Differential modulation of natural killer cell cytotoxicity by 17β-estradiol and prolactin through the NKG2D/NKG2DL axis in cervical cancer cells

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Abstract. Natural killer (NK) cells play a crucial role in cervical cancer (CC). As estrogens and prolactin (PRL) have been reported to be involved in CC, the present study attempted to elucidate the effects of both hormones on NK cells in CC. For this purpose, NKL cells, as well as CC-derived cell lines (HeLa, SiHa and C33A) and non-tumorigenic keratinocytes (HaCaT cells) were stimulated with 17β-estradiol (E2; 10 nM), PRL (200 ng/ml), or both (E2 and PRL) for 48 h. The expression of hormone receptors (estrogen receptor α and β, G protein-coupled estrogen receptor 1 and PRL receptor) and NK cell activating receptors [natural killer group 2D (NKG2D), natural cytotoxicity triggering receptor 3, natural cytotoxicity triggering receptor 2 and natural cytotoxicity triggering receptor 1] were measured using western blot analysis and flow cytometry, respectively. In the HeLa, SiHa, C33A and HaCaT cells stimulated with the hormones, the expression of NKG2D ligands [MHC class I polypeptide-related sequence A/B (MICA/B)] on the membrane and the soluble form of MICA was evaluated using flow cytometry and ELISA. Cytotoxicity assay was performed using GFP-transfected K562 cells as target cells. E2 reduced NKL cell-mediated cytotoxicity, while PRL exerted the opposite effect. NKL cells expressed different hormone receptor forms, of which PRL only induced a decrease in NKG2D expression compared to the untreated control NKL cells. PRL increased MICA/B expression in HeLa cells and E2 and PRL reversed this effect. However, in SiHa cells, the concurrent incubation with the two hormones decreased MICA/B expression. E2 and PRL, either alone or in combination, decreased soluble MICA secretion in all CC cell lines, while E2 solely increased soluble MICA secretion in SiHa cells. On the whole, the present study provides evidence that E2 and PRL mediate the mechanisms through which NK and CC cells mediate a cytotoxic response and these have an antagonistic effect on NK cell-mediated cytotoxicity.

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Introduction

Cervical cancer (CC) is one of the most common cancers among women worldwide and the second cause of cancer mortality in developing countries (1). Human papillomavirus (HPV) is the leading risk factor for CC development (2). However, different types of lesions may be observed in the cervix prior to cancer establishment, including grade 1, 2 and 3 cervical intraepithelial neoplasia, as well as invasive carcinoma (3). Apart from HPV infection, other risk factors have been reported to be involved in the transformation process from normal to malignant cells, including smoking, oral contraceptive use and steroid sex hormones, among others (4-6). The tumor microenvironment (TME) is crucial for the carcinogenic process, and hormones are a key factor in this context. In addition, cells that belong to the innate immune system are located in the TME, having the ability to kill tumor cells (7). 17β-estradiol (E2) and prolactin (PRL) have been reported to be present in the TME (8-10); however, their role on immunological mechanisms generated in the response to CC is poorly understood.

Estrogens are sex hormones that belong to the cholesterol-derived steroids group, whose three primary forms are estrone (E1), E2 and estriol (E3), of which E2 has been reported to exhibit an increased biological activity (11). The functions of E2 are mediated through the estrogen receptors (ER)α and β,

and the G protein-coupled estrogen receptor 1 (GPER) (12,13). Of note, ERα, ERβ and GPER have been shown to be overexpressed in CC tissues compared with that in the premalignant lesion and normal cervical epithelium (14,15). Studies using mice have demonstrated that a temporary presence of E2 promotes CC, being ERα signaling-dependent (16,17).

PRL is a lactogenic polypeptide hormone synthesized primarily by the pituitary gland (18). Additionally, other studies have demonstrated an extrapituitary PRL production by some tissues and organs. PRL exerts its functions through its binding to the PRL receptor (PRLR) (19,20). Previous studies have demonstrated the expression of a 60 kDa PRL in CC tissues and CC-derived cells. This PRL variant may regulate various processes, including apoptosis, cytokine production and metabolism in THP-1 and CC-derived cells (14,21,22).

PRLR is a member of the class I cytokine receptor superfamily; it presents with various isoforms, one long, one intermediate, and two short isoforms, with an average weight of 85-90, 65 and 40-50 kDa respectively (23). High PRL levels have been reported in the serum of patients with CC (24). There is also evidence of the increased expression of PRLR in premalignant lesions, CC tissues and CC-derived cell lines (21). The stimulation of CC-derived cells with PRL induces the expression of anti-apoptotic gene through the signal transducer and activator of transcription (STAT)-3 (25). This evidence confirms the importance of PRL in CC pathogenesis and some relevant events in the progression of the disease.

Natural killer (NK) cells are a major component of the innate immunity against tumors and viral infections. They constitute 5 to 15% of all lymphocytes and are phenotypically defined by the expression of CD56 and the absence of CD3 (26). NK cells are equipped with a repertoire of receptors that can both stimulate (activating receptors) or prevent (inhibitory receptors) their reactivity (27). The natural cytotoxicity receptors (NCRs), including natural cytotoxicity triggering receptor 3 (NKp30), natural cytotoxicity triggering receptor 2 (NKp44) and natural cytotoxicity triggering receptor 1 (NKp46), have been reported to induce NK cell activation; however, their corresponding ligands have not yet been well defined (28). Another activating receptor is natural killer group 2D (NKG2D), a type 2 transmembrane protein, whose ligands include MHC-I chain-related protein A and B (MICA and MICB) and the UL16 binding proteins (ULBP) from 1 to 6 (29). In 2012, a previous study revealed that NKG2D receptor expression in NK cells decreased when interacting directly with CC cell lines (30). Another study revealed that the expression of NKp30 and NKp46 receptors was decreased in squamous intraepithelial lesions and CC; however, NKG2D was only decreased in CC, and was negatively associated with NK cell cytotoxic activity (31). Of note, tumors evade the immune system through the liberation of MICA and MICB from the cellular membrane to create a soluble form (32). This process has been reported to be mediated by various metalloproteinases (33). The soluble form of MICA and MICB has been found to be associated with the internalization and degradation of NKG2D and the consequent decrease in the NK cell-mediated cytotoxicity (34,35). In cancers, such as CC, which is related to a viral infection, it is crucial to understand whether the factors included in the TME, including hormones, may modify the mechanisms that favor the malignancy of the disease.

