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Research Article

Increase in HLA-G1 proteolytic shedding by tumor cells: a regulatory pathway controlled by NF-κB inducers

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Abstract. HLA-G is expressed by tumors, in which it contributes to the evasion of immunosurveillance. NF-κB appears to be a candidate for regulating HLA-G expression, since it is considered to be a hallmark of cancer. We investigated the role of NF-κB in modulating HLA-G expression in HLA-G-positive tumor cells, JEG-3 (choriocarcinoma), FON (melanoma), and M8-HLA-G1 (HLA-G1-transfected melanoma). The treatment of tumor cells with two NF-κB inducers, tumor necrosis factor-α and phorbol 12-myristate 13-acetate, decreased HLA-G1 cell surface expression but increased intracytoplasmic HLA-G proteins. Reduction in HLA-G1 cell surface expression is driven by NF-κB and involves a proteolytic shedding process dependent on metalloproteinase activity. In contrast, an increase in intracytoplasmic HLA-G proteins involves post-transcriptional mechanisms that are independent of NF-κB. These results, and the fact that soluble HLA-G1 reduces the cytotoxicity of the NKL cell line, lead us to propose a novel regulatory pathway for HLA-G expression by tumor cells that may have particular relevance in tumor escape.

Keywords. HLA-G, TNF-α, PMA, proteolytic shedding, tumor.

HLA-G molecules play a major role in immune tolerance and are frequently expressed in malignant lesions [1, 2]. The NF-κB transcription factor plays a pivotal role in both innate and adaptive immunity, leading to the induction of multiple genes that regulate immune and inflammatory responses [3, 4]. Moreover, NF-κB is constitutively activated in various cancers [4]. Therefore, in attempting to identify mechanisms involved in the regulation of HLA-G expression in tumor cells, we investigated the role of NF-κB as a potential HLA-G regulator.

HLA-G molecules are selectively expressed in healthy tissues of the placenta, thymus, and cornea and are secreted by erythroblasts from primitive to definitive hematopoiesis [1, 5]. However, during malignant transformation, HLA-G expression may be extended to various tissues in both tumors and tumor-infiltrating cells [2]. Increased soluble HLA-G levels are also observed in serum and in ascites from patients with malignancies [6, 7]. Functionally, HLA-G inhibits the cytotoxicity of natural killer (NK) cells and T lymphocytes [8–11] and inhibits alloproliferative responses [12–15] through interaction with inhibitory receptors such as KIR2DL4 (CD158d), ILT2 (CD85j), and ILT4 (CD85d) [16–20]. HLA-G molecules are generated by alternative splicing of the HLA-G primary transcript. Four of them, HLA-G1, HLA-G2, HLA-G3, and HLA-G4, are membrane-bound proteins, whereas HLA-G5, HLA-G6, and HLA-G7 are soluble forms [1]. Functionally shed forms of HLA-G1 have also been described [21]. HLA-G molecules are involved in maternofetal tolerance [22, 23], embryo implantation

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[24–29], and graft acceptance [30–34], and based on these immunotolerant properties may be employed by tumor cells to escape the immune system [35–37].

NF-κB is a ubiquitous transcription factor present in the cytoplasm as homo- or heterodimers of a family of structurally related proteins. Most predominant is the p50-RelA dimer, present in an inactive form in association with the inhibitory protein IκB-α [38]. NF-κB responds to a wide variety of agents [39], the most well known of which are phorbol esters [40] and tumor necrosis factor-α (TNF-α), which is produced by many tumor cells [41]. These agents lead to phosphorylation and degradation of IκB-α by the 26S proteasome, thus releasing the NF-κB dimer [42]. This free NF-κB translocates from the cytoplasm to the nucleus, where it mediates transcription of target genes involved in immunity [43], inflammation [44], apoptosis [45] and the cell cycle [46]. NF-κB is also associated with cancers [4], especially with hematopoietic malignancies, cancers of the breast, colon, ovary, prostate, liver, and pancreas, and melanoma [47]. In particularly, activation of NF-κB is a crucial mediator of inflammation-induced tumor growth and progression, and may predict the metastatic potential of tumors [48].

NF-κB factors are known to regulate the expression of classical HLA class I molecules because of the presence of two κB sites within the proximal promoter gene region [49]. This is not the case for HLA-G [50], which exhibits an unusual promoter region, since HLA class I cis-acting regulatory elements are disrupted or non-functional [51, 52]. Despite the fact that the HLA-G proximal promoter gene is unresponsive to NF-κB [53], we cannot exclude the presence of other κB target sites outside this promoter region, or the possible indirect effect of NF-κB-mediated post-transcriptional or post-translational events in modulating HLA-G expression. Since NF-κB may be a target candidate in cancer treatment, it is critically important to define whether such NF-κB-targeted therapy strategies might modulate HLA-G, thereby favoring immune tolerance of tumor cells.

In the present report, we demonstrate that the NF-κB inducers TNF-α and phorbol 12-myristate 13-acetate (PMA) reduce HLA-G1 expression at the cell surface in choriocarcinoma and melanoma cell lines. Such a decrease in HLA-G1 surface expression is regulated by NF-κB and involves proteolytic shedding of membrane-bound HLA-G1 molecules. On the other hand, intracytoplasmic HLA-G protein expression in these tumor cell lines is enhanced by post-transcriptional mechanisms that are independent of NF-κB activation.

Materials and methods

Antibodies and reagents. The following antibodies were used for flow cytometry studies: anti-HLA-G MEM-G/9 (mouse IgG1; Exbio, Prague, Czech Republic) and 87G (mouse IgG2a; Exbio) [54]; anti-HLA-A, -B, -C SV99-86 (mouse IgG kindly provided by Dr. S. Ferrone, Roswell Park Cancer Institute, Buffalo, N.Y.); anti-CD54-FITC (anti-ICAM-1; Immunotech, Marseille, France) and PE-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (Immunotech) as a secondary antibody. The following antibodies were used for immunoblotting: IκB-α (C-21) sc-371 (Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-pan HLA-G mAb 4H84 (mouse IgG1; M. McMaster, University of California, San Francisco, Calif.), anti-tubulin mAb (Sigma, Saint-Quentin Fallavier, France), horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (Sigma). For ELISA, we used anti-HLA-G MEM-G/9, a rabbit polyclonal anti-human β2-microglobulin conjugated to horseradish peroxidase (DAKO, Trappes, France) as a detection antibody. IκB-α inhibitor BAY 11-7082 [55], metalloproteinase inhibitor GM6001 was supplied by Calbiochem (Darmstadt, Germany); PMA, cycloheximide, interleukin (IL)-2, PI3K inhibitor LY-294.002, and MEK inhibitor PD-98.059 were from Sigma; complete protease inhibitor cocktail was from Roche (Meylan, France); TNF-α was purchased from Santa Cruz Biotechnology and interferon (IFN)-β from Tebu-Bio (Perray en Yvelines, France). PMA and BAY 11-7082 were reconstituted in dimethyl sulfoxide (DMSO).

