PGE$_2$ inhibits natural killer and γδ T cell cytotoxicity triggered by NKR and TCR through a cAMP-mediated PKA type I-dependent signaling

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ABSTRACT

Natural killer (NK) and unconventional γδ T cells, by their ability to sense ligands induced by oncogenic stress on cell surface and to kill tumor cells without a need for clonal expansion, show a great therapeutic interest. They use numerous activating and inhibitory receptors which can function with some independence to trigger or inhibit destruction of target cells. Previous reports demonstrated that PGE$_2$ is able to suppress the destruction of some tumor cell lines by NK and γδ T cells but it remained uncertain if PGE$_2$ interferes with the different activating receptors governing the cytolytic responses of NK and γδ T cells. In this report, using the model of specific redirected lysis of the mouse FcγR$^+$ cell line P815, we clearly demonstrate that the major NK receptors (NKR): NKG2D, CD16 and natural cytotoxicity receptors (NCR: NKp30, NKp44, NKp46) and γδ T cell receptors TCR Vγ9Vδ2, NKG2D and CD16 are all inhibited by PGE$_2$. As is the case with γδ T cells, we show that PGE$_2$ binds on E-prostanoid 2 (EP2) and EP4 receptors on NK cells. Finally, we delineate that the signaling of the blockade by PGE$_2$ is mediated through a cAMP-dependent activation of PKA type I which inhibits early signaling protein of cytotoxic cells. In the discussion, we focused on how these data should impact particular approaches in the treatment of cancer.

1. Introduction

NK cells and the unconventional γδ subset of T lymphocytes have the ability to respond early to oncogenic transformation without needing clonal expansion, to produce inflammatory mediators such as IFN-γ and TNF-α and to kill appropriate cellular targets. Due to these abilities, they actively participate in the immunosurveillance of tumors and are enrolled in cancer immunotherapies [1,2]. In contrast with αβ T lymphocytes whose activation is controlled principally through TCR signaling, the activation of NK and γδ T cells is controlled through the recognition – by their multiple activating and inhibitory receptors – of ligands on the cell surface of target cells [3]. Most of circulating γδ T lymphocytes from humans and primates express the HLA-unrestricted TCR Vγ9Vδ2 which is specific for small non-peptide phosphorylated metabolites (so-called phosphoantigens, PAg) which are frequently up-regulated in human tumor cells [4]. NK and γδ T cells highly express NKG2D (a lectin-like type II transmembrane homodimer), a receptor allowing them to respond to ligands (MICA, MICB and ULBP 1–4) up-regulated during oncogenic transformation and stress [5–7]. CD16 (a FcγRIII receptor) expressed by NK and γδ T cells plays an important role in antibody dependant cell cytotoxicity (ADCC) which is of particular clinical interest with the increasing number of anti-cancer therapies using monoclonal antibodies [8–10]. Finally, NK cells also express NCR (NKp30, NKp44 and NKp46) that are critically involved in tumor cell recognition [11]. In a marginal way, under particular conditions of activation, γδ T cells also express NKp44 [12].

Despite the ability of immune cells to infiltrate and kill tumor cells, the spontaneous clearance of established tumors by endogenous immune mechanisms is rare and the different cellular approaches of immunotherapy tested so far have obtained limited results. Indeed, tumor cells and their microenvironment often produce numerous immunomodulatory molecules that can negatively influence the functions of immune cells [13,14]. PGE$_2$ is a major inhibitory factor produced by tumor cells or their surrounding microenvironment [15]. The rate-limiting enzyme in PGE$_2$ synthesis is cyclooxygenase-2 (COX2). It is over-expressed in many cancers leading to an overproduction of PGE$_2$ often linked to an adverse clinical outcome, a high tumor grade and metastatic dissemination [16,17].

The negative impact of tumor-derived PGE$_2$ on tumor immunity has been mainly explained by its ability to inhibit directly the proliferation and effector functions of CD4$^+$ and CD8$^+$ T cells

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[18,19] and to promote their differentiation in regulatory T cells [20]. PGE$_2$ has also been involved in the inhibition of NK and γδ T cell cytolytic functions [21–24]. Nevertheless, studies that analyzed the regulation of NK and γδ T cell functions by PGE$_2$ only focused on its ability to regulate their cytotoxicity against target cells. But, this cytotoxicity reflects the integration of multiple signals transduced by the different activating receptors expressed by NK and γδ T cells. In order to improve current immunotherapies, it is crucial to establish if PGE$_2$ can interfere with the different activating receptors governing the cytolytic responses of NK and γδ T cells. In this study we demonstrate that PGE$_2$ inhibits the destruction of target cells by NK and γδ T cells activated either through TCR Vγ9Vδ2 (for γδ T cells), NCR (for NK cells), CD16 or NKG2D (for both) receptors. We also delineate the mechanism which mediates the inhibitory signal of PGE$_2$.