Both estrogen and PRL receptors have been identified in human cell lines and murine NK cells. However, the expression of GPER in these cells remains unclear (36,37). There is evidence to indicate that estrogens have been linked to a decrease in NK cell cytotoxicity using human and murine models, while PRL exert opposite effects in human NK cell lines (NK-92 and YT cell lines) (37). In addition, the effects of these hormones may affect the regulation of proteins that belong to cytotoxicity processes, including activating receptors and their ligands (37-43).

Riera-Leal *et al* (14), observed the effects of E2 and PRL on CC-cell line metabolism and concluded that the two hormones increased cell metabolism, with PRL to a lesser extent than E2. However, PRL appears to exert a more prominent effect over E2 when simultaneously applied.

In the CC TME, E2 and PRL are present. Thus, the present study aimed to investigate the effects of the E2 and PRL stimuli, concurrently or separately applied on NKL cells and CC-derived cell lines, as well as to evaluate the expression of different molecules related to NK cell-mediated cytotoxicity, including NCR, NKG2D and MICA/B.

Materials and methods

Cell culture and hormone stimuli. The HeLa, SiHa, C33A, MCF7 (all from ATCC) and HaCaT (CLS Cell Lines Service GmbH) cell lines were cultured in DMEM medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin G (10,000 U/ml), and streptomycin (10,000 μg/ml) (Gibco; Thermo Fisher Scientific, Inc.). Similarly, the NKL (kindly donated by Dr Adriana Aguilar Lemarroy) and K562 (ATCC) cell lines were grown in supplemented RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). All cell lines were incubated at 37˚C and 5% CO_2 until 80% of confluence was obtained. NKL, HeLa, SiHa, C33A and HaCaT cell cultures were stimulated for 48 h with PRL (200 ng/ml) isolated from HeLa cell supernatant, E2 (10 nM; Sigma Aldrich; Merck KGaA), or both (E2 and PRL). HeLa, SiHa, C33A, HaCaT and K562 cell lines were authenticated by Multiplexion GmbH, using the multiplex human cell line authentication test.

Isolation and purification of the 60 kDa-weighted PRL. The isolation of PRL from the HeLa cell supernatant was performed using magnetic beads (Protein G Microbeads MultiMACS™; Miltenyi Biotec GmbH) following the manufacturer's protocol. The 60 kDa PRL was purified employing the 50 kDa molecular cut-off filters (Amicon® Ultra 0.5 ml centrifugal filters; cat. no. UFC505024; MilliporeSigma). The procedure for the filtration was performed as follows: 14,000 x g for 30 min (filtration phase); 1,000 x g for 2 min (recovery phase) at 4˚C. Once purified, the correct identification of the 60 kDa PRL was determined using a 12% polyacrylamide gel for electrophoresis at 95V for 90 min. Subsequently, silver nitrate (cat. no. 209139; MilliporeSigma) staining was performed for 20 min at room temperature to visualize the 60-kDa band belonging to PRL. Finally, quantification of the purified protein was performed utilizing Thermo Scientific NanoDrop 2000c Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

NK cell cytotoxicity assay. The cytotoxicity of NK cells against the Green Fluorescent Protein (GFP)-transfected K562 cell line (kindly donated from Dr Adriana Aguilar Lemarroy) was evaluated in a propidium iodide (PI) flow cytometry assay. K562 cells were seeded with a constant number (150,000) with different effector (NK cells) to target cell ratios [effector:target (E:T) 1:1, 5:1 and 10:1]. The target cells were incubated alone to measure untreated control cell death. Co-cultures between NKL and GFP-transfected K562 cells (GFP-K562) (lymphoblasts derived from chronic myeloid leukemia), characterized by its absence or decrease of MHC-I molecules, in complex medium were performed for 4 h at 37˚C and 5% CO_2. The cells were washed twice with 1% PBS and incubated in the same buffer with PI (cat. no. P4170; MilliporeSigma) for 20 min at room temperature in darkness. The reading was performed using Attune® NxT acoustic focus cytometer with the FACS Diva v3.1.2 software (BD Biosciences). The cytotoxic activity was expressed as the % of specific lysis by using the following formula:

$$\%\ specific\ lysis = \frac{100\ x\ (\%\ sample\ lysis - \%\ basal\ lysis)}{100 - \%\ basal\ lysis}$$

Degranulation assay. CD107a was used as a marker of NKL degranulation upon target recognition. A total of 30,000 NKL cells (effector) were co-cultured with 30,000 K562 cells (target) cells at an E:T ratio of 1:1 in 96-well plates for 4 h. At the start of the incubation period, a 1:400 dilution of anti-CD107a-PE (cat. no. 555801; BD Biosciences) was added to each well. Monensin (BioLegend, Inc.) was used as a protein transport blocker an added for 1 h into the co-culture. To identify viable NKL cells from the target cells, a 1:50 dilution of CD45 antibody (BioLegend, Inc.; cat. no. 304027) and Zombie NIR dye (BioLegend, Inc.; cat. no. 423105) were used. The reading was performed using Attune® NxT acoustic focus cytometer with the FACS Diva Software v3.1.2 (BD Biosciences).

Western blot analysis. Total proteins were extracted from NKL and MCF7 cell lines (obtained from ATCC) using RIPA lysis and extraction buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.), and the coomasie plus (Bradford) assay (cat. no. 23238; Thermo Fisher Scientific, Inc.) was used for protein quantification. A total of 50 μg protein was mixed with loading buffer and then denatured at 95˚C for 5 min. Electrophoresis was performed on 10% polyacrylamide gels at 110 V for 60 min, and subsequently, a PVDF-membrane electrical transference (Bio-Rad Laboratories, Inc.) was performed for 90 min at 240 V. The membranes were incubated overnight at 4˚C with a blocking solution of 1X PBS and 5% blotting-grade blocker (cat. no. 1706404; Bio-Rad Laboratories, Inc.). The dilution of the primary antibodies used was 1:500 for ERα, ERβ and PRLR (cat. nos. sc-8002, sc-373853, sc-20992, respectively; Santa Cruz Biotechnology, Inc.) and GPER (cat. no. ab39742; Abcam) and 1:10,000 for β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) in blocking solution consisting of 1X PBS and 5% blotting-grade blocker, and incubated overnight at 4˚C. The membranes were washed five times for 7 min with PBS and Tween-20 (cat. no. P1379; MilliporeSigma) and incubated for 90 min with a dilution of 1:10,000 anti-mouse or anti-rabbit secondary antibodies (cat. nos. sc-2005, sc-2357; Santa Cruz Biotechnology, Inc.) at room temperature. Subsequently, the membranes were washed 6 times for 10 min. Luminol and horseradish peroxidase reagents (Immobilion; Merck KGaA) were used to perform the chemiluminescence process. β-actin expression was used as an internal control. The Microchemi 6.0 (DNR Bio-Imaging Systems Ltd.) was used to visualize the membranes and GelQuant software V1.7.8 (BiochemLabSolutions) was utilized for densitometric measurement.