Cell lines and culture. The JEG-3 choriocarcinoma cell line (American Type Culture Collection) was maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Cergy Pontoise, France) at 37 °C under 5% CO_2. The FON melanoma cell line derived from a primary melanoma lesion (kindly provided by Dr. F. Faure, Institut Curie, Paris, France) was cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine (Invitrogen). Both JEG-3 and FON (FON^{G1+}) naturally express HLA-G molecules at the cell surface [56]. We used also FON^{G1-} cells, which do not express HLA-G1 at their membranes and strongly express the HLA-G2 isoform [56]. M8 melanoma cells transfected with HLA-G1 (M8-HLA-G1) as described previously [57] were cultured in RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, and 100 µg/ml hygromycin B. The NKL cell line, kindly provided by E. H. Weiss (Munich, Germany) was maintained in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine and 50 U/ml IL-2 (Sigma-Aldrich). This cell line established from a patient with an aggressive NK cell leukemia expresses a large range of NK antigens and exhibits cytolytic activity [58]. All maintenance media contained 10 µg/ml gentamicin and 0.25 µg/ml fungizone (Invitrogen).

Cell treatments. For NF-κB activation, cells were exposed in complete medium, or in medium without serum in some experiments, to 50 ng/ml recombinant human TNF-α (Santa Cruz Biotechnology) or to 10 ng/ml PMA (Sigma) for 12 h. For NF-κB inhibition, cells were treated with 5 µM BAY 11-7082 (Calbiochem), which inhibits the phosphorylation of IκB-α [55, 59, 60]. For metalloproteinase inhibition, we treated cells with commonly used reagents: EDTA [21] at 10 mM or GM6001 [61] at 10 µM (Calbiochem). Cycloheximide at 20 µg/ml (Sigma) was used for protein synthesis inhibition. Inhibition of PI3K/Akt and MAPK/ERK pathways was carried out as previously reported [62, 63] by using, respectively, LY-294.002 at 50 µM (Sigma) and PD-98.059 at 30 µM (Sigma). Control experiments were carried out in the presence of the solvent reagent alone: DMSO for PMA, BAY 11-7082, and PD-98.059; ethanol for LY-294.002; H_2O for TNF-α. After treatment, cell viability was checked with trypan blue exclusion. The responsiveness of the HLA-G gene upon treatment for 12 h was validated by stimulating cells with 2000 U/ml IFN-β.

Flow cytometry. After washing the cells in PBS containing 2% FBS, they were incubated with primary antibodies for 20 min at 4 °C, followed by two washes. When necessary, PE-conjugated $F(ab')_2$ fragment goat anti-mouse IgG was used as a secondary antibody. Isotype-matched control antibodies were systematically used to evaluate non-specific binding. Flow cytometry analysis was carried out using an Epics XL cytometer (Beckman Coulter) and EXPO32 software (Beckman Coulter). Specific fluorescence indexes (SFIs) were calculated by dividing the mean fluorescence obtained with specific antibody by the mean fluorescence obtained with the isotypic control antibody. The effect of treatment was calculated by the variation rate in the SFI: ΔSFI = (SFI T – SFI NT)/SFI NT, where SFI T = SFI of treated cells and SFI NT = SFI of untreated cells.

Western blot analysis. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, and complete protease inhibitor (Roche, Basel, Switzerland) for 20 min at 4 °C. The lysates were centrifuged at 14,000 g for 20 min at 4 °C to remove the insoluble debris. Equal amounts of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were analyzed by immunoblot, as previously described [64]. Intensities of the HLA-G bands were analyzed using AlphaEaseFC software (fluorchem 8800 imaging) and normalized according to the amounts of tubulin.

ELISA. Shed HLA-G1 (sHLA-G1) concentrations in the culture supernatants of M8-HLA-G1 cells were measured by sandwich ELISA, according to the workshop procedure (Essen, Germany, 2005) [65]. Briefly, 100 µl of the culture supernatants (in duplicate) was incubated with 10 µg/ml capture antibody (MEM-G/9) overnight at 4 °C. The detection antibody (anti-human β2-microglobulin conjugated to horseradish peroxidase) was added for 1 h at 37 °C, followed by addition of the substrate (tetramethylbenzidine; Sigma) for 30 min in the dark. The reaction was stopped with 1 N HCl. The optical density was measured at 450 nm. The ELISA detection limit was 5 ng/ml.

Quantitative and classical RT-PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. QRT-PCR targeting all HLA-G mRNA was carried out with an ABI Prism 7000 SDS (Applied Biosystems, Courtabœuf, France) in a duplex PCR with GAPDH as an endogenous control, as previously described [66].
Classical RT-PCR was carried out by coamplification of HLA-G cDNAs using the G.257F/G.1004R pan-HLA-G primers and β-actin according to the 13th HLA workshop, as previously described [57].

NKL cytotoxicity. Analysis of NKL cytotoxicity was performed by ^{51}Cr release assays using M8-pcDNA target cells incubated with culture supernatants from M8-HLA-G1 cells either treated or not with TNF-α (50 ng/ml) for 12 h. NKL cells were previously stimulated with IL-2 (Sigma) at 100 U/ml for 18 h at 37 °C under 5% CO_2 and then incubated for 1 h with culture supernatants. M8-pcDNA target cells were labeled for 1 h at 37 °C with 100 µCi ^{51}Cr and incubated with NKL cells for 4 h at 37 °C and 5% CO_2 at effector/target cell ratios of 20 : 1, 10 : 1 and 5 : 1. After incubation, 50 µl of the supernatants from cell cocultures were harvested for scintillation counting (Wallac 1450 Microbeta; EGG Instruments, Evry, France). All experiments were performed in triplicate in a final volume of 200 µl. The percentage of specific lysis was calculated as follows: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. For maximum and spontaneous release, targets cells were incubated with 100 µl of HCl 0.1 M or with 80 µl of medium, respectively. Blockage experiments of sHLA-G1 were carried out by adding either 87G anti-HLA-G or IgG2a-isotype antibodies (irrelevant) in culture supernatants from TNF-α-treated M8-HLA-G1 cells during 30 min before cytotoxic assay.

Statistical analysis. Statistical significance was assessed using a paired t-test and differences were considered to be statistically significant at $p < 0.05$.

Results

NF-κB inducers decrease HLA-G1 cell surface expression on tumor cells. TNF-α or PMA were added to cultures of JEG-3 choriocarcinoma and FON melanoma cells, both of which constitutively express HLA-G1 at the cell surface. In Figure 1, NF-κB activation through TNF-α and PMA treatment of JEG-3 and FON cells is verified by SDS-PAGE analysis of IκB-α, the inhibitory subunit of NF-κB. As expected, IκB-α degradation was observed only a few minutes after these treatments.

