NK Cells Expressing a Progesterone Receptor Are Susceptible to Progesterone-Induced Apoptosis[1]

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It has been proposed that progesterone (P4) induces the suppression of immune responses, particularly during pregnancy. However, knowledge about the mechanisms involved has remained largely elusive. We demonstrate herein that peripheral blood NK (PBNK) cells express both classical progesterone receptor (PR) isoforms and are specifically affected by the actions of P4 through two apparently independent mechanisms. Progesterone induces caspase-dependent PBNK cell death, which is reversed by two different anti-progestins, ZK 98.299 and RU 486, supporting the involvement of classical PR isoforms. It was suggested that $CD56^{bright}CD16^{-}$ killer Ig-like receptor $(KIR)^{-}$ NK cells might represent precursor cells, which, upon activation, acquire the features of a more mature NK subset expressing KIR receptors. The present study demonstrates that PR expression seems to be restricted to more mature KIR^{+} PBNK cells. The expression of PR had a functional counterpart in the suppressive effect of P4 on IL-12-induced IFN-γ secretion. This cytokine suppression was mainly observed in KIR^{+} PBNK cells, without affecting the high secretion of IFN-γ by $CD56^{bright}$ PBNK cells. The lack of PR expression on $CD56^{bright}KIR^{-}$ PBNK cells provides an additional phenotypic marker to test the idea that they might represent the PBNK precursors selectively recruited into the endometrium where they differentiate to become the uterine NK cells. Additionally, these findings may be relevant to NK cell function in viral immunity, human reproduction, and tumor immunity. *The Journal of Immunology,* **2008, 180: 5746–5753.**

As suggested by their name, NK cells occur naturally and are part of the innate immune system. Unlike T and B cells, they do not require sensitization to become activated, and they represent the first line of host defense against viral infections and tumors. NK cells produce immunoregulatory cytokines that contribute to early host defense against several types of viruses (1), bacteria (2), and parasites (3). In humans, NK cells represent ~10% of the PBMC. NK cells can be divided into two distinct subsets based on their expression of CD56. Most peripheral blood NK (PBNK)[3] cells show low levels of CD56 ($CD56^{dim}$) and high levels of CD16 expression, while a small subset (~10%) expresses high levels of CD56 ($CD56^{bright}$) and low, or no, CD16.

Progesterone (P4) has been considered for years to be a natural immunosuppressant. However, little is known about the mechanisms that control this effect. An in vivo immunosuppressive effect of P4 was demonstrated by prolonged survival of xenografts following P4 implants that were at concentrations typically found in the placenta. Similarly, in vitro studies also demonstrated the ability of P4 to inhibit lymphocyte activation and proliferation in response to alloantigens or mitogens. It was proposed that P4 effects on T cells were mediated through the blockade of a K^{+} channel, which rapidly inhibited the Ca^{2+} signaling pathway, thus inhibiting production of the major proliferative cytokine for T cells. This mechanism would require a high concentration of P4, which is not blocked by classical antagonists (4). This rapid mechanism resembles nongenomic effects described for steroids as early as 60 years ago. These mechanisms were described as nongenomic because the effects were not abrogated by transcriptional inhibitors and seemed to occur without requiring the hormone to bind intracellular receptors (5).

Most members of the steroid hormone family can exhibit rapid effects. For example, the increases in Ca^{2+}, inositol triphosphate, and diacylglycerol are inhibited by neomycin or pertussis toxin, but not by the classical hormone antagonists, suggesting the involvement of G protein-bound membrane receptors. In particular, P4 has been shown to interact with other membrane receptors, including the oxytocin receptor (6) and the nicotinic acetylcholine receptor (7). Membrane progestin receptors named α, β, and γ have been cloned and encode membrane proteins with seven transmembrane domains. Their transcripts show distinct distributions in reproductive, neural, kidney, and intestinal tissues. However, several synthetic progestins and antiprogestins, with relatively high binding affinities for nuclear progesterone receptor (PR), displayed no binding affinity for the recombinant human γ protein (8). Additionally, a membrane-associated progesterone-binding protein called 25-Dx has been observed in brain regions involved in reproductive behavior (9).

Classic PR is a ligand-activated nuclear transcription factor. After binding to P4, the PR dissociates from chaperone proteins, dimerizes, and binds to specific DNA sequences (10), enhancing transcription of target genes (11, 12). Two isoforms have been

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[3] Abbreviations used in this paper: PBNK, peripheral blood NK; P4, progesterone; PR, progesterone receptor; KIR, killer Ig-like receptor; RT, room temperature; PI, propidium iodide; DEX, dexamethasone; ZK, ZK 98.229; RU, RU 486; PBST, PBS with Tween 20; E2, 17β-estradiol; GR, glucocorticoid receptor; DC, dendritic cell; uNK, uterine NK cells; MPA, medroxiprogesterone acetate.

described in human cells, PR_B and PR_A (13), with molecular masses of 114–120 and 94 kDa, respectively. P4-induced genomic effects mediated by PR are usually blocked by antiprogestins, such as RU 486 (mifepristone) and ZK 98.29 (onapristone) (14, 15).

Herein we show that PBNK cells express the classical PR and are specifically affected by the actions of P4 via two apparently independent mechanisms. P4 induces significant caspase-mediated, dose-dependent cell death and a significant reduction in the secretion of IFN- γ. More importantly, we demonstrate that expression and functional responses to P4 are restricted to more mature PBNK cells.

Materials and Methods

Subjects

Buffy coat obtained from 1 U of blood from volunteers' donations was collected from 10 male and 10 nonpregnant female healthy donors (mean age 34 years, range 27–42 years) and processed immediately. In all of these subjects, we excluded any infectious, endocrine, and anatomic abnormalities. No subjects were taking oral contraceptives, and all were off any therapy at the time of the study. This investigation was approved by the Investigation and Ethics Committee at the Hospital de Clínicas José de San Martín, and informed consent was obtained from all subjects.

Isolation of PBMCs

PBMCs were obtained from buffy coats through a Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation.