Flow cytometry. For the NKL cell lines with and without hormonal stimulation, the cell density was adjusted to $2x10^5$ cells in total. The cells were washed with 1X PBS and centrifuged at 1,800 x g for 10 min at 4˚C. Subsequently, cells were incubated with anti-NKG2D, anti-NKp30, anti-NKp44 and anti-NKp46 antibodies at 1:100 dilution (cat. nos. 130-123-948, 130-121-995, 130-120-623 and 130-126-054, respectively; Miltenyi Biotec GmbH) at 4˚C for 30 min in the dark. The cells were washed again and centrifuged at 1,800 x g for 5 min at 4˚C. The cells were then fixed with 1 ml 0.05% PBS-formaldehyde solution. Following the same procedure, CC-derived and HaCaT cell lines were labeled against anti-MICA/B antibodies (cat. no. 130-100-889; Miltenyi Biotec GmbH) for analysis using flow cytometry. The percentages and mean fluorescence intensity (MFI) were determined with appropriate protocols and controls to electronically compensate the overlapping signals using the Attune® NxT Software v3.1.2 acoustic focus cytometer (Invitrogen; Thermo Fisher Scientific, Inc.).

Soluble MICA quantification in cell culture supernatants. Soluble MICA levels were analyzed using the Human MICA ELISA kit (cat. no. RAB0358-1KT; MilliporeSigma) in the supernatant of HeLa, SiHa, C33A and HaCaT cell lines stimulated with E2 and PRL, according to the manufacturer´s instructions. The results were obtained from two independent experiments using the appropriate absorbance values (450 nm).

Statistical analysis. Data capture was performed using the statistical program GraphPad 8.0.2 (GraphPad Software, Inc.). Statistical analysis to compare the expression patterns of hormone receptors, activating receptors, ligands and differences in the cytotoxicity activity were carried out, using the ANOVA test followed by the Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Antagonistic effects between E2 and PRL on NK cell-mediated cytotoxicity. To evaluate the effect of hormones on NK cell-mediated cytotoxicity, NKL cells were stimulated with E2 (10 nM) or PRL (200 ng/ml) either alone or in combination for 48 h and subsequently co-cultured with GFP-K562 cells at various E:T ratios (1:1, 5:1 and 10:1) for 4 h. The identification of dead target cells was characterized as GFP^+PI^+ by flow cytometric analysis (Fig. 1A).

Comparing the effect of hormones against untreated control cells (without stimulation), it was demonstrated that stimulation with E2 tends to decrease the lysis of GFP-K562 cells; however, PRL stimulation tended to increase cytotoxicity