HLA-G expression on JEG-3 and FON cells following TNF-α stimulation (n = 15 and n = 8, respectively) or PMA stimulation (n = 18 and n = 10, respectively) was analyzed by flow cytometry. We observed that cell exposure to NF-κB inducers for 12 h generated a significant and reproducible decrease in HLA-G1 cell surface expression on JEG-3 and FON cells (Fig. 2a, b). The TNF-α-induced decrease in HLA-G1 surface expression was also observed on M8 melanoma cells transfected with HLA-G1 cDNA (M8-HLA-G1; Fig. 2a, n = 7). Conversely, an increase in classical HLA-A, -B, and -C molecules was observed at the cell surface of M8-HLA-G1 and FON cells. The observed variations in HLA-G1 and classical class I cell surface expression were not affected by treating M8-HLA-G1 and FON with TNF-α either in serum-free medium (Fig. 3b) or in medium containing serum (Fig. 3a), thus excluding a role for serum.

To investigate the involvement of NF-κB in the modulation of HLA-G expression by TNF-α or PMA in JEG-3, FON, and M8-HLA-G1 cells, we examined the effect of BAY 11-7082, a chemical inhibitor of IκB-α phosphorylation that sequestrates NF-κB in the cytoplasm. The efficiency of inhibition by BAY 11-7082 has been demonstrated previously [55, 59, 60] and was validated in our experiments by blockage of PMA-induced up-regulation of ICAM-1 (data not shown) [67]. We observed that pre-treatment with BAY 11-7082 reverses or moderates the HLA-G1 cell surface decrease mediated by TNF-α or PMA (Fig. 4). In some experiments, BAY 11-7082 treatment even boosted constitutive HLA-G1 surface expression. The same observations were made with M8-HLA-G1 cells, using specific inhibitors of PI3K/Akt and MAPK/ERK pathways (namely, LY and PD), which are involved in NF-κB activation [68, 69] (Fig. 5). The decrease in HLA-G1 at the surfaces of JEG-3, FON, and M8-HLA-G1 cells upon TNF-α and PMA treatment appears to be mediated by NF-κB activation.

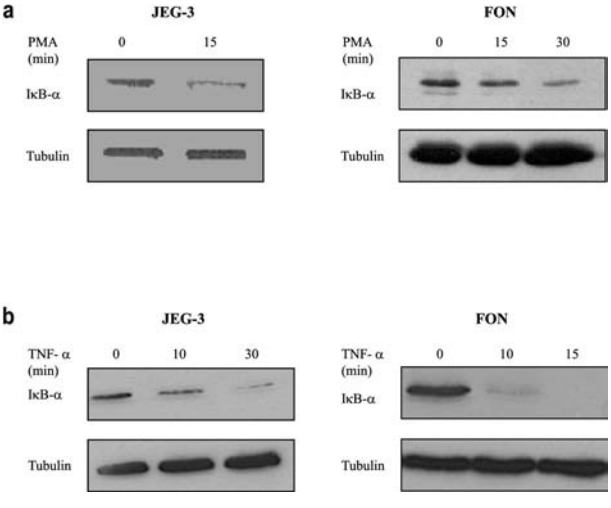

Figure 1. Activation of NF-κB in JEG-3 and FON cells by PMA (*a*) or TNF-α (*b*) treatment. Lysates were prepared and assessed by Western blot analysis using anti-IκB-α antibody to analyze NF-κB activation and anti-tubulin antibody to control protein loading. IκB-α degradation peaking at 10–15 min certifies that NF-κB activation has been rapidly achieved in both cell lines upon treatment.

Stimulation with TNF-α and PMA enhances the intracytoplasmic HLA-G protein content. Next, we examined the effect of TNF-α and PMA on the total HLA-G protein expression in JEG-3, FON, and M8-HLA-G1 cells by Western blot analysis. We found an up to twofold increase in the total HLA-G1 protein level expression after 12 h of induction by both activators in each cell line (Fig. 6a). Notably, this result was still observed in the presence of BAY 11-7082, suggesting that the increase in HLA-G protein level is independent of NF-κB activation (data not shown). To analyze whether treatment with NF-κB inducers also modified the expression of the HLA-G2 isoform, we used FON cells lacking HLA-G1 (FON^{G1-}), but that strongly express this isoform [56]. In this context, we noted a similar effect on HLA-G2 expression as seen before with HLA-G1 (Fig. 6b). Quantification of HLA-G2 bands revealed an increase close to fourfold after treatment by PMA. Thus, TNF-α or PMA treatments involve at least two distinct pathways to regulate HLA-G expression. One is dependent on NF-κB activation and controls the presence of HLA-G1 at the cell surface; the other is independent of the NF-κB pathway and enhances the level of HLA-G proteins in the cell.

Modulation of HLA-G expression involves post-transcriptional processes. The fact that TNF-α modulates HLA-G1 expression in M8 melanoma cells transfected with HLA-G1 cDNA without the HLA-G gene promoter region suggests that the process that controls HLA-G expression is not transcriptional. To confirm this in cells that constitutively express HLA-G mRNA, we carried out quantitative RT-PCR on JEG-3 cells, which demonstrated no significant change in HLA-G transcript levels in cells treated or untreated with TNF-α and PMA (Fig. 7a) whereas an increase in HLA-G gene transcriptional activity was induced by IFN-β in JEG-3 cells (Fig. 7c). In addition, classical RT-PCR revealed no change in the alternative splicing of the HLA-G primary transcript, thus