2. Materials and methods

2.1. Reagents

PGE$_2$ and Brefeldin A were obtained from Sigma–Aldrich (Saint-Louis, MO). Butaprost-free acid, sulprostone, 1-hydroxy PGE$_1$, Rp-8Br-cAMP, 8-HA-cAMP and 6-benz-cAMP and purified rabbit polyclonal antibodies (pAbs) directed against EP2, EP3 and EP4 were from Cayman Chemical (Ann Arbor, MI). Monoclonal antibodies (mAbs) directed against TCR Vδ2 (clone B6), IFN-γ, TNF-α and CD107a were purchased from BD Bioscience (San Jose, CA). Anti-TCR Vγ9, anti-CD16 (clone 3G8), anti-CD3, anti-CD56 mAbs and goat F(ab)′$_2$ anti-rabbit IgG were from Beckman–Coulter (Fullerton, CA). Purified mouse anti-CD3 mAb (clone OKT3) was purchased from eBioscience (San Diego, CA). Purified mAbs against NKp30 (clone 210845), NKp44 (clone 253415), NKp46 (clone 195314) and NKG2D (clone 149810) were from R&D Systems (Minneapolis, MN). The rabbit anti-Lck, anti-Y505 Lck, anti-PLCγ, anti-Y783 PLCγ, anti-Y493 ZAP70, anti-ERK1/2 pAbs, the rabbit anti-ZAP70 mAb, and the mouse anti-Y202/204 ERK1/2 mAb (all used at 1/1000) were purchased from Cell Signaling Technology (St. Quentin en Yvelines, France). Affinity purified secondary antibodies were purchased from Jackson ImmunoResearch Europe Ltd. (Newmarket, UK).

2.2. Cell isolation and culture

Fresh blood samples were collected from healthy donors, and peripheral blood mononuclear cells (PBMC) were prepared on a Ficoll–Paque density gradient (Amersham Biosciences AB, Uppsala, Sweden) by centrifugation (800 × g, 30 min at room temperature [RT]). Primary Vγ9Vδ2 T cell lines were generated as described [25] from PBMC culture stimulated with BrHPP (200 nM, Innate Pharma, Marseilles, France) and IL-2 (300 U/mL, Sanofi-Aventis, Labège, France) in complete RPMI-1640 medium, e.g. with penicillin, streptomycin (Cambrex Bio Science, Verviers, Belgium), sodium pyruvate, and 10% of heat-inactivated FCS (Invitrogen Corp., Paisley, UK). These cell lines comprised more than 95% TCR Vγ9Vδ2$^+$ cells. Highly pure CD3$^-$CD56$^+$ NK cells (>90%, as checked by flow cytometry) were negatively selected by magnetically activated cell sorting (NK cell isolation kit II, Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. NK cells were then activated with 100 U/mL IL-2 (Sanofi-Aventis, Labège, France) in complete RPMI medium for 2 days before use.

2.3. ^{51}Cr-release cytotoxic assays

FcγR$^+$ target cells were labelled with 100 μCi ^{51}Cr–sodium bichromate (Amersham Biosciences AB, Uppsala, Sweden). After 3 washes, ^{51}Cr-labelled P815 cells were incubated for 30 min at RT

for redirected lysis experiments with mouse mAbs directed against NKp30 (5 μg/mL), NKp44 (5 μg/mL), NKp46 (5 μg/mL), NKG2D (15 μg/mL), CD16 (5 μg/mL) or TCR Vδ2 (5 μg/mL). A standard ^{51}Cr-release assay was then conducted for 4 h with Vγ9Vδ2 T cells or NK cells in the presence of increasing doses of PGE$_2$ with an effector/target cell ratio of 5/1. The percentage of specific lysis was calculated as follows: [(^{51}Cr-release) – (spontaneous release)]/ [(maximum release) – (spontaneous release)] × 100.

2.4. Cytokine production

Vγ9Vδ2 T cells were stimulated for 5 h with increasing doses of BrHPP in the presence or absence of PGE$_2$ (1 μg/mL) and 10 μM Brefeldin A (Sigma–Aldrich, Saint-Louis, MO). Cells were stained with anti-TCRγ9-FITC, fixed with PBS 2% paraformaldehyde, stained for 30 min in PBS 1% saponin with mAbs directed against IFNγ and TNFα as described previously [21].