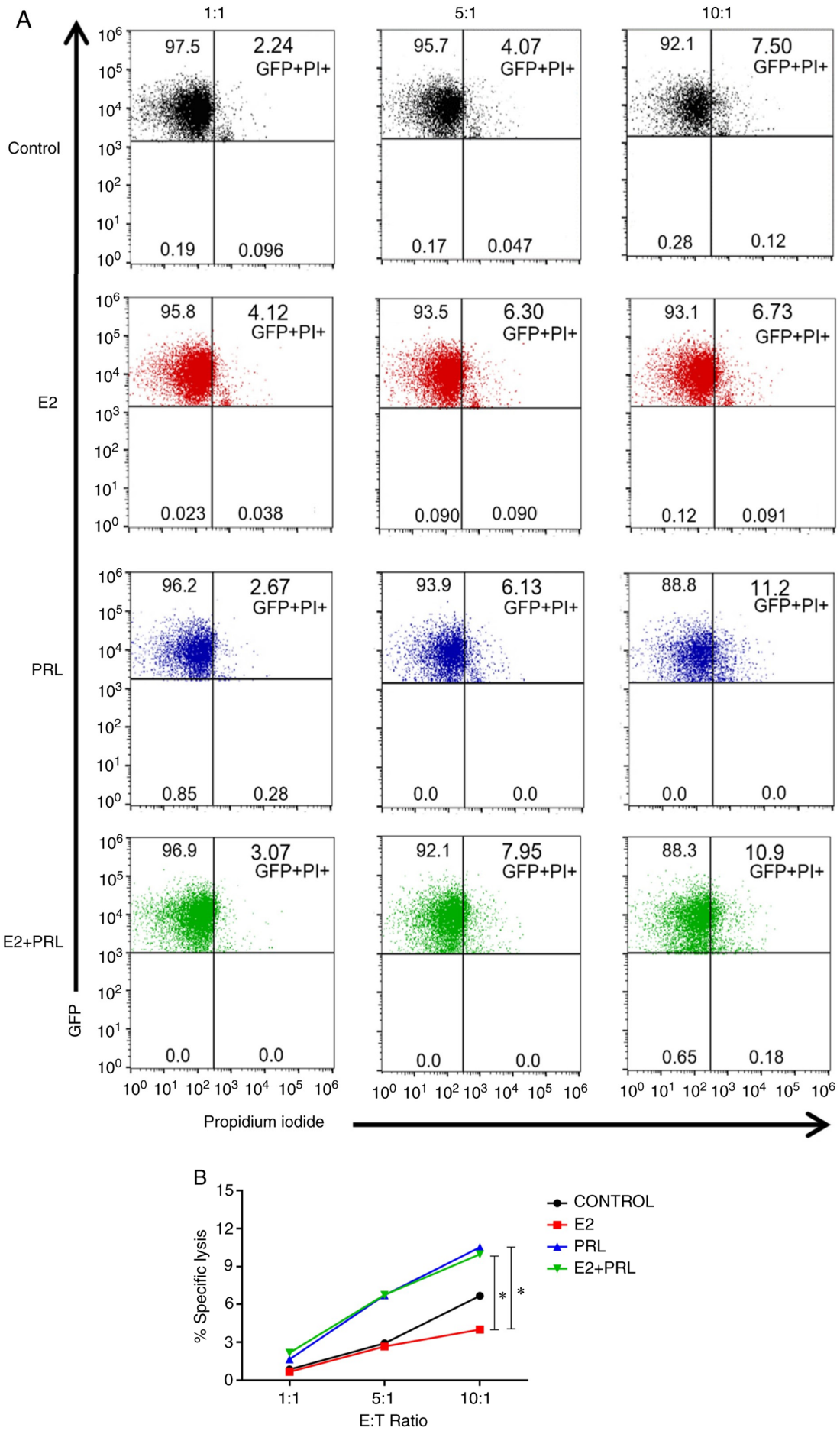

Figure 1. Continued.

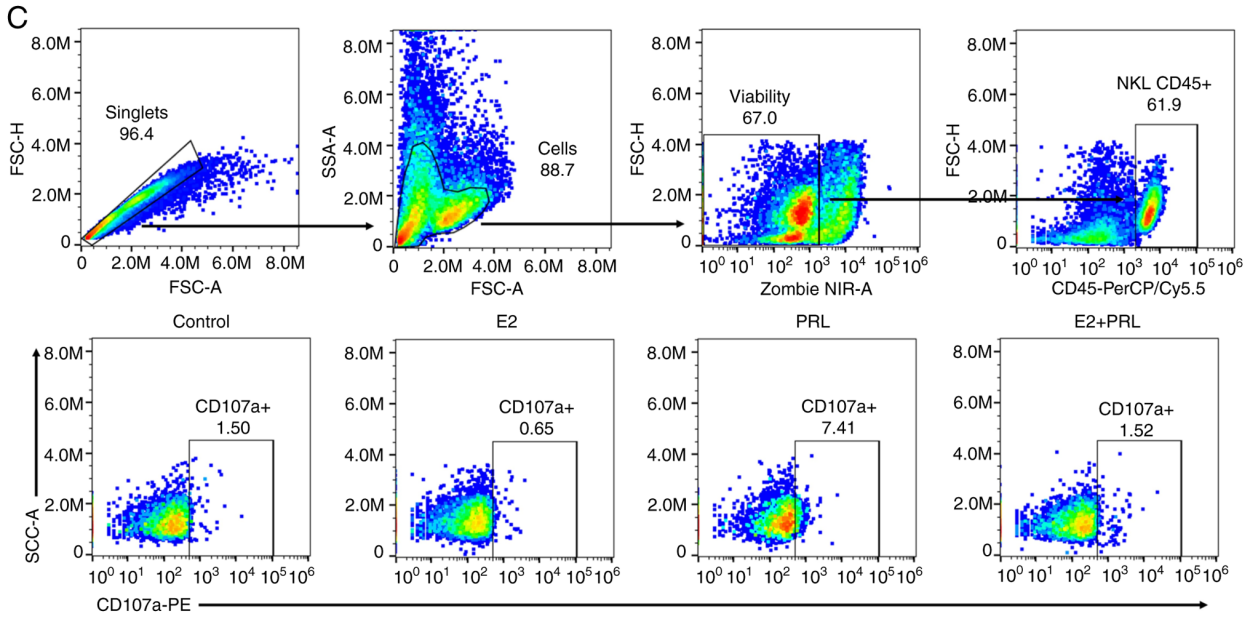

Figure 1. Differential effects of hormones on NK cytotoxic activity. NKL cells were stimulated with E2 (10 nM), PRL (200 ng/ml), both (E2 + PRL) or untreated (control) for 48 h and incubated in the presence of GFP-transfected K562 cells at different E:T ratios (1:1, 5:1 and 10:1). The dead target cells are characterized as GFP^+PI^+. (A) Representative dot plot of three independent experiments. (B) Graphic of specific lysis of target cells, data shown represent the mean from 3 independent experiments. (C) NKL cells were incubated with K562 at 1:1 E:T ratio. Zombie NIR dye, CD45 and CD107a were used to identify viability, NKL population and degranulation respectively. Statistical analysis was performed using ANOVA (*P<0.05). NK, natural killer; E2, 17β-estradiol; PRL, prolactin; GFP, green fluorescent protein; PI, propidium iodide; E:T, effector:target; NIR, near infrared.

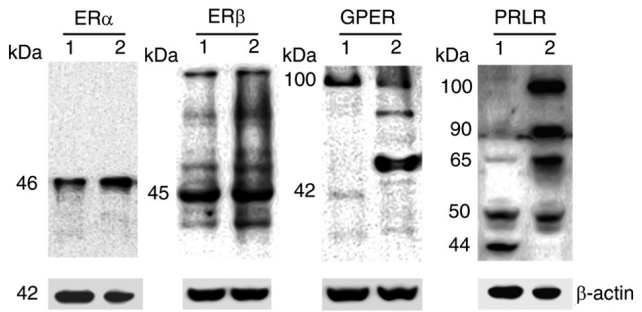

Figure 2. Expression of estrogen and prolactin receptors in NKL cells. The expression of hormonal receptors was evaluated in protein extracts from NKL and MCF7 cell lines. Western blot analysis revealed the expression of all four hormonal receptors (ERα, ERβ, GPER and PRLR) and their different isoforms in NKL (lane 1) and MCF7 cells (lane 2). ERα presented a unique 46-kDa band, ERβ presented bands of approximately 32, 45 and 56 kDa, GPER exhibited bands of 42 and 100 kDa and PRLR presented bands of ~44, 50, 65 and 90 kDa. β-actin was used as a loading control. NK, natural killer; ER, estrogen receptor; GPER, G protein-coupled estrogen receptor 1; PRLR, prolactin receptor.

against GFP-K562 cells. Notably, at a 10:1 ratio, stimulation with PRL exerted a positive effect on the cytotoxicity on NKL cells, contrary to E2, which exerts an antagonistic effect as compared to PRL (P<0.05). Notably, the combined effect of the two hormones exerted similar effects as those observed with PRL alone, as regards NKL cell-mediated cytotoxicity (P<0.05) (Fig. 1B). It was revealed that the hormones located within the TME may have the ability to regulate the cytotoxicity of NK cells with antagonistic outcomes.