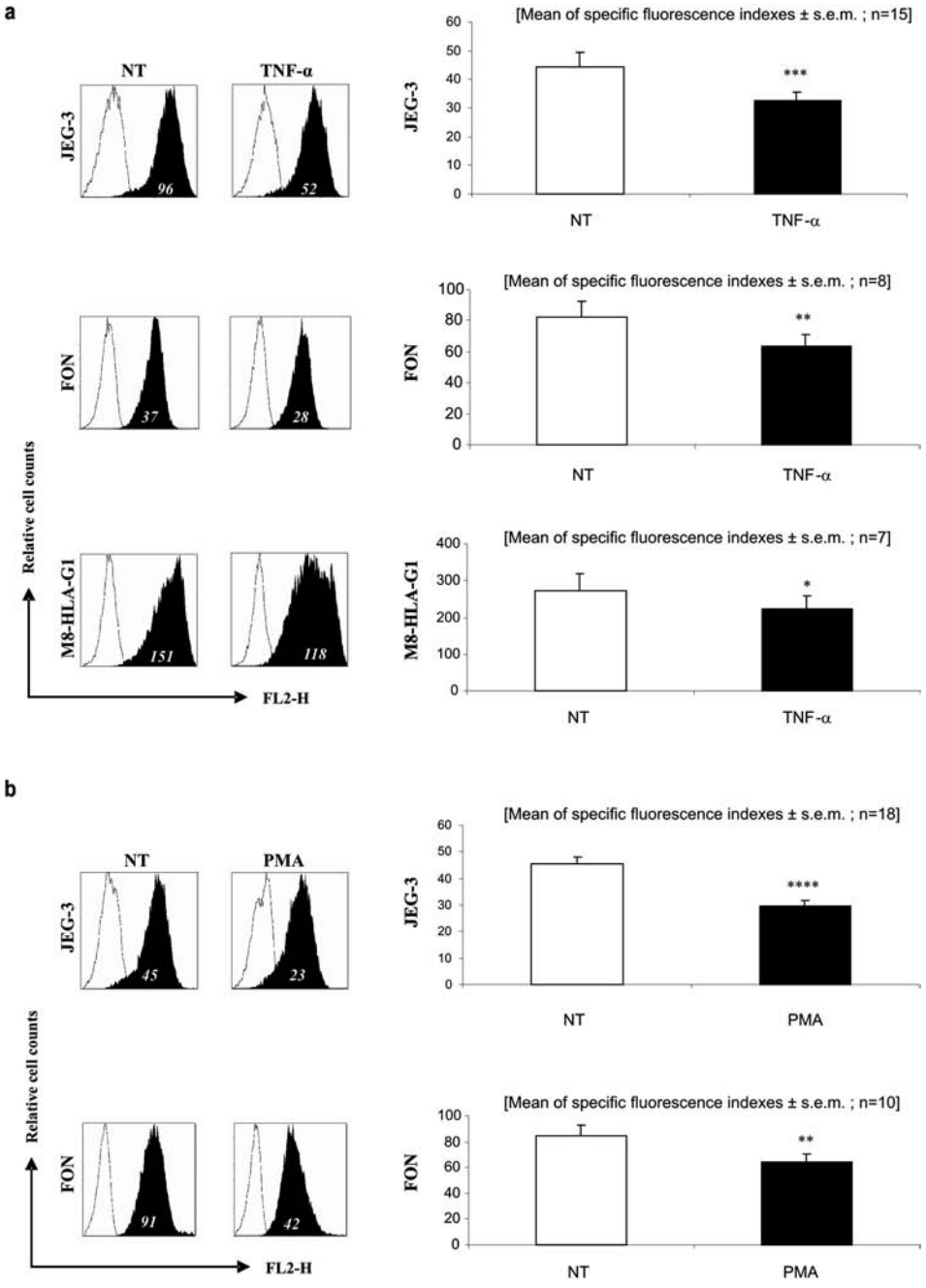

Figure 2. Stimulation of JEG-3, FON, and M8-HLA-G1 cells by NF-κB inducers decreases cell surface HLA-G1 expression. Flow cytometric analysis of cell surface expression of HLA-G1 after 12 h stimulation or not (NT) with 50 ng/ml TNF-α (*a*) or 10 ng/ml PMA (*b*). Cells were stained with MEM-G/9, a specific anti-HLA-G antibody [54]. Representative flow cytometric analyses are presented on the left. The specificity of MEM-G/9 staining (filled curve) was established using an isotype-matched control (empty curve). The inserted numbers represent the SFI. Histograms on the right are the mean of the fluorescence intensity of all experiments; error bars indicate the SE. Significant differences (*$p < 0.01$; **$p < 0.005$; ***$p < 0.001$; ****$p < 0.0001$) are observed in the HLA-G1 cell surface expression of TNF-α- and PMA-stimulated cells, compared with unstimulated cells (NT).

supporting the involvement of a regulatory mechanism acting at a post-transcriptional level (Fig. 7b). Then, to determine whether the modulation of HLA-G proteins upon NF-κB stimulation involved ongoing HLA-G or was dependent on other protein synthesis, JEG-3 cells were treated with TNF-α in the presence or absence of the protein synthesis inhibitor cycloheximide. The results in Figure 8 indicate that a TNF-α-mediated decrease in HLA-G1 cell surface expression involves de novo protein synthesis.

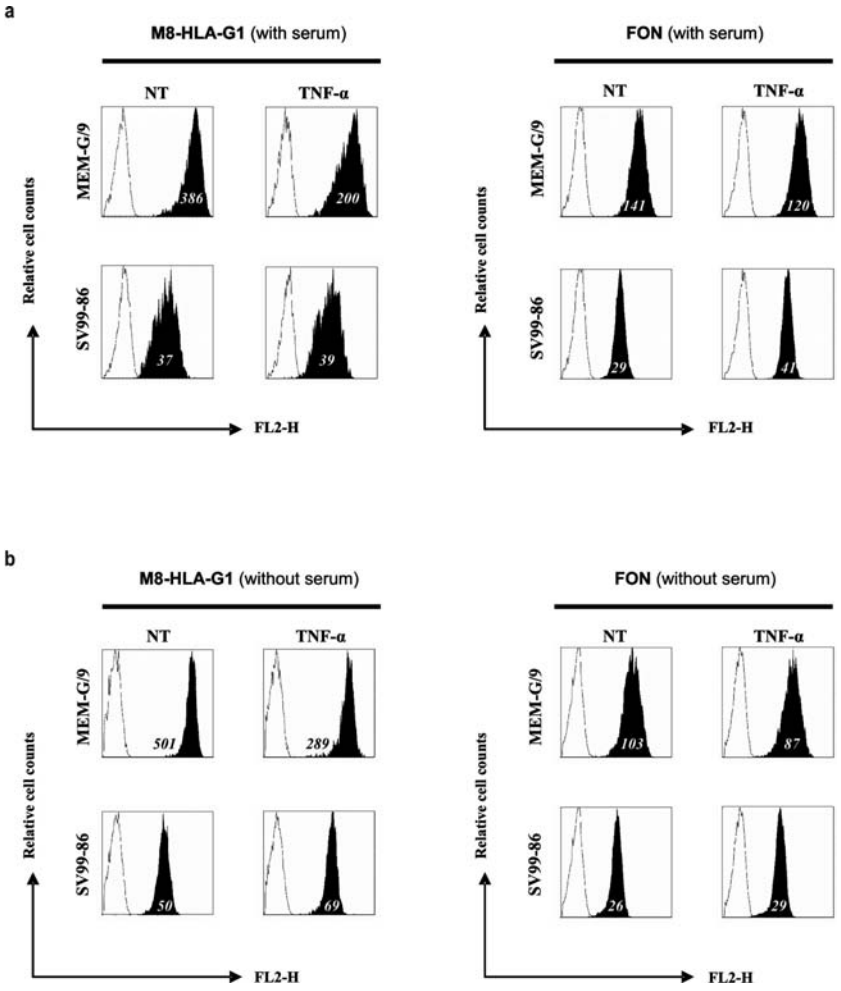

Figure 3. Differential effect of TNF-α on HLA-G and classical HLA class I expression is independent of the presence of serum in the culture medium. Flow cytometric analysis of HLA-G1 and classical HLA class I molecules at the cell surface of M8-HLA-G1 and FON treated or not (NT) with TNF-α in the presence (*a*) or absence (*b*) of serum (FCS) in the culture medium. Representative histograms (n = 2 of each) obtained with MEM-G/9 (anti-HLA-G antibody), SV99-86 (anti- HLA-A, -B, -C antibody) and isotype control staining are shown.