2.5. CD107a degranulation assays

P815 were incubated for 30 min RT with mouse mAbs directed against NKp30 (5 μg/mL), NKp44 (5 μg/mL), NKp46 (5 μg/mL), NKG2D (15 μg/mL), CD16 (5 μg/mL) or TCR Vδ2 (5 μg/mL). 10^5 Vγ9Vδ2 T cells or NK cells were incubated with 10^5 P815 in complete RPMI medium in the presence of 5 μg/mL anti-CD107a-PC5 or IgG1-PE-Cy5 antibodies. Soluble PGE$_2$, PKA type I subunit agonists 8-HA-cAMP and 6-benz-cAMP or EP1–4 receptor agonists 1-hydroxy PGE$_1$, butaprost-free acid and sulprostone were added at the beginning of the test at the indicated concentrations. In some experiments NK and Vγ9Vδ2 T cells were pre-incubated with PKA type I inhibitor Rp-8Br-cAMP (1 mM) for 1 h in complete RPMI medium. Cells were pelleted by gentle centrifugation (110 × g for 1 min), left in co-incubation for 5 h and washed with PBS/EDTA 0.5 mM. Cells were then stained for 15 min at 4 °C with anti-TCR Vγ9-FITC for Vγ9Vδ2 T cells or with anti-CD3-PE and anti-CD56-PC7 for NK cells. Rates of Vγ9Vδ2 T cells or NK cells expressing CD107a were then determined by flow cytometry in each experiment.

2.6. PGE$_2$ receptor expression

Total RNA was isolated from freshly isolated NK cells by TRIzol^{TM} reagent and was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp., Paisley, UK). cDNA encoding human EP1, EP2, EP3, EP4 and hypoxanthine phosphoribosyl transferase (HPRT) were amplified by PCR as previously described [21]. The size of the fragments for EP1, EP2, EP3, EP4 and HPRT were 455 bp, 409 bp, 451 bp, 377 bp and 369 bp respectively. The corresponding receptor proteins were detected by flow cytometry using rabbit primary pAbs directed against EP2, EP3 and EP4 (Cayman Chemical, Ann Arbor, MI) and FITC-conjugated goat F(ab)′$_2$ anti-rabbit IgG secondary antibody.

2.7. Western blot

Vγ9Vδ2 T cells were pre-treated 30 min with PGE$_2$ (1 μg/mL) and then incubated with anti-CD3 mAb for TCR cross-linking and PGE$_2$ during different times. Cells were washed with cold PBS and lysed in lysis buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM Na$_3$VO$_4$, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) for 5 min on ice, followed by centrifugation at 13,000 × g for 5 min at 4 °C. For each lysate, 30 μg of total protein were boiled for 5 min at 95 °C in the presence of 5% β-mercaptoethanol. Proteins were separated in a 10% SDS-PAGE and transferred onto nitrocellulose membranes

(Amersham Biosciences AB, Uppsala, Sweden). Nonspecific binding to the membrane was blocked for 1 h at RT with 10% nonfat milk in PBS containing 0.1% Tween 20 (PBST). Membranes were incubated overnight at 4 °C with specific primary antibody diluted at an appropriate concentration in PBST containing 1% nonfat milk. Membranes were then washed 3 times at RT and bound immunoglobulins were detected with horseradish peroxidase-conjugated secondary antibody for 30 min at RT in 1% nonfat milk dissolved in PBST. Membranes were then washed with PBST and bound Abs were detected by the enhanced chemiluminescence system ECL kit (Amersham Biosciences AB, Uppsala, Sweden).

2.8. Statistical analysis

Significant differences were assessed by Student's t-test (for normal distributions) or Mann–Whitney's rank sum (for other distributions) two-tailed tests with $a = 0.01$ with the SigmaStat software (Systat Software Inc., San Jose, CA).

3. Results

3.1. PGE$_2$ inhibits the responses of γδ T cells mediated by TCR Vγ9Vδ2, NKG2D and CD16

First of all, we wished to determine if the inhibition of the functions of γδ T cells by PGE$_2$ could be overcome by strong activation of TCR Vγ9Vδ2. In this purpose, we first co-incubated γδ T cells with the FcγR$^+$ mouse mastocytoma cell line P815 coated with increasing doses of anti-TCR Vδ2 mAbs. We analyzed the level of expression of CD107a after cytolytic degranulation [26] of γδ T cells. We observed that the percentage of CD107a$^+$ γδ T cells was significantly inhibited by PGE$_2$ even when P815 cells were coated with the highest dose of anti-TCR Vδ2 (Fig. 1a). Then, analysis of the intracellular production of IFN-γ and TNF-α by Vγ9Vδ2 T cells stimulated with increasing doses of the synthetic PAg BrHPP [25] showed that PGE$_2$ at 1 μg/mL inhibited the production of these cytokines even at 25 times as much as the EC$_{50}$ of BrHPP (Fig. 1b). Thus, a strong activation of TCR Vγ9Vδ2 did not thwart inhibition of γδ T cell functions by PGE$_2$. Since other activating receptors such as NKG2D and CD16 also participate in the recognition and destruction of target cells by γδ T cells [5,10], we wanted