To confirm the cytotoxicity assay results, a degranulation assay in NKL cells was performed, and it was observed that CD107a expression tended to decrease in NKL cells stimulated with E2 compared to the untreated control cells. It was also demonstrated that PRL stimulation was able to induce CD107a expression compared to the unstimulated cells, which is consistent with the antagonistic effects shown by the cytotoxicity assay. The E2 + PRL stimulation did not cause a marked change in degranulation marker expression in NKL cells (Fig. 1C).

Expression of hormonal receptors in NKL cells. Once it was observed that hormones can regulate NKL cell-mediated cytotoxicity, the expression of the estrogen receptors (ERα, ERβ and GPER) and the PRL receptor were characterized using western blotting with protein extracts from NKL cells.

The expression of the four hormonal receptors is depicted in Fig. 2. The expression of ERα is denoted by the presence of a single 46-kDa band. The bands indicating the expression of ERβ are 32, 45 and 56 kDa. Of note, the 45-kDa band was thicker in the two cell lines. To the best of our knowledge, this is the first study to demonstrate the presence of GPER in NK cells. GPER was expressed as 42- and 100-kDa bands. Notably, the highest GPER expression was the 100-kDa band, which has been related to glycosylation of this receptor. Finally, PRLR was observed in several bands of approximately 44, 50, 65 and 90 kDa in agreement with the various isoforms of the receptor. Of note, the expression of PRLR was higher in the 50-kDa band, which corresponds to a short isoform of this receptor. When compared with that of the MCF7 cell line, which was used as a positive control, the expression pattern of ERα and ERβ was similar to that observed in the NKL cells. In the MCF7 cells, a higher expression level of the normal form of GPER was observed compared with that in the NKL cells. Finally, it was observed that MCF7 cells expressed the long isoforms of PRLR in a greater proportion in comparison

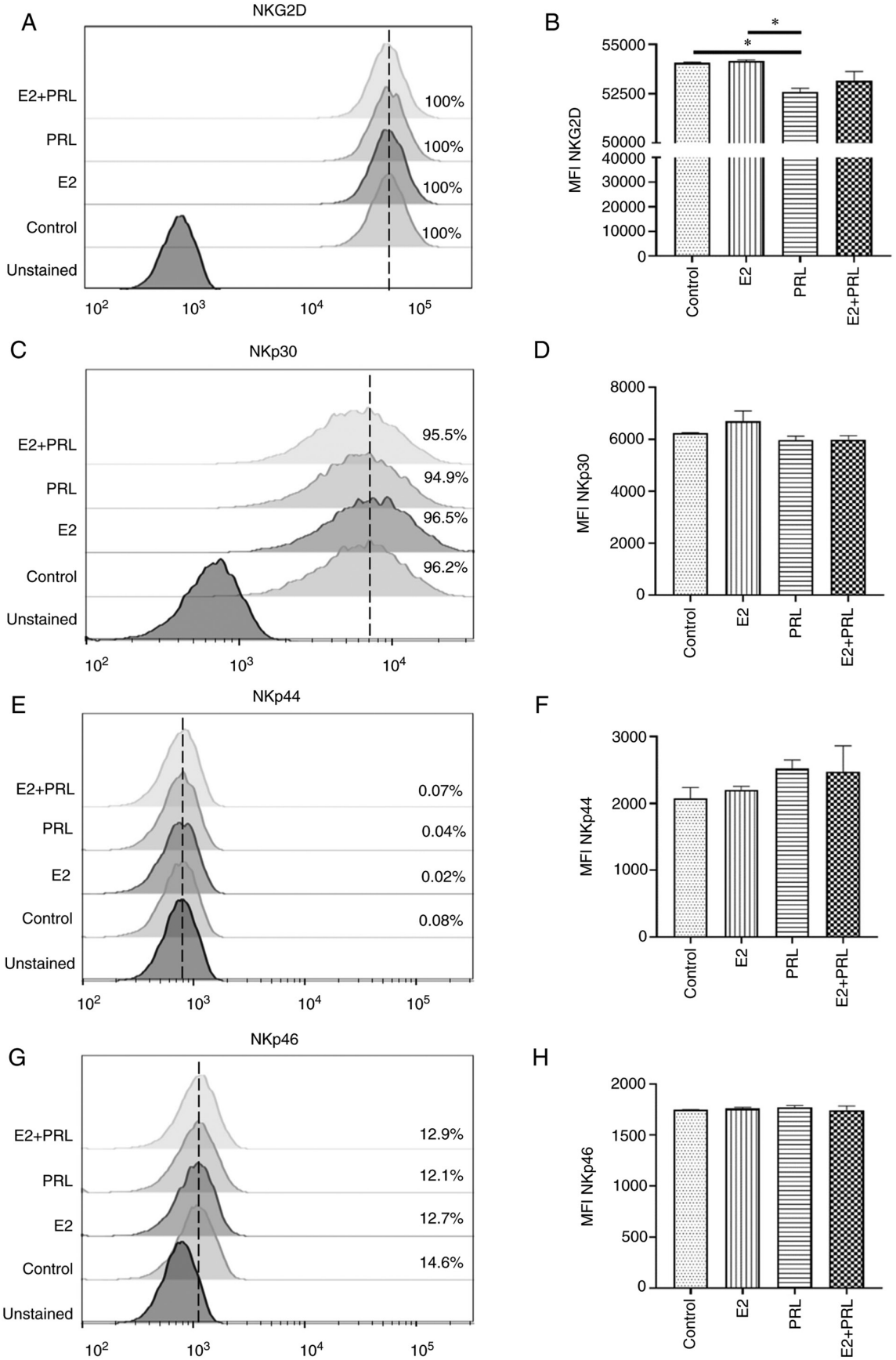

Figure 3. NCR and NKG2D regulation by hormones in NKL cells. The expression of (A) NKG2D, (C) NKp30, (E) NKp44, and (G) NKp46 was analyzed using flow cytometry on NKL cells stimulated with E2, PRL, both or untreated (control). The dotted line (---) indicates the maximum peak located in the untreated control cells. The MFI was expressed as the mean ± SD. (B, D, F and H) Histograms of the MFI representative of each group. All statistical analyses were performed using ANOVA ($^*P<0.05$). NCR, natural cytotoxicity receptors; NKG2D, natural killer group 2D; NKp30, natural cytotoxicity triggering receptor 3; NKp44, natural cytotoxicity triggering receptor 2; NKp46, natural cytotoxicity triggering receptor 1; E2, 17β-estradiol; PRL, prolactin; MFI, mean fluorescence intensity; SD, standard error.