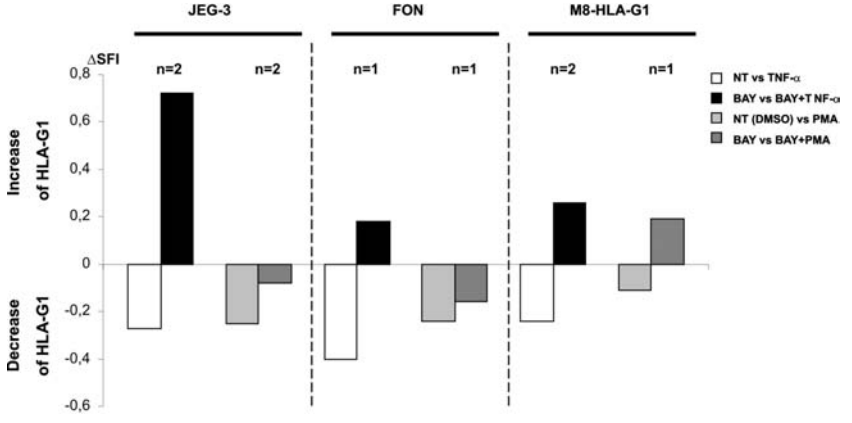

Figure 4. Decrease in HLA-G1 on JEG-3, FON, and M8-HLA-G1 cell surfaces after TNF-α treatment is NF-κB dependent. Flow cytometry analysis of HLA-G1 (MEM-G/9 antibody) at the cell surface of HLA-G-positive cells treated or not (NT) with TNF-α and PMA in the presence or absence of NF-κB inhibitor BAY 11-7082. Each diagram represents ΔSFI obtained from 1 (n = 1) or the mean of 2 (n = 2) independent experiments. NF-κB inhibitor BAY 11-7082 alone was used as a control for cells pre-treated with BAY 11-7082 for 1 h before stimulation with TNF-α (BAY versus BAY+TNF-α) or PMA (BAY versus BAY+PMA) for 12 h. DMSO is a control solvent for PMA. Negative or positive values of ΔSFI indicate the decrease or increase, respectively, in HLA-G1 cell surface expression following cell treatments. ΔSFI obtained in the presence of BAY 11-7082 indicates that the NF-κB inhibitor reverses or reduces the decrease in HLA-G1 cell surface expression upon TNF-α and PMA treatment.

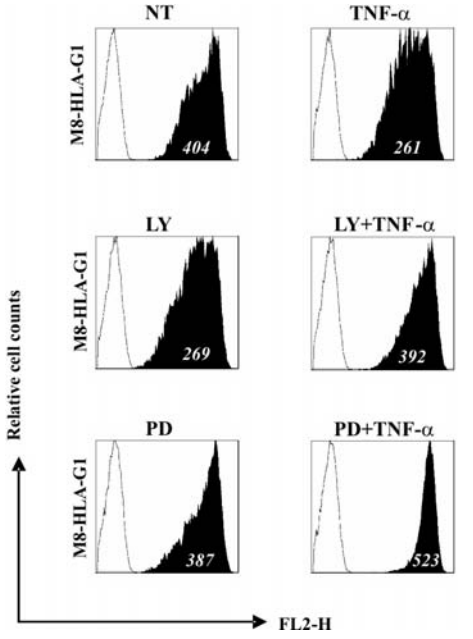

Figure 5. Decrease in HLA-G1 at the cell surface of M8-HLA-G1 by TNF-α is dependent on PI3K/Akt and MAPK/ERK pathways. Flow cytometry analysis carried out with the MEM-G/9 antibody on untreated (NT) M8-HLA-G1 cells compared with cells treated with TNF-α to validate the effect of TNF-α alone (top of Figure). HLA-G cell surface expression was analyzed on cells treated with PI3K inhibitor (LY: LY-294.002) or MEK inhibitor (PD: PD-98.059) alone and compared with cells pre-treated with LY (one representative experiment; n = 2) or PD (n = 1) for 1 h and then stimulated by TNF-α for 12 h. The inserted numbers are the SFI and indicate that LY and PD reverse the TNF-α-induced decrease in HLA-G1 at the cell surface.

The increase in the entire HLA-G protein content following TNF-α and PMA treatments and the concomitant decrease of HLA-G1 expression at the cell surface of tumor cell lines are therefore controlled by post-transcriptional/translational mechanisms. To investigate such mechanisms, we first analyzed whether β2-microglobulin molecule levels were modified after TNF-α treatment of JEG-3 and FON cells, but found no change (data not shown). Next, to investigate whether NF-κB activation by TNF-α or PMA influences proteolytic shedding of HLA-G1 cell surface molecules, JEG-3 and FON cells were treated with the metalloproteinase inhibitor GM6001. We observed that this inhibitor markedly reduced the TNF-α-induced decrease in HLA-G1 cell surface expression (Fig. 9A). In addition, FON treatment with EDTA, another metalloproteinase inhibitor, exerted the same effect with either TNF-α or PMA induction (Fig. 9b). These results suggest that the decrease in HLA-G1 cell surface expression is caused by an increase in proteolytic shedding of the molecule.

Finally, we investigated by ELISA the proteolytic shedding of membrane-bound HLA-G1 molecules (sHLA-G1) in culture medium. For this purpose, we chose to work with M8-HLA-G1, which expresses high levels of HLA-G1. The use of this cell line also excludes HLA-G5 detection by antibodies used in the ELISA. Figure 10 shows that both TNF-α and PMA treatments significantly enhance proteolytic shedding of cell surface HLA-G1