NK cell purification

NK cells were enriched by using the RosetteSep human NK cell enrichment cocktail (Stem Cell Technologies) following the manufacturer's protocols. In brief, in the first step leukocyte concentrates were incubated with Abs against cell-surface Ags on human hematopoietic cells (CD3, CD4, CD19, CD36, CD66b) and glycoforin A on RBCs. In the second step, the sample was diluted with twice the volume of PBS containing 2% FCS (Natocor), and NK cells were purified through a Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation. The enriched cells were removed from the Ficoll-Hypaque/plasma interface, washed twice, and resuspended in complete culture medium (RPMI 1640, Sigma-Aldrich) supplemented with 10% heat-inactivated charcolized FCS, 200 mM L-glutamine (Sigma-Aldrich), and gentamicin. From each individual donor, we obtained $40–80 \times 10^6$ PBNK cells containing >90% purified NK cells (as determined by flow cytometry analysis).

Killer Ig-like receptor (KIR)$^+$ and KIR$^-$ NK cell isolation

Enriched PBNK cells were incubated with a combination of the following mouse anti-KIR mAbs: KIR2DL2/L3/S2 (clone CHL, kindly provided by S. Ferrini), KIR2DL1/S1/S3 (clone HP-3E4) and KIR2DL1/S1 (clone HP-MA4) (kindly provided by M. Lopez Botet), KIR3DL2/L1 (5.133, kindly provided by M. Colonna), and KIR2DS4, which was purchased from R&D Systems. In a second step, cells were mixed with goat anti-mouse IgG magnetic beads (Dynal Biotech). KIR$^-$ PBNK cells were obtained by immunomagnetic negative selection, and the KIR$^+$ PBNK cells were detached from the magnetic beads using DETACHaBEAD (Dynal Biotech). After an overnight incubation at 37°C, cells were first incubated with a purified mouse anti-human KIR mix and afterward with a FITC goat anti-mouse (DakoCytomation). The purity of KIR$^+$ and KIR$^-$ PBNK cells ranged between 85–95% and 86–90%, respectively.

CD4$^+$ T cell isolation

CD4$^+$ T cells were obtained by negative selection using the Dynal CD4$^+$CD25$^+$ Treg Kit (Dynal Biotech) according to the manufacturer's protocols. In brief, PBMCs were incubated with Abs against B cells, NK cells, monocytes, CD8$^+$ T cells, erythrocytes, and CD45RA cells, which were depleted by mixing with Dynal Dynabeads (immunomagnetic negative selection). The enriched cells were washed twice and resuspended in complete culture medium. The purity of isolated CD4$^+$ T cells was between 87 and 94%, as determined by flow cytometry.

CD20$^+$ B cell isolation

PBMCs were incubated with mouse anti-human-CD20 IgG mAb (BD Biosciences) and then washed twice with culture medium. In the second step, cells were mixed with goat anti-mouse IgG magnetic beads (Dynal Biotech). After positive selection, cells were detached by adding DETACHaBEAD to yield 90% CD20$^+$ cells.

CD3$^+$ T cell isolation

PBMCs were incubated with mouse anti-human-CD3 IgG mAb (BD Biosciences) and then washed twice with culture medium. Afterward, cells were mixed with goat anti-mouse IgG magnetic beads (Dynal Biotech). After positive selection, cells were detached by adding DETACHaBEAD to yield >90% of CD3$^+$ cells.

Cell lines

T47D human breast carcinoma cells and K562 human chronic myelogenous leukemia cells were maintained in complete culture medium. Both cell lines were obtained from the American Type Culture Collection.

Flow cytometry

PBMCs or purified cells were stained with FITC or PE-labeled mAb specific for CD56, CD16, CD3, CD4, and CD20 (BD Biosciences). Negative control samples were incubated with an isotype-matched Ab. Cells was analyzed on a FACSCalibur cytometer using WinMDI software (BD Biosciences). Dead cells were excluded by forward and side scatter characteristics. Statistical analyses are based on at least 30,000 events gated on the population of interest.

Intracellular staining for detection of endogenous PR

Purified PBNK cells ($1–2 \times 10^6$) were fixed in 1 ml of PBS with 1% paraformaldehyde containing 0.05% Tween 20. After an overnight incubation at 4°C, cells were treated twice with 0.5 ml of RNase-free DNase at 100 U/ml (Promega). Staining steps were performed for 1 h at room temperature (RT). Cells were incubated with a mouse anti-human PR mAb (Ab-7, Lab Vision) and washed with FACS staining buffer (PBS supplemented with 3% charcolized FCS, 0.50% Tween 20, and 0.05% azide). PR mAb binding was detected using Alexa Fluor 488 goat anti-mouse-IgG (Molecular Probes) and washed as described above. Cell-surface staining was then performed using the anti-human mAb PE-CD56, PE-CD4, PE-CD3, or PE-CD20 (BD Biosciences) for 20 min at RT followed by washing with PBS. Cells were analyzed using a FACSCalibur cytometer.

The presence of PR was also determined using a rabbit anti-human PR Ab (C-20, Santa Cruz Biotechnology), and an anti-rabbit IgG FITC conjugate (BD Biosciences) was used as a secondary Ab following the technique described above.

Confocal laser scanning microscopy

Purified PBNK cells or CD4$^+$ T cells were stained with Ab-7 mAb (working dilution 1/50) and incubated for 1 h at RT. Cells were washed twice with FACS staining buffer before the addition of Alexa Fluor 488-labeled goat anti-mouse IgG. After washing twice, cells were blocked with 10% mice serum followed by a standard CD56 or CD4 surface staining. Cells were allowed to adhere onto polylysine-coated glass coverslips for 30 min. The nuclear staining was detected in blue by 4′,6-diamidino-2-phenylindole (DAPI) staining. The coverslips were mounted with 1,4-diazabicyclo[2.2.2]octane (DABCO) on glass slides and observed on a digital Eclipse E800 Nikon C1 confocal microscope system with Nikon Plan Apo 60×/1.40 Oil.

