of efficient blocker antibodies. But, we cannot exclude the implication of other unknown receptors for HLA-G in this activation.

Because it is well described that hypersignaling via the PI3K/AKT/mTOR axis might have an important role in cancer disease [38], and because PI3K and ERK are implicated in NK cytotoxicity function [10], we checked in this work the implication of PI3K and MEK in NF- κ B activation. We demonstrated that pre-incubation of NK cells with specific inhibitors of PI3K and MEK do not change NF- κ B level activation. We concluded that PI3K and MEK seem not to

be implicated in this activation. But interestingly, another study has demonstrated the implication of Akt [34] and MAPK/ERK [37] in NF- κ B activation. These disparities may be due to differences in model cells and/or working conditions.

The limitation of the present study is the absence of NK function's regulation dependent from NF- κ B activation upon soluble HLA-G stimulation. In fact, the strong inhibitory effect of HLA-G molecule on NK cytotoxicity is completely independent of NF- κ B (Fig. 5). This result was in agreement with Mami-Chouaib's study where NF- κ B inhibition abrogates NK cytotoxicity [19]. Concerning immunomodulatory function, we hypothesize that the two forms of HLA-G, shedding HLA-G1 as well as soluble HLA-G5, might have the similar one. In fact, it was demonstrated that both proteins inhibit NK-mediated cytotoxicity [20, 22]. This could be explained by the same conformation shared by the two molecules. In fact, the unique structural difference founded between them is the cleaved amino acids (1 to 2 kDa) [22]. Further investigations will undoubtedly shed light on specific functions of HLA-G forms.

Because NK cells could secrete cytokines without degranulation [6], we proposed that activated NF- κ B pathway by soluble HLA-G could be implicated in cytokine modulation and chemokine synthesis. The presence of NF- κ B binding sites in numerous cytokine promoters, such as for IFN- γ , TNF- α , and IL-8 [7, 13, 33], and the results given by Rajagopalan study, which reported that soluble HLA-G protein can enhance pro-inflammatory and pro-angiogenic cytokines and also chemokines [25], underline this putative role of NF- κ B activation. There is still a need to increase our knowledge about NF- κ B activation after soluble HLA-G stimulation in such announced functions and also in other functions like apoptosis or inhibition of NK proliferation.

In summary, the present study corroborated our previous study showing that membranous HLA-G1 activates classical NF- κ B pathway in NK cell [9]. Here, we have proved that HLA-G1 shedding form as well as soluble HLA-G5 form can activate NF- κ B in NK cells. This activation is thought to be relevant in heterogeneous population of NK cells. We reported, elsewhere, that the activation of NF- κ B does not require PI3K or MEK kinase function. Finally, further studies are needed to explore the role of NF- κ B activation by soluble HLA-G forms in NK cells,

giving new insights on HLA-G inhibitory function mechanism that might be exploited in future for therapeutic benefit.

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