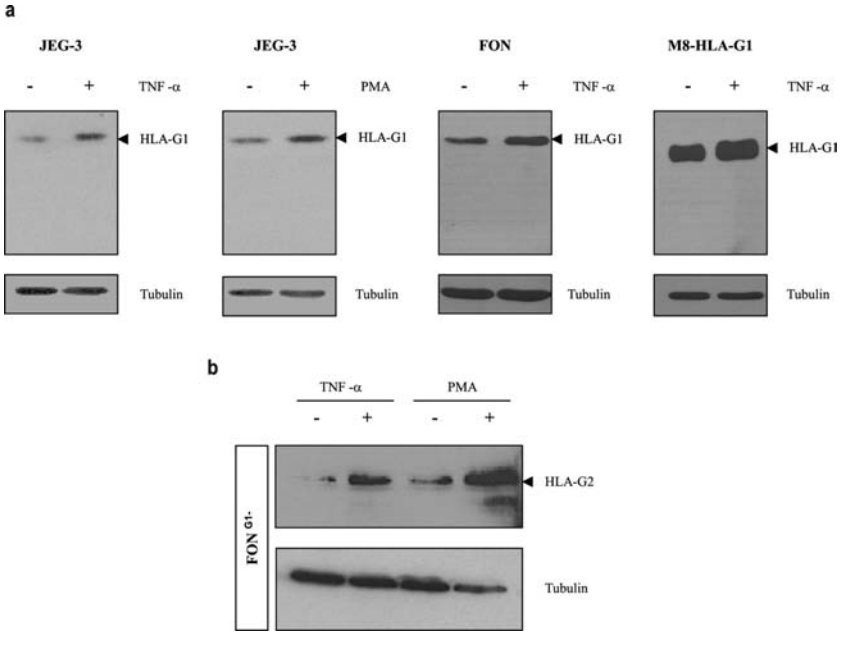

Figure 6. Increase in the intracytoplasmic HLA-G protein pool after 12 h exposure to NF-κB inducers in tumor cell lines. Representative Western blot analysis carried out with JEG-3 (n = 3 with TNF-α; n = 3 with PMA), FON (n = 1 with TNF-α), and M8-HLA-G1 (n = 1 with TNF-α) cells (*a*) and FON^{G1-} cells (n = 1 with TNF-α; n = 1 with PMA) (*b*). Cell lysates were separated on 10% SDS-PAGE and immunoblotted with 4H84 antibody to detect all HLA-G isoforms and anti-tubulin antibody to control protein loading. PMA or TNF-α treatments carried out for 12 h increase HLA-G expression, mostly HLA-G1 in JEG-3 and FON, and HLA-G2 in FON^{G1-}. – untreated cells; + treated cells. DMSO was added to the control cell medium for PMA.

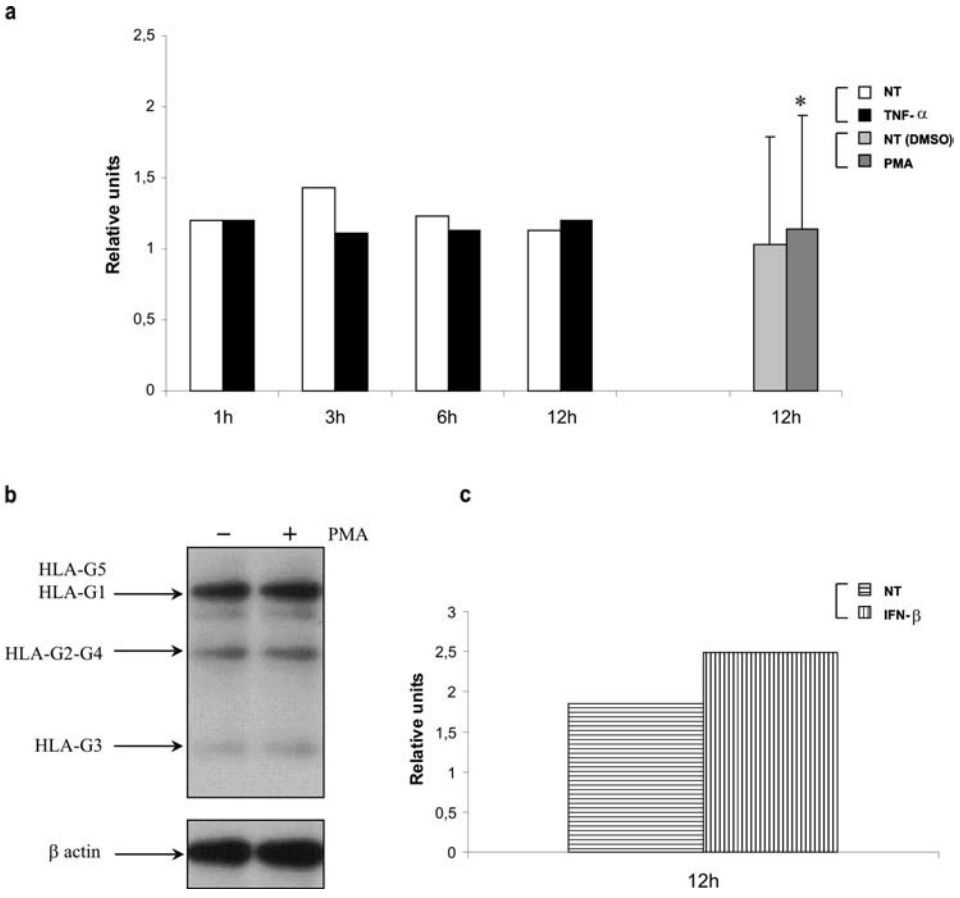

Figure 7. The quantities of HLA-G transcripts and pattern of HLA-G alternative splicing are not affected by TNF-α and PMA. (*a*) Quantitative HLA-G-specific RT-PCR carried out on JEG-3 cells treated or not with NF-κB inducers. Cells were treated or not (NT) with TNF-α for 1 h (n = 1), 3 h (n = 1), 6 h (n = 1), and 12 h (n = 2) without affecting the HLA-G transcript level. The changes observed following PMA treatment for 12 h (n = 8) are not statistically significant (*p > 0.05). Means of the level of HLA-G transcripts ± SE are shown. (*b*) Representative Southern blots (n = 4) obtained by classical RT-PCR carried out on JEG-3 samples treated (+) or not (–) with PMA for 12 h. β-actin levels were used as an internal control. Alternatively spliced transcripts are indicated. We noted no qualitative or quantitative change in HLA-G mRNA following PMA treatment. (*c*) Quantitative HLA-G-specific RT-PCR performed on JEG-3 cells treated or not with 2000 U/ml IFN-β for 12 h (positive control of modulation of HLA-G transcription).

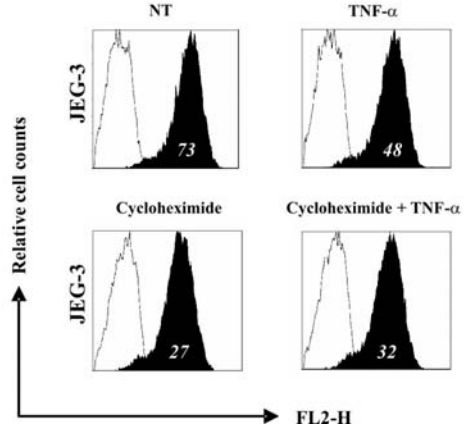

Figure 8. Inhibition of protein synthesis reduces the TNF-α-induced decrease in HLA-G1 at the surface of JEG-3 cells. Representative flow cytometry experiments (n = 2) carried out with MEM-G/9 antibody on JEG-3 cells pre-treated with cycloheximide at 20 µg/ml for 1 h and then stimulated by TNF-α for 12 h. The inserted numbers represent the SFI. TNF-α efficiency was also checked (top of Figure).

proteins. Altogether, these results indicate that the decrease in HLA-G1 at the cell surface of tumor cells upon TNF-α and PMA treatments corresponds to an increase in HLA-G1 proteolytic shedding.