Apoptosis assay

Assays were performed on purified NK cells or following their sorting into KIR$^+$ and KIR$^-$ PBNK cells. Total purified PBNK cells or KIR$^+$ and KIR$^-$ PBNK cells (2×10^5) were cultured in complete culture medium for 48 h in a 96-well U-bottom plate (BD Biosciences) in the absence or presence of different concentrations of P4 (1×10^{-6}, 1×10^{-7}, and 1×10^{-8} M; Sigma-Aldrich) in a final volume of 200 μl. Percentage of apoptosis and necrosis was determined by using FITC-labeled annexin V and propidium iodide (PI) (BD Biosciences), respectively, and analyzed with a FACSCalibur cytometer using WinMDI software. To test whether P4 triggered caspase activation, cells were also incubated with this steroid hormone in the presence of 50 μM caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and capase-9 inhibitor (Z-LEHD-FMK), all from R&D Systems. The effect of dexamethasone (DEX) on purified PBNK cells was tested by culturing PBNK cells in the absence or presence of different concentrations of DEX (1×10^{-7} and 1×10^{-9} M, Sigma-Aldrich). To investigate whether the apoptotic effect of P4 could be blocked by antiprogestins, we incubated purified PBNK cells with P4 in two different doses (1×10^{-6} and 1×10^{-8} M) in the presence or absence of two antiprogestins: ZK 98.299 (ZK; Schering) at 1×10^{-5} and $1 \times$

10^{-6} M concentration and RU 486 (RU; Sigma-Aldrich) at 1×10^{-7} M concentration. Cells were cultured for 48 h. Results were analyzed as described above.

Cytotoxicity assays

A 4-hr standard ^{51}Cr (PerkinElmer) release assay was performed by using as target cells, 5×10^3 ^{51}Cr-labeled human erythroleukemic cell line, K562, per well mixed with NK cells as effector cells. PBNK cells cultured for 24 h with 1×10^{-8} M P4 or with medium alone were used at E:T ratios of 20:1, 10:1, 5:1, and 1:1. The percentage of cytotoxicity (^{51}Cr release) was calculated as follows: 100 × [(Experimental release − Spontaneous release)/(Maximum release − Spontaneous release)]. Maximum release was obtained from target cells lysed with 2% Triton X-100. Spontaneous release was always <15% of maximum release.

PBNK cell stimulation by IL-12 and IL-15

In some experiments, total PBNK cells or KIR$^+$ and KIR$^-$ PBNK cells were stimulated with 10 ng/ml IL-12 and/or 10 ng/ml IL-15 (PeproTech) and were used for apoptosis assays or cytokine quantification.

Cytokine quantification

Total purified PBNK cells or KIR$^+$ and KIR$^-$ PBNK cells (2×10^5) were stimulated with 10 ng/ml IL-12 and/or 10 ng/ml IL-15 (PeproTech), and secretion of IFN-γ was measured by an ELISA test. The concentrations in culture supernatant were assessed by using mAbs and recombinant cytokines from Pierce according to the manufacturer's instructions. Briefly, flat-bottom 96-well microtiter plates were coated with mouse anti-human IFN-γ mAb at 2 μg/ml in PBS and incubated overnight at 4°C, followed by blocking with PBS containing 2% BSA for 90 min at RT. Samples and IFN-γ standard (human rIFN-γ) were serially diluted and incubated at RT for 90 min. Subsequently, biotinylated anti-IFN-γ mAb was added at 0.75 μg/ml for 1 h at RT. Avidin-alkaline phosphatase conjugate (Sigma-Aldrich) was then added for 30 min. Alkaline phosphatase substrate solution was added and the plates were read in an ELISA reader at 405-nm wavelength. Washing steps (Tris 50 mM and 0.2% Tween 20) were included between each step of the ELISA. A standard curve was plotted and regression analysis was applied. The sensitivity of this assay was 15 pg/ml.

Western blot

Cells were lysed on ice for 30 min in buffer A (20 mM Tris-HCl (pH 7.4), 1.5 mM EDTA, 0.25 mM DTT, 20 mM Na_2MoO_4, 10% glycerol). Protease inhibitors (0.5 mM PMSF, 0.025 mM *N*-carbobenzyloxy-L-phenylalanyl chloromethyl ketone (ZPCK), 0.0025 mM N_α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLC), 0.025 mM *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.025 mM N_α-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME); Sigma-Aldrich) were added before preparing the extracts. The homogenate was centrifuged for 20 min at 12,000 rpm at 4°C. The supernatant was immediately stored at −70°C or in liquid nitrogen and used later in the immunoblot assays. Protein concentration was determined by the method of Lowry et al. (16). The samples (100 μg total protein/lane) were separated on 8% discontinuous polyacrylamide gels (SDS-PAGE) using the Laemmli buffer system (17). The proteins were dissolved in sample buffer (6 mM Tris (pH 6.8), 2% SDS, 0.002% bromophenol blue, 20% glycerol, and 5% mercaptoethanol) and boiled for 4 min. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane and blocked overnight in 5% dry skimmed milk dissolved in 0.1% PBS with Tween 20 (PBST) (0.8% NaCl, 0.02% KCl, 0.144% Na_2PO_4, 0.024% KH_2PO_4 (pH 7.4), 0.1% Tween 20). The membranes were washed several times with PBST and then incubated with PR Ab-7/hPRa 7 (Ab7, NeoMarkers) or with PR C-19 (Santa Cruz Biotechnology) at RT for 2 h, at a concentration of 2 μg/ml in PBST. Blots were probed with sheep anti-mouse or donkey anti-rabbit Ig, HRP-conjugated whole Ab (Amersham Life Sciences). The chemiluminescence signal was generated with luminol (A8511-5G, Sigma-Aldrich), 4-hydroxycinnamic acid (p-coumaric acid; C9008-25G, Sigma-Aldrich), and hydrogen peroxide (Merck). Rainbow protein m.w. markers were from Cell Signaling Technology. The blots were exposed to medical x-ray film (CP-BU New, Agfa).

Statistical analysis

We performed statistical analyses using GraphPad Prism version 3.0 for Windows (GraphPad software). The significance of differences between the different groups was determined by one-way ANOVA and by the Mann-Whitney *U* test. p values of <0.05 were considered statistically significant.