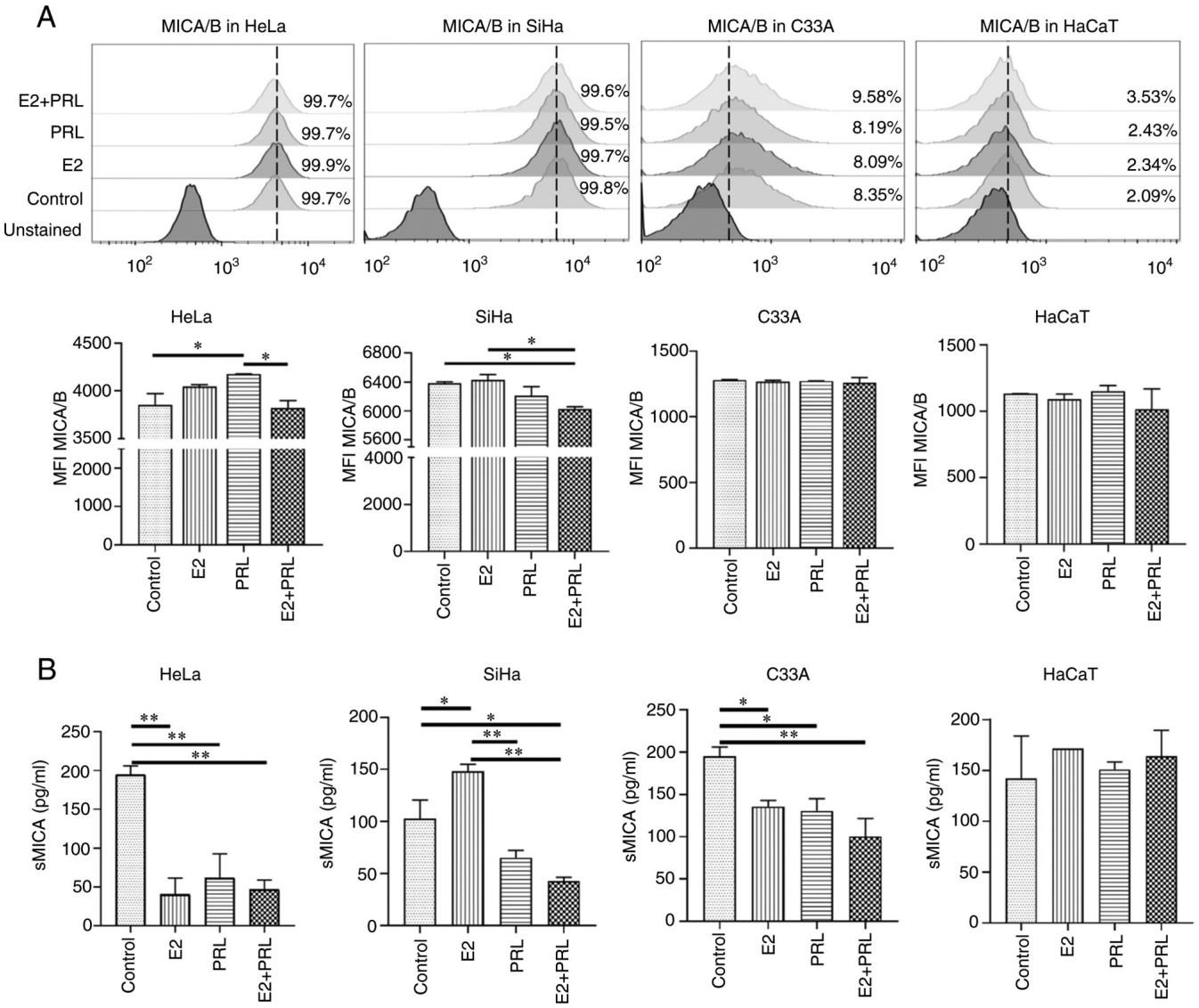

Figure 4. Modulation of MICA/B by hormones in different CC cell lines. (A) Cell surface MICA/B expression was evaluated in HeLa, SiHa, C33A and HaCaT cells stimulated with E2, PRL, both or untreated (control) using flow cytometry. The MFI is expressed as the mean ± SD. (B) The expression of the soluble form of MICA was measured by ELISA assays from HeLa, SiHa, C33A and HaCaT supernatants after 48 h stimuli with E2 (10 nM) and PRL (200 ng/ml). The data shown represent the mean ± SD of absorbance values (450 nm) from two independent experiments. All statistical analyses were performed using ANOVA (*P<0.05 and **P<0.01). MHC class I polypeptide-related sequence A/B; CC, cervical cancer; E2, 17β-estradiol; PRL, prolactin; MFI, Mean Fluorescence Intensity; sMICA, soluble MICA; SD, standard error.

to the NKL cells. The presence of all hormone receptors and their isoforms contribute to a more detailed understanding of the hormonal effects on NK cells.

Expression of NCR and NKG2D in NKL cells stimulated with E2 and PRL. To evaluate the effect of hormone stimuli on activating receptors including NKp30, NKp44, NKp46 and NKG2D expression on NKL cell surface, cells were stimulated with E2 and PRL for 48 h and flow cytometric analysis was performed (Fig. 3). In the untreated control cells (without stimulation), the percentage of NKG2D and NKp30 receptors (100 and 96.2%, respectively) was higher than that for NKp46 (14.6%). As was expected, the percentage of positivity for the NKp44 receptor was zero, as previously reported (44). The histograms demonstrated that the percentages of positive NKL cells for the different receptors were not markedly altered due to hormonal stimuli (Fig. 3A, C, E and G). However, PRL stimuli induced a significant decrease in the expression of NKG2D compared to the untreated control cells or E2 stimuli (P<0.05; Fig. 3B). The expression of NKp30, NKp44 and NKp46 receptors in NKL cells was not significantly altered by E2 and PRL stimuli (Fig. 3D, F, H). The downregulation of NKG2D due to PRL stimulation demonstrated that it may be able to modulate signaling pathways involved in NK cell cytotoxicity.