TNF-α-induced HLA-G1 proteolytic shedding inhibits the cytotoxicity of NKL cells. To determine the functional relevance of sHLA-G1 produced upon TNF-α treatment, we tested its effect on NK cell cytolytic activity, using NKL as effector and M8-pcDNA as target cells. For this purpose, cells were cocultured with supernatants of M8-HLA-G1 cells treated or not with 50 ng/ml TNF-α for 12 h. Supernatants from TNF-α-induced cells produced a decrease in the cytolytic activity of NKL cells estimated at 18.6 ± 2.7% (mean ± SE, n = 9, p < 0.018) compared with supernatants without treatment. The most illustrative experiment is shown in Figure 11a. To further demonstrate that TNF-α-induced sHLA-G1 is involved in the decrease of NKL cell cytotoxicity against M8 cell

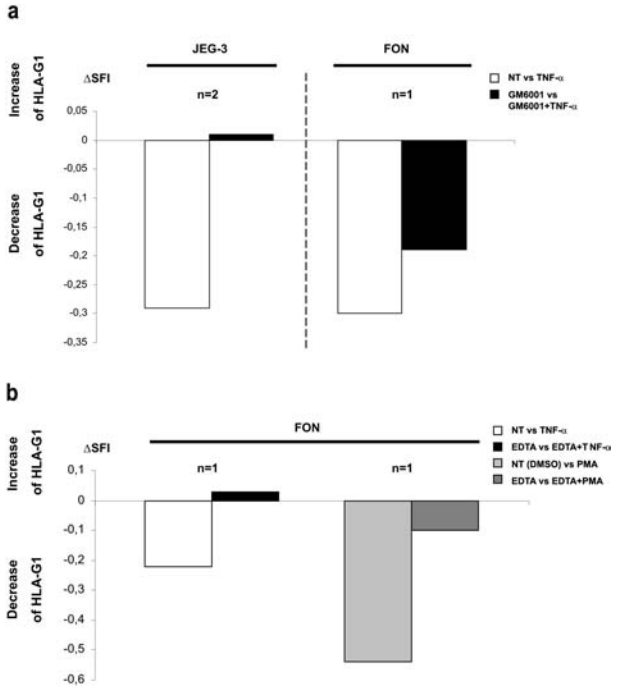

Figure 9. Metalloproteinases inhibition reduces the TNF-α-induced decrease in HLA-G1 at the JEG-3 and FON cell surfaces. (*a*) Flow cytometry analysis of the effect of the metalloproteinase inhibitor GM6001 on the expression of HLA-G1 cell surface expression was checked by comparing ΔSFI values between NT versus TNF-α and GM6001 versus GM6001+ TNF-α. (*b*) Flow cytometry analysis of the effect of the metalloproteinase inhibitor EDTA on the expression of HLA-G1 cell surface expression was checked by comparison of ΔSFI: NT versus TNF-α and EDTA versus EDTA+ TNF-α. Each diagram represents the ΔSFI obtained from 1 (n = 1) or the mean of 2 (n = 2) independent experiments. Negative and positive values for ΔSFI indicate the decrease and the increase, respectively, in HLA-G1 cell surface expression following cell treatments. ΔSFI obtained in the presence of GM6001 or EDTA indicates that metalloproteinase inhibitors reverse or reduce the decrease in HLA-G1 cell surface expression upon TNF-α and PMA treatments.

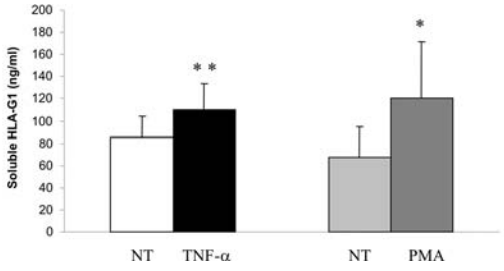

Figure 10. Soluble HLA-G1 resulting from proteolytic shedding in M8-HLA-G1 cells is enhanced by TNF-α and PMA. M8-HLA-G1 cells were treated or not (NT) with TNF-α (n = 8) or PMA (n = 3) for 12 h and the supernatants subjected to ELISA to evaluate sHLA-G1. The observed variations between mean sHLA-G1 concentrations of treated and untreated cells are statistically significant using the paired t test: **$p < 0.001$ and *$p < 0.01$ for TNF-α and PMA treatments, respectively. Error bars indicate the SE.

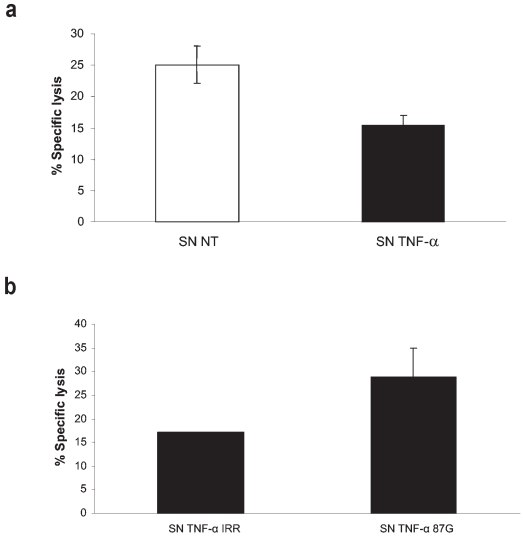

Figure 11. Soluble HLA-G1 resulting from TNF-α -induced proteolytic shedding in M8-HLA-G1 cells impaired NKL cytotoxicity function. (*a*) Illustrative experiment of ^{51}Cr release assay (from n = 9) performed with IL-2-stimulated (100 U/ml) NKL added to chromium-labeled M8-pcDNA cells at effector/target ratios of 10 : 1. Coculture was performed either in the absence (SN NT) or in the presence (SN TNF-α) of supernatant containing TNF-α-induced soluble HLA-G1. (*b*) One experiment of sHLA-G1 blockage with anti-HLA-G (87G) (triplicate) or irrelevant (IRR) (duplicate) antibodies performed on supernatants obtained after TNF-α stimulation of M8-HLA-G1 cells.

lines, we performed one experiment using anti-HLA-G 87G as blocking antibody. Figure 11B shows that 87G lead to a significant enhancement of NK lysis in comparison with irrelevant antibodies. Thus, the cytolytic potential of NKL cells is impaired when the culture medium contains TNF-α-induced sHLA-G1.

Discussion

This study provides new insight into mechanisms that regulate HLA-G expression in tumor cells. We previously demonstrated that HLA-G gene transcription activity is dependent on cis-acting epigenetic changes, including CpG demethylation and histone acetylation [70]. Here, we show that post-transcriptional/translational mechanisms, some of which are controlled by NF-κB, modulate HLA-G expression in three tumor cell lines, two constitutively expressing HLA-G, the other being transfected with HLA-G1 cDNA.