Results

P4 triggers dose-dependent cell death in CD56$^+$ PBNK cells

In preliminary experiments, we observed that P4 induced apoptosis in a subset of PBMC, which we subsequently identified as CD56$^+$. To confirm this effect, purified PBNK cells from healthy donors (median purity of 90%) were cultured for 48 h in the presence or absence of decreasing concentrations of P4. Fig. 1*a* shows the results from five independent experiments where PBNK cells were cultured with decreasing concentrations of P4. When the effect of P4 was compared with the cell death of PBNK cells cultured in medium alone (mean ± SEM, 9.25 ± 0.95%), we observed a significant dose-dependent increase of cell death: 31.13 ± 1.74%, $p < 0.0002$; 21 ± 0.89%, $p = 0.0007$; and 14.75 ± 0.95%, $p = 0.0013$ with 1×10^{-6}, 1×10^{-7}, and 1×10^{-8} M P4, respectively. As an additional control, cells cultured in the presence of 1×10^{-7} M concentration of 17β-estradiol (E2) showed a cell death frequency similar to that observed in cells cultured with medium alone (p = NS).

Anti-progestins inhibit the apoptotic effect of P4

PBNK cells were cultured for 48 h with the lower concentration of P4 and with or without antiprogestins. As indicated in Fig. 1*b*, the addition of ZK (1×10^{-6} M) inhibited the apoptotic effect of 1×10^{-8} M P4 by restoring the viability of PBNK cells to values similar to the controls without this sex hormone. The results include data expressed as the percentage of induced cell death ± SEM of triplicate samples obtained in five independent experiments using five different donors.

Progesterone delivers inhibitory signals through a caspase-dependent pathway

We examined whether caspase-3, caspase-8, or caspase-9 inhibitors were able to overcome the apoptosis triggered by P4. PBNK cells were cultured for 48 h with P4 (1×10^{-8} M) in the presence or absence of 50 μM caspase-3, caspase-8, or caspase-9 inhibitors. Data from four independent experiments were analyzed by flow cytometry and cell death was measured by PI and annexin V staining. As depicted in Fig. 1*c*, all three inhibitors were able to block the P4-induced apoptosis (data are expressed as mean percentage of dead cells ± SEM). The addition of caspase inhibitors did not affect cellular proliferation in the absence of P4.

The effects of glucocorticoid hormones are also mediated by an intracellular glucocorticoid receptor (GR). In the absence of ligand, the GR is located in the cytoplasm, but upon ligand-induced activation, the receptor translocates to the nucleus, where it can initiate transcription of specific target genes by binding to glucocorticoid-responsive elements in the promoter region of target genes and interact with transcriptional coactivators. To rule out the possibility that P4 could be exerting the apoptotic effect by a glucocorticoid-like effect, we compared P4 and DEX capacity on PBNK cell apoptosis and the ability of two different antiprogestins to reverse the hormone-induced effect. RU antagonist is known to induce a receptor conformation that is able to bind DNA (14) with very high affinity, thus antagonizing both the PR and the GR. In contrast, ZK, a type II low-affinity antagonist that induces a conformation that does not bind DNA (15), has been considered as a pure P4 antagonist. Purified PBNK cells were cultured for 48 h in the presence of P4 (1×10^{-8} M) or DEX (1×10^{-9} M). As depicted in Fig. 1, *b* and *d*, ZK (1×10^{-6} M) only abrogated P4-induced apoptosis (ZK vs P4, $p = 0.002$; ZK vs DEX, p = NS). In contrast, RU (1×10^{-6} and 1×10^{-7} M) was able to inhibit the apoptosis induced by both P4 and DEX (RU vs P4, $p < 0.038$; RU vs DEX, $p < 0.028$).