Modulation in the expression of NKG2D ligands in CC-derived cells by hormonal stimuli. Since CC cells induce the expression of stress ligands, including MICA and MICB (NKG2D receptor ligands in NK cells), the present study then evaluated the effects of E2 and PRL on MICA/B expression in CC-derived cell lines (HeLa, SiHa and C33A) and a non-tumorigenic immortalized keratinocyte cell line (HaCaT). As previously described in the literature, it was observed that, in the untreated control cells, the HPV-18 and

HPV-16 positive cell lines (HeLa and SiHa, respectively) presented a higher percentage of cells expressing MICA/B (99.7 and 99.8%, respectively) (45), contrary to HPV-negative cells (C33A and HaCaT, 8.35 and 2.09%, respectively; Fig. 4A). Stimulation of HeLa cells with PRL increased the expression of MICA/B compared to the untreated control cells ($P<0.05$); however, the simultaneous stimulation with E2 and PRL reversed this effect ($P<0.05$). By contrast, in SiHa cells, the concurrent stimulation with E2 and PRL decreased MICA/B expression compared to the untreated control cells and E2 stimulus ($P<0.05$; Fig. 4A). Hormonal stimuli did not induce changes in MICA/B expression in the C33A or HaCaT cells.

Among the escape mechanisms of tumor cells towards immunological recognition is the release or secretion of soluble forms of activating ligands. In line with this, it has been discovered that MIC molecules can be released into the extracellular matrix and thereby promote an immune escape strategy for tumor cells (32). For this reason, in the present study, the levels of soluble MICA (sMICA) in CC-derived and HaCaT cell line supernatants, stimulated for 48 h with E2, PRL or both was evaluated (Fig. 4B). In comparison to the untreated control cells, stimulation with E2 or PRL alone, and E2 and PRL in combination, decreased the liberation of MICA into the supernatant of all CC-derived cell lines ($P<0.05$), apart from the SiHa cells, where E2 stimulation resulted in increased sMICA levels ($P<0.05$). This effect was abrogated by stimulation with PRL alone, and with E2 and PRL in combination ($P<0.01$) in SiHa cells. Notably, the hormone stimuli had no effect on MICA secretion in the HaCaT cell supernatant. Both the membrane and soluble form of the MICA ligand are regulated by E2 and PRL in CC-derived cell lines.

Discussion

CC represents one of the main health issues in women. Of note, 604,000 new cases worldwide were estimated in 2020. The main risk factor associated with CC is HPV infection, which is present in >99% of patients with CC (1). However, it has been revealed that HPV infection alone is not sufficient for CC to manifest (16). In this sense, the hormonal role constitutes an important factor for the carcinogenesis of this type of tumor. Hormones, including 17β-estradiol and PRL are related to the genesis, persistence and development of CC (14,16,21,46), since in addition to being present in the TME of this cancer type, they can contribute to anti-apoptotic, proliferative, invasive, survival effects and metabolic adaptation of CC cells (10,15,21,22,25,47). In addition, they can regulate the expression of HPV oncogenes (48). The functionality of these hormones within the TME may also depend on a bilateral regulation between the two hormones, since there are studies demonstrating the possible regulation of PRLR by E2, as well as the regulation of estrogen receptors exerted by PRL effects (49-51).

In the TME there are also cells of the innate immune system, including NK cells, which have the potential to kill cells transformed and infected by HPV (52). As regards CC, studies have revealed that there is a poor infiltration of NK cells, and therefore this may be associated with a decrease in their cytotoxic activity against tumor cells (53,54). In both *in vitro* models and patients, it has been observed that CC cells are capable of regulating NK cell cytotoxicity given that tumor-infiltrating NK cells decrease the expression of perforins, activating receptors and IFN-γ, and on the other hand increasing the expression of inhibition receptors (54,55). Likewise, the expression of activating receptors, such as NKG2D, and the expression of cell stress ligands with CC have been related (30,31,45,56). The present study demonstrated that E2 decreased the cytotoxicity of NKL cells, as well as CD107a expression, which is consistent with the findings in the studies by Hao *et al* (57,58) underlining that various concentrations of E2 may have a negative effect on proliferative capacity, IFN-γ expression and the cytotoxic effects of the NK cells extracted from mouse spleens against the YAC-1 target cells. A possible explanation for this phenomenon is the indirect decrease in granzyme B levels, due to the effect of E2, where it has been demonstrated that estrogen induces the expression of inhibitory proteinase 9, a potent inhibitor of granzyme B (38,58).

Subsequently, when confirming the effect of E2 on the cytotoxicity of NKL cells, the present study analyzed the possible isoforms of the estrogen receptors that these cells express, with the aim of determining the pathway through which E2 may exert such an effect. As regards ERα, it has been revealed that it presents with three main isoforms, known as ERα66, ERα46 and ERα36, named for their characteristic molecular weights (59). NKL cells express the 46 kDa isoform of ERα, which has also been previously detected in lymphocytes with $CD3^+$ $CD8^+$ and $CD3^-CD56^+$ phenotypes obtained from peripheral blood (60). This isoform is characterized by the lack of the first 173 amino acids of the amino terminal AF-1 domain and has been associated with an inhibitory role on tumor cell growth, as in breast cancer cells ERα46 may inhibit the estrogenic effects of ERα66, inducing in turn cell proliferation and cell cycle progression. It has been suggested that these effects occur due to a functional competition between both isoforms (59,61,62). In relation to ERβ, in other human cancer models it has been demonstrated that this receptor presents with various isoforms, known as ERβ1, β2, β3, β4 and β5 whose molecular weights range from 50 to 59 kDa (63). NKL cells strongly express ERβ, represented as a 45-kDa band and a weaker expression of a 56-kDa band. Similarly, in peripheral blood lymphocytes the expression of ERβ with weights lower than 56 kDa has been detected (60). Furthermore, in breast cancer cells the presence of ERβ isoforms with molecular weights of around 44 kDa has also been observed. This may be attributed to the fact that exons 5 and 6 of the ERβ mRNA are eliminated, thereby generating a protein of lower molecular weight (60,64). To date, the possible role of these ERβ isoforms with molecular weights <50 kDa is unknown; therefore, further studies are warranted to achieve a better understanding at the functional level of these variants.