First, analysis of HLA-G gene transcriptional activity upon treatment with TNF-α or PMA, both of which stimulate NF-κB activity, confirms previous data demonstrating that the HLA-G gene is not targeted by the classical NF-κB pathway, thus excluding the presence of a putative functional target site outside the proximal promoter region of HLA-G. Secondly, we observed a decrease

in HLA-G1 at the cell surface of three tumor cell lines treated with either TNF-α or PMA. This effect was specific to HLA-G, since expression of classical HLA class I molecules was enhanced, a result in agreement with previous work showing that TNF-α induces the expression of HLA class I molecules [71, 72]. Third, we found an enhancement of intracytoplasmic HLA-G proteins in the three cell lines upon exposure to TNF-α and PMA. Fourth, we observed that NF-κB inducers increase the amounts of sHLA-G1 in M8-HLA-G1 cell culture medium. The enhancement of sHLA-G1 is of particular relevance, as it impairs NK cell cytolysis against melanoma target cells (M8-pcDNA).

The use of the NF-κB inhibitor BAY 11-7082 indicates that this transcription factor drives the decrease in cell surface HLA-G1. Not only does the addition of BAY 11-7082 block or moderate this effect, but in some cases it could enhance HLA-G cell surface expression. Such a result may be related to previous data showing that IL-10 could up-regulate HLA-G cell surface expression of peripheral blood monocytes [73]. Indeed, IL-10 has recently been demonstrated to inhibit NF-κB activity, a mechanism that involves suppression of the PI3K/Akt pathway and IκB kinase activity in dendritic cells [74].

On the other hand, TNF-α and PMA treatments increase the intracytoplasmic cell content of at least HLA-G1 and HLA-G2. This occurred independently of the NF-κB pathway, since BAY 11-7082 did not block the increase in the HLA-G protein expression level. Signaling induced by TNF-α and PMA is known to activate a number of pathways that are different from that of NF-κB, which therefore might be involved in the observed enhancement of HLA-G expression [75]. Then, when NF-κB activation is blocked, these NF-κB-independent pathways might drive the observed HLA-G enhancement upon PMA and TNF-α treatments. In the absence of up-regulation of HLA-G mRNA levels following TNF-α and PMA treatments, the mechanisms involved might therefore affect HLA-G protein stability and/or the efficiency of HLA-G mRNA translation. Nevertheless, the factors and mechanisms involved remain to be investigated.

We next analyzed how cell surface HLA-G1 molecules decrease while the intracytoplasmic content of HLA-G1 proteins increases. One hypothesis could be that TNF-α and PMA treatments of HLA-G-positive tumor cell lines may affect the stability/degradation of HLA-G proteins on the cell surface. However, this hypothesis is not supported by the fact that ELISA, performed with MEM-G/9 and anti-β2m antibody, can only detect conformational sHLA-G1 molecules in cell culture medium. Second, previous work carried out with antigen-presenting cells revealed that even though HLA-G1 could be detected in total cell extract, HLA-G1 could not be detected at the cell surface [76], suggesting that either HLA-G1 is retained in the cell or is secreted by proteolysis [21]. A possible defect in HLA-G1 transport to the cell surface upon TNF-α and PMA treatment is not supported by the fact that NF-κB activation may up-regulate several antigen-processing machinery components as well as β2-microglobulin proteins [77, 78], which more likely favors cell surface expression. In particular, we did not reveal any down-regulation in the level of β2-microglobulin after treatment with NF-κB inducers (data not shown). Conversely, our data strongly support the notion that the NF-κB-mediated HLA-G1 decrease at the cell surface is generated by the enhancement of HLA-G1 proteolytic shedding following an increase in metalloproteinase activity. Indeed, we observed an inhibition in the TNF-α-induced decrease in cell surface HLA-G1 using metalloproteinase inhibitors, and an increase in levels of sHLA-G1 forms in the supernatant of M8-HLA-G1 cells treated with TNF-α or PMA. It is of note that the HLA-G1 cell surface decrease was also obtained in serum-free culture medium, suggesting that proteases are solely coming from the NF-κB modulated cells rather than being present in the cell culture medium. In accordance with our observations, metalloproteinases have recently been demonstrated to be responsible for the release of sHLA-G1 at the cell surface of LCL 721.221 and K562 transfectants [21]. NF-κB was demonstrated to be involved in the up-regulation of metalloproteinases particularly MMP-9 [79]. Moreover, several studies have monitored the induction of the MMP-9 gene by TNF-α and the implication of PMA in the activation of MMPs [80, 81]. These data are in agreement with results obtained in stimulating JEG-3 cells with cycloheximide. In fact, we found that this treatment diminished the decrease in HLA-G1 proteins at the cell surface, suggesting that having blocked protein synthesis, we also stopped metalloproteinase production. Therefore, the decrease in HLA-G1 at the cell surface upon TNF-α and PMA treatment is consistent with NF-κB-mediated enhancement of HLA-G1 proteolytic shedding.

Previous studies have mentioned that PMA, which activates protein kinase C *in vivo* and *in vitro,* is an extremely potent mouse skin tumor promoter [82]. TNF-α, a pro-inflammatory cytokine that plays a critical role in diverse cellular events, such as septic shock, cell proliferation, differentiation, and apoptosis, also acts to promote tumor growth and progression [83]. Accordingly, several reports have detected abnormally high levels of TNF-α protein in the blood of cancer patients exhibiting various tumor types [84]. On the other hand, high levels of sHLA-G have been detected in the serum of melanoma patients, as well as in various lymphoproliferative disorders, such as chronic B lymphocytic leukemia and non-Hodgkins B lymphoma [6, 7, 85]. In particular, plasma levels of soluble HLA-G were recently reported to be higher in patients with HLA-G-positive B cell chronic lymphocytic leukemia than in patients with HLA-G-negative leukemia. In addition, the authors observed that HLA-G was

associated with unfavorable outcome [86]. Since metalloproteinases are known to be involved in invasion and genesis of cancer cells [87], those that are generated by NF-κB activators could therefore play an important role in tumor invasion by generating proteolytic shedding of HLA-G molecules. sHLA-G1 produced by this process could act by interacting with the inhibitory receptors of immune cells, notably NK cells that are the major component of the innate immune system through their rapid activation and their potent cytolytic response against tumor cells. In agreement, our results show that the soluble shedding form of HLA-G1 produced by TNF-α is able to inhibit NK cell cytotoxicity *in vitro*. This can be reversed by the addition of 87G blocking antibody.

In conclusion, the data presented here introduce a new concept concerning the post-transcriptional regulation of HLA-G expression via NF-κB inducers. The increase of HLA-G1 expression and its release in the form of proteolytic shedding could potentially have an impact on the progression of tumor cells and should be considered in new therapeutic strategies aimed at targeting NF-κB.

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