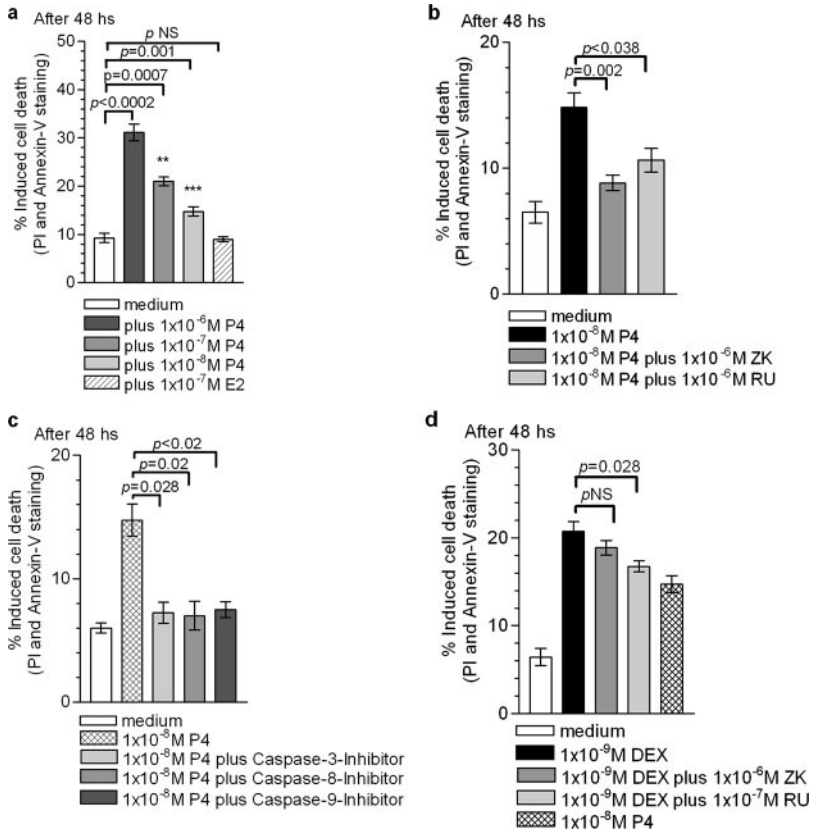

FIGURE 1. Progesterone-induced apoptosis in PBNK cells. *a*, Purified PBNK cells (2×10^5, median purity of 90%) were cultured in a final volume of 200 μl for 48 h in the presence or absence of decreasing concentrations of P4 (1×10^{-6}, 1×10^{-7}, and 1×10^{-8} M). Early and late apoptosis of the purified PBNK cells were determined by annexin V and PI staining of triplicates. Results obtained from five independent experiments, using five healthy donors, are expressed as the mean percentage of cell death ± SEM. Indicated *p* values were obtained by comparing different concentrations of P4 or E2 vs medium only. **, *p* values obtained by comparing P4 at 1×10^{-6} M vs P4 at 1×10^{-7} M ($p < 0.001$) and ***, 1×10^{-7} M vs 1×10^{-8} M ($p < 0.0007$). *b*, Purified PBNK cells were cultured for 48 h with a lower concentration of P4 (1×10^{-8} M) in the presence of both anti-progestins. ZK (1×10^{-6} M) or RU (1×10^{-6} M) inhibited the apoptosis induced by P4 (mean percentage of cell death ± SEM = 15.50 ± 1.05% for P4, 9.0 ± 0.57% for P4 plus ZK, and 10.63 ± 0.94% for P4 plus RU). Results and *p* values depicted in the figure are representative of five independent experiments with five donors. *c*, Purified PBNK cells were cultured for 48 h with P4 (1×10^{-8} M) in the presence or absence of 50 μM of the following inhibitors: caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and capase-9 inhibitor (Z-LEHD-FMK). All three inhibitors were able to block P4-induced apoptosis. The means ± SEM are representative of triplicates from four independent experiments performed with four donors. Cell death was also measured by flow cytometry after PI and annexin V staining. *d*, Purified PBNK cells were cultured for 48 h in the presence of P4 ($1 \times 10^{-}8$ M) or DEX (1×10^{-9} M). The addition of RU was able to abrogate the apoptosis induced by P4 and DEX (RU vs P4, $p < 0.038$; RU vs DEX, $p < 0.028$). In contrast, ZK acted mainly as an inhibitor of the P4-induced apoptosis (ZK vs P4, $p = 0.002$; ZK vs DEX, $p =$ NS), with little or no effect on its capability to abrogate the DEX-induced apoptosis. Results are expressed as the mean percentage of induced cell death ± SEM of triplicate samples obtained in five independent experiments.

PR expression

The experiments described in the preceding section were performed using equal numbers ($n = 5$) of male and female blood donors. Thus, if the apoptotic effect of P4 was acting through a PR, gender should not affect PR expression. Presence of PR was assessed by Western blot, FACS, and confocal imaging. All assays included as a positive control the breast cancer cell line T47D, which expressed high PR levels.

Western blots were performed by using a range of $40-80 \times 10^6$ purified PBNK cells obtained from single donors ($n = 6$). In four of the samples we were able to identify the presence of PR_A (85 kDa) and PR_B (120 kDa) isoforms. However, in two individuals we mainly detected the PR_B isoform (Fig. 2*a*). This finding might be related to the previously described polymorphisms of the PR gene that could affect the PR_A/PR_B ratio (18). Although all samples assayed were positive, the expression of PR in purified PBNK cells was lower than in the breast cancer cell line.

We next performed confocal microscopy of highly purified PBNK cells or $CD4^+$ cells previously stained with Ab-7 mAb. As illustrated in Fig. 2*b*, in addition to anti-CD56 membrane staining, PBNK cells clearly showed diffuse intracellular staining with Ab-7 mAb. In contrast, purified $CD4^+$ cells only showed surface staining with the anti-$CD4^+$ mAb, without any evident expression of the PR.

Flow cytometry analysis indicated that Ab-7 mAb was detected on most cells of the breast cancer cell line T47D (86.63 ± 1.59%) and also on purified $CD56^+$ NK cells, although with a lower mean fluorescence intensity in the latter (Fig. 2*c*). The analysis of PBNK cells from six individuals showed expression of PR that ranged between 50 and 71% of the cells (63.63 ± 2.57%). In contrast, we found very low frequency of PR expression on purified T and B cells.

Expression of PR on NK cells according to their expression of KIR

Several studies have reported the absence of PR in endometrial $CD56^{bright}$ NK cells (19–21). Because of its biological implications, we investigated whether our detection of PR expression

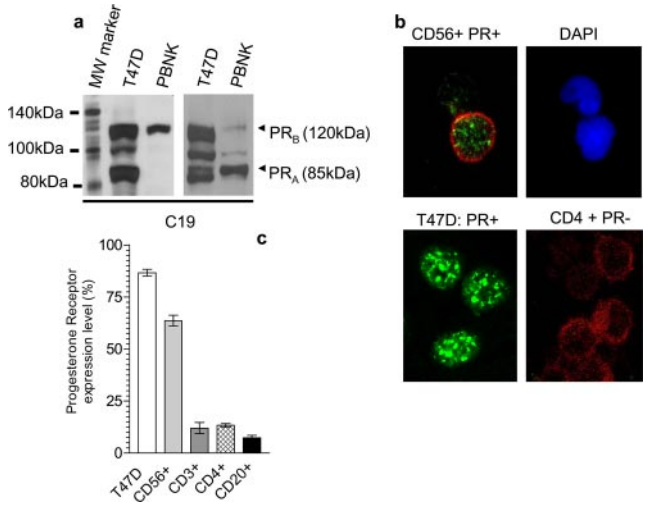

FIGURE 2. Presence of PR on PBNK cells by Western blot, confocal microscopy, and flow cytometry. *a*, Western blot analysis obtained using the rabbit polyclonal anti-PR C19 Ab. The T47D cell line shows the presence of a 118-kDa band, representative of the PR_B isoform, and a 90-kDa band corresponding to the PR_A isoform. Bands with a molecular mass of 120 and 85 kDa were also detected (although with lower intensity) in four different extracts from highly purified PBNK cells (*right side*). Also depicted (*left side*) is the Western blot from one of two individuals that mainly expressed the higher m.w. isoform. *b*, For confocal microscopy, the T47D cell line, highly purified PBNK cells, and $CD4^+$ cells were exposed to 1×10^{-8} M P4 for 2 h and processed as described in *Materials and Methods*. Cells were incubated with anti-PR Ab-7 mAb for 1 h at RT before the addition of Alexa Fluor 488-labeled goat anti-mouse IgG (green). This procedure was followed by standard CD56 or CD4 surface staining (red), and cells were allowed to adhere to the polylysine-coated glass coverslips for 30 min for image analysis and observation in a digital Eclipse E800 Nikon C1 confocal microscope system with Nikon Plan Apo 60×/1.40 Oil. Green staining of the anti-PR Ab-7 mAb is present in both purified PBNK cells and the T47D cell line, although with higher intensity in the breast cancer cell line, but showing no reactivity with $CD4^+$ cells. Nuclear staining is detected in blue by DAPI staining. Results are from one representative experiment with three donors. *c*, The presence of PR was also detected by flow cytometry analysis as described above (see *Materials and Methods*). Anti-PR Ab-7 mAb reacted with 86.63 ± 1.59% of the T47D cell line. The expression of PR in PBNK cells investigated in six individuals ranged between 50 and 71% (63.63 ± 2.57%). In contrast, PR showed very low immunoreactivity in peripheral blood purified $CD3^+$, $CD4^+$, or $CD20^+$ cells. Data are presented as means ± SEM.

might be restricted to a particular NK subset. KIR were reported to be expressed on a considerable fraction of $CD56^{dim}CD16^+$ PBNK cells, but they were absent on the $CD56^{bright}CD16^-$ PBNK subset (22). We were able to confirm that expression of KIR was restricted to the $CD56^{dim}$ subset, and we also demonstrated that expression of PR was mostly restricted to the $CD56^{dim}$ PBNK cells, in particular those expressing KIR (Fig. 3). However, we noticed that expression of PR on the KIR^+CD56^{dim} PBNK cells might differ between different samples. PR expression ranged between 45 and 90%, which might be caused by differences in the level of maturation of different individual samples. In any case, those PR not expressed on KIR^+ cells were detected on $CD56^{dim}KIR^-$ PBNK cells (data not shown). Additionally, results from the five independent sorting experiments confirmed that PR was either expressed at very low frequencies or was absent on $CD56^{bright}KIR^-$ PBNK cells. As an extension of this observation, we investigated whether induction of apoptosis was also related to the expression of PR. As illustrated in Fig. 4, this was indeed the case, as P4-induced apoptosis was mainly detected in KIR-enriched $CD56^{dim}$ PBNK cells.

Progesterone and NK function

After homing to the secondary lymphoid organs, human dendritic cells (DCs) are able to interact and exert a potent stimulatory effect on NK cells, mainly through the actions of IL-12 and IL-15 (23, 24). After demonstration of its proapoptotic activity on PBNK cells, we investigated whether P4 was also able to interfere with the main functions of these cells.

To identify variations in IFN-γ production in distinct PBNK cell subsets, NK cells sorted according to their KIR expression were cultured in the presence of IL-12, IL-15 alone, or IL-12 plus IL-15 in combination with P4. As depicted in Fig. 5, the addition of P4 significantly inhibited the secretion of IFN-γ. Of note, the addition of P4 to PBNK cells stimulated with IL-15 only induced a small inhibition of this cytokine secretion. Similarly, P4-PBNK-treated cells cultured in the presence of IL-12 plus IL-15 only induced a marginal reduction in the production of IFN-γ.

It was important to exclude the possibility that the functional effect on cytokine secretion was independent from its apoptotic effect. In preliminary experiments, we demonstrated that cell death induced by P4 was abrogated when IL-15 was added simultaneously with P4. The frequency of P4-induced cell death was 12.2 ± 0.73%, which decreased significantly to 5.8 ± 0.37% with the addition of this cytokine ($p = 0.008$) (Fig. 4*a*). The same

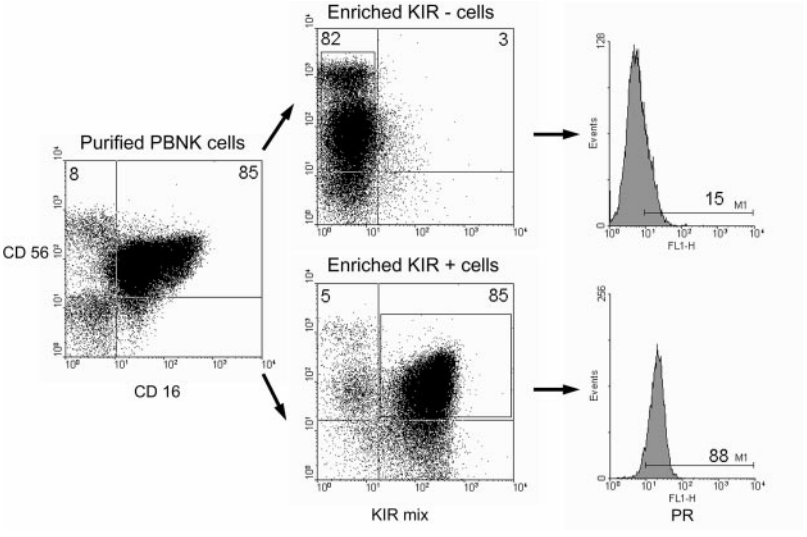

FIGURE 3. Differential PR expression in $CD56^{bright}KIR^-$ and $CD56^{dim}KIR^+$ PBNK cells. Purified PBNK cells were sorted according their KIR expression into what we describe as enriched KIR^- and enriched KIR^+ cells. Cells were left to recover overnight and were stained with anti-PR Ab-7 mAb. Enriched KIR^+ cells are illustrated (*bottom*), which concentrate most of the expression of PR (88% in this particular experiment). In contrast, little expression of PR can be observed in the enriched KIR subset (*upper*). The expression of PR was investigated in five independent experiments. In the enriched KIR^+ cells, its expression ranged between 45 and 88%. In contrast, the expression of PR in the KIR^- cells always was <15%.

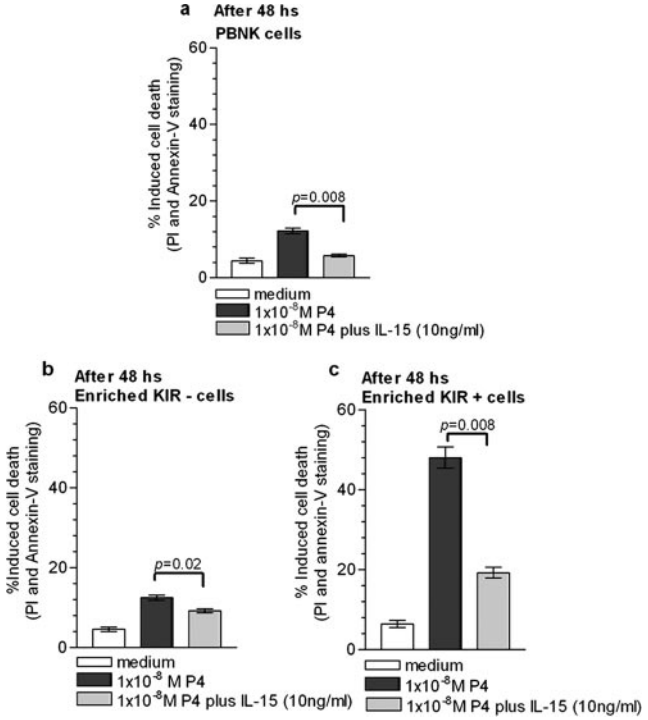

FIGURE 4. Differential apoptotic effects on unsorted PBNK cells and enriched KIR$^+$ or KIR$^-$ NK cells. Unsorted PBNK or enriched KIR$^+$ or KIR$^-$ cells (2×10^5) (range of purity 85–95% and 86–90%, respectively) were cultured in a final volume of 200 μl for 48 h in the presence or absence of P4 at 1×10^{-8} M and P4 plus IL-15. *a*, Unsorted PBNK cells: The frequency of P4-induced cell death was 12.2 ± 0.73%, which decreased significantly to 5.8 ± 0.37% with the addition of IL-15 ($p = 0.008$). *b*, Enriched KIR$^-$ cells show a reduced frequency of P4-induced apoptosis when cultured simultaneously with IL-15 (12.5 ± 0.64% vs 9.25 ± 0.48%, $p = 0.02$). *c*, Enriched KIR$^+$ cells: The increase of apoptotic P4-induced KIR$^+$ cells suffers a significant reduction when simultaneously cultured with IL-15 (48.0 ± 2.67% vs 19.2 ± 1.35%, $p = 0.008$). Results are representative of three independent experiments using different healthy donors.

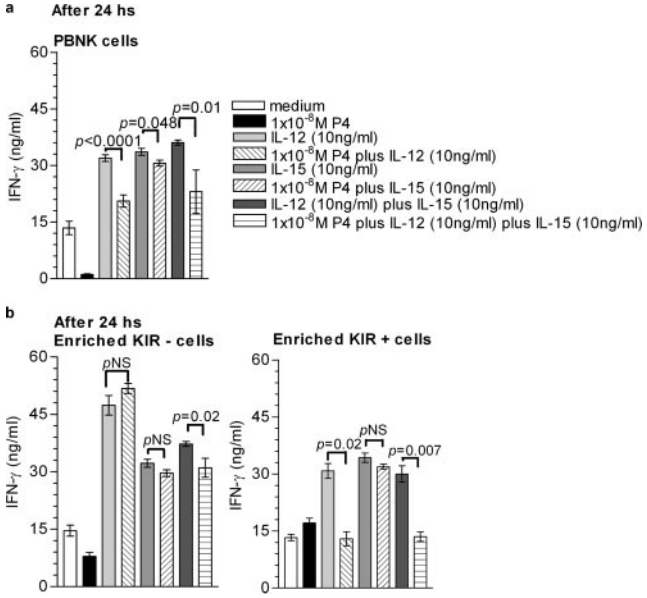

FIGURE 5. Differential effect of progesterone on IFN-γ secretion. *a*, PBNK cells were stimulated for 24 h with different combinations of IL-12, IL-15, and P4. The secretion of IFN-γ induced by 10 ng/ml IL-12 was greatly reduced by the simultaneous addition of P4 ($p < 0.0001$). Despite its antiapoptotic effect, the addition of P4 to PBNK cells stimulated with 10 ng/ml IL-15 also suppressed secretion of this cytokine. Finally, the simultaneous addition of IL-15, IL-12, and P4 showed the suppression of IFN-γ secretion. Results are representative of five independent experiments. *b*, The addition of P4 to enriched KIR$^+$ cells stimulated with 10 ng/ml IL-12 significantly suppressed IFN-γ secretion ($p = 0.028$) (*right panel*). In contrast, P4 did not affect IFN-γ production of the KIR$^-$-enriched NK cells (*left panel*). Similar to the effect observed on unsorted PBNK cells, the addition of IL-15 to enriched KIR$^+$ or KIR$^-$ PBNK cells induced a nonsignificant effect on the inhibition induced by P4 on IL-12 stimulated cells. Results are representative of three independent experiments.

figure also depicts the effect of IL-15 into the two subsets of PBNK cells. As illustrated in Fig. 4, *b* and *c*, enriched KIR$^-$ cells and KIR$^+$ cells also showed a diminished frequency of P4-induced apoptosis when cultured simultaneously with IL-15 (12.5 ± 0.64 vs 9.25 ± 0.48%, $p = 0.02$; and 48.0 ± 2.67 vs 19.2 ± 1.35%, $p = 0.008$, respectively). Consequently, we asked whether IL-15 was also able to abrogate the suppression of IFN-γ secretion observed when IL-12 was incubated together with P4. As depicted in Fig. 5*a*, addition of IL-15 did not prevent the suppression of IFN-γ secretion induced by P4 in IL-12-activated PBNK cells. We then asked whether this effect was related to the NK subset expressing PR. As expected, the enriched KIR$^-$ cells (containing most of the CD56^{bright} cells) stimulated with IL-12 secreted high levels of IFN- γ. However, this production was not affected by the addition of P4.

Interestingly, we observed that the suppressor effect of P4 was mainly induced in the KIR$^+$-enriched CD56^{dim} PBNK cells. Similar to the effect observed on unsorted PBNK cells, the addition of IL-15 to enriched KIR$^+$ or KIR$^-$ PBNK cells induced a nonsignificant effect on the inhibition induced by P4 on IL-12 stimulated cells (Fig. 5*b*).

We next designed experiments to verify whether the apoptotic effect of P4 might affect NK activity. Four independent standard ^{51}Cr-release assays were performed using fixed numbers of the K562 cell line as target cells and resting or P4-incubated PBNK cells as effectors cells. Experiments were performed at four E:T ratios: 1:1 (5×10^3 PBNK cells), 5:1 (2.5×10^4 PBNK cells), 10:1 (5.0×10^4 PBNK cells), 20:1 (1×10^5 PBNK cells). PBNK cells were cultured for 24 h in medium alone or with 1×10^{-8} M P4, and the number of effector cells was adjusted by ignoring the apoptotic ones (by using the trypan blue staining). All experiments using PBNK cells treated with P4 at the different E:T ratios showed a cytotoxic activity that did not differ from the untreated controls (percentage lysis in all E:T ratios for untreated cells vs P4-treated PBNK cells, p = NS). The results suggest that a subset of NK cells not affected by P4 might preserve its cytotoxic activity.

Discussion

In the present study, we found that progesterone induced apoptosis and the suppression of IFN-γ secretion in PBNK cells. What seems to be relevant from a biological point of view is that these functional effects appeared in the PBNK cell subset expressing the PR. As already described, the PR is known to include two functionally different isoforms, PR_A and PR_B, transcribed from distinct estrogen-inducible promoters within a single copy of the PR gene. The present study demonstrated the presence of the PR in extracts of highly purified PBNK cells. Additionally, confocal microscopy confirmed intracellular expression of the PR restricted to PBNK cells. We observed the absence of PR on CD56^{bright} PBNK cells, which is in line with several previous reports indicating the lack of PR on the CD56^{bright} uterine NK (uNK) cells (19–21). In this context, our demonstration of the presence of PR restricted to the

$CD56^{dim}$ subset, in particular to the cells expressing KIR receptors, provides new insight into the role of progesterone in the physiology of PBNK cells. For instance, this could explain the decrease in IFN-γ production associated with the high concentrations of P4 present during pregnancy (25).

We also demonstrated the presence of the PR in PBNK cells, not only in nonpregnant women, but also in men. This was not an unexpected finding, as the PR was already found to be expressed in men (26), and progesterone secretion and serum progesterone levels showed no great quantitative differences between men and women, at least outside of the luteal phase (27).

We demonstrated that P4-induced apoptosis could be abrogated by the addition of antiprogestins. Interestingly, we found that while RU was able to abrogate P4- or DEX-induced apoptosis, ZK (14, 15) was only able to block P4-induced apoptosis, supporting the specific involvement of PR in P4-induced apoptosis. Although multiple mechanisms have been proposed for glucocorticoid-induced cell death in lymphocytes, most of them seem to invoke caspase-independent pathways (28–31). In contrast, our results seem to support the hypothesis that P4 binds to a canonical PR and mediates its effect through a caspase-dependent pathway.

Immune cells in the human endometrium undergo remarkable changes during the course of each menstrual cycle. This is the case for natural regulatory T cells, which suffer an expansion at the late follicular phase that declines in the absence of embryo implantation (32). Similarly, uNK cells increase in number during the middle to late secretory phase, where they are found in close contact with endometrial glands and spiral vessels, and they are present in large numbers in early pregnancy. At this stage, they seem to play a central role in the process of placentation by increasing blood flow from the mother to the fetus, and combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and affect reproductive success (33). $CD56^{bright}$ PBNK cells appear in the uterus 3–5 days after ovulation, coincident with the onset of stromal cell decidualization. Although the source of these uNK cells is not clearly elucidated, a subset of blood $CD56^{bright}$ cells exhibits an enhanced capacity to adhere to decidual vascular endothelium during the periovulatory period of the menstrual cycle (34). $CD56^{bright}$ cells differ in their KIR repertoire from $CD56^{dim}$ NK cells (22). More importantly, it was recently demonstrated that $CD56^{bright}CD16^{-}KIR^{-}$ PBNK cells might represent precursor cells, which, upon activation, acquire the features of a more mature NK subset expressing KIRs, a phenomenon that apparently occurs in the secondary lymphoid organs (35). Our findings that $CD56^{bright}CD16^{-}KIR^{-}$ PBNK cells lack PR provides an additional phenotypic marker to test the idea that they might represent the PBNK precursors selectively recruited into the endometrium, where they differentiate to become the uNK cells.

The high secretion of IFN-γ by KIR^{-}-enriched PBNK cells was unaffected by P4, providing further support for a putative effect of P4 on those PBNK cells expressing the PR.

Our results also seem to indicate that the suppression of IFN-γ secretion induced by P4 observed in the $CD56^{dim}KIR^{+}$ PBNK cells might be independent of its apoptotic effect. Furthermore, a recent study observed the convergence of IFN-γ and P4 signaling pathways in endometrial stromal cells and demonstrated that progesterone was able to antagonize the signaling pathways of IFN-γ (36).

Cytokine secretion and killing have been suggested to be mediated by two different subsets of human NK cells. However, while abundant IFN-γ production by IL-12-cultured PBNK cells was strongly inhibited by the addition of P4, the killing capacity of viable PBNK cells was unaffected by treatment with P4.

Although the molecular mechanisms by which P4 causes suppression of the immune response have remained largely elusive (37), the proapoptotic effects of P4 in NK cells together with the induced suppression of IFN-γ secretion could at least partially explain their immunosuppressive effects.

NK cells are known for their central role in protection against viruses, in particular with the Herpesviridae family of viruses. This is supported by those rare cases of pure NK immunodeficiency, which are mainly associated with herpesvirus infection (38, 39). In addition to these reports, we could include previous reports indicating that prolonged exposure to P4 prevented induction of a protective mucosal response to herpes simplex virus type 2 (40).

Studies have indicated the induction of clinically beneficial effects by treatment of lung, colon, or ovary cancer with antiprogestins (41, 42). In this context, growth control induced by medroxyprogesterone acetate (MPA) on estrogen and progesterone receptors positive for mammary tumors might also be included (43). As NK cells are known to play an important role in destroying tumor cells, our results provide further support for a potential role for the presence of the PR on PBNK cells in tumor immunity.

In summary, we present for the first time evidence for the differential expression of the PR on different subsets of PBNK cells, which allows us to suggest that P4 regulates NK cell function and that classical PRs are mediating these effects. Our observation that the PR is absent from $CD56^{bright}$ PBNK cells provides a differential phenotypic marker to identify the PBNK cell subset that might reach the uterus endometrial layer during the menstrual cycle and pregnancy. We also postulate a putative cellular mechanism to explain the immunosuppressive effect of progesterone on PBNK cells.

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Disclosures

The authors have no financial conflicts of interest.

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