Another receptor through which E2 has been reported to exert its effects is the G protein-coupled estrogen receptor, GPER, which has been reported to be associated with non-genomic pathways through kinase-dependent signaling for rapid gene regulation (65). Recent findings have revealed that GPER is overexpressed in biopsies of patients with CC and its agonistic activation increases mitochondrial permeability,

as well as apoptosis, as well decreases the proliferation of CC cells (15). NKL cells express a weak band of 42 kDa and a strong band of around 100 kDa. Currently, no evidence has been reported concerning the presence of GPER in these cells; however, the high weight of GPER has been related to glycosylated forms and/or dimerization of this receptor (66,67). It has been demonstrated that GPER glycosylation may occur mainly in an asparagine residue known as Asn^{44} and this post-translational modification has been associated with its location in the plasma membrane, where GPER can regulate the rapid non-genomic response of estrogens (68,69).

By contrast, in the present study it was observed that stimulation with PRL induced an increase in NKL cell-mediated cytotoxicity and CD107a expression. This is in line with previous studies by Sun *et al* (37,70), where NK cells extracted from mice treated with PRL and also from cell lines including NK-92 were used. This increase in NK cell-mediated cytotoxicity may be attributed to the fact that PRL, in conjunction with IL-2 and IL-15, may increase the expression of IFN-γ, perforins and Fas-L (37). Considering that PRL has been reported to exert its effects through its receptor, it was decided to visualize the possible isoforms by which PRL could exert this effect on cytotoxicity. PRLR is expressed in a number of isoforms, including a long (between 80 and 90 kDa), an intermediate (65 kDa) and 2 short isoforms (between 40 and 55 kDa) (23). The variant with the highest expression in NKL cells was the short isoform corresponding to the 50-kDa band, characterized by the lack of the Box 2 region, which is crucial for the interaction with proteins containing an SH2 domain, including STAT proteins, leading to a negative regulation on the effects triggered by the long isoform of the PRLR (18,23). Further more detailed studies are required to determine whether the different PRLR isoforms may have a functional effect on NK cells. Notably, the concurrent stimulation of E2 and PRL also increased the cytotoxicity of NKL cells, as observed with PRL alone. It was observed that PRL may overcome the effects of E2 and as previously mentioned, this may be attributed to both hormones having a bilateral regulation (49-51). This is in line with Riera-Leal *et al* (14), who observed in a context of CC cell metabolism, that PRL may have a greater impact over the estrogenic effects induced by E2.

Subsequently, the present study aimed to evaluate whether the differences in the cytotoxicity of NKL cells by hormones may be attributed to changes in the expression of activation receptors, including NCR and NKG2D. The data obtained did not indicate that these effects were related to the NCR, since it was observed that the hormones did not modify the expression of NKp30, NKp44 and NKp46. By contrast, it was observed that PRL may decrease the expression of NKG2D in NKL cells. This is in line with a previous study by Ma *et al* (71) in 2010, where it was demonstrated that the expression of NKG2D may decrease in T lymphocytes from patients with prolactinoma.

When the change in the expression of NKG2D by these hormones was observed, it was decided to evaluate MICA and MICB ligands of this receptor, which are expressed in the membrane, as well as in soluble forms. MICA and MICB are known as stress proteins and these ligands have been reported to be elevated in CC patient biopsies and to be also overexpressed in CC-derived cell lines, including SiHa, HeLa, CALO and INBL (45,54,71). The results of the present study are consistent with the findings from the study by del Toro-Arreola *et al* (45), with MICA/B being expressed mainly in HaCaT, C33A, HeLa and SiHa cells. Furthermore, sMICA was detected in cell supernatants at relatively similar levels. Of note, it was demonstrated that PRL may increase MICA/B expression on the HeLa cell surface, while decreasing sMICA release in the supernatant of all CC-derived cells. In the context of the interaction that exists in the CC microenvironment, the increase in cytotoxicity which was observed under the effect of PRL may be explained by the increase in MICA/B in the membrane, which can bind to NKG2D; this is also supported by the decrease in sMICA. To the best of our knowledge, there no studies available to date that relate the effect of PRL with the release of MIC molecules. However, it has been revealed that metalloprotease 9, which has the ability to cleave MICA, decreases its expression due to the effects of PRL, possibly explaining the aforementioned result (72,73). By contrast, in the present study, E2 decreased sMICA in HeLa and C33A cells, whereas an opposite effect was observed in SiHa cells, possibly indicating that the effects of E2 vary depending on the cell type. Although an effect of E2 on MICA/B surface expression, when stimulating the cells with both hormones was not detected, it was observed that sMICA expression decreased in all CC-derived cell lines. This may indicate that the joint effect of the hormones may be related to the increase in the cytotoxicity of NKL cells and also supporting the regulatory effect of one hormone on the other.

In conclusion, the results of the present study suggested that E2 and PRL, which are overexpressed in the CC microenvironment, may antagonistically regulate the cytotoxicity of NK cells. Furthermore, NKL cells express different variants of the hormone receptors by which their effects may be exerted. By contrast, hormones regulate the expression of molecules, including the NKG2D receptor, MICA/B ligands and their soluble forms, which may be involved in the cytotoxicity of NK cells. This knowledge revealed an overview that may help in understanding further the mechanism by which these hormones may contribute to the development of CC. It would be of interest to evaluate the possible molecules involved in pathways triggered by E2 and PRL on NK cell-mediated cytotoxicity in future studies, using next-generation RNA sequencing, ultimately aiming to identify novel therapeutic targets involved in CC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AGP and CDHS performed the experiments. MGC assisted in the flow cytometry data analysis and in the interpretation of the results. ARdA, JCVP, IGRL, AAL and MGC contributed to the statistical analysis and the critical review of the manuscript. JSZN participated in the ELISA. AGP and MGC confirm the authenticity of all the raw data. ALPS designed the study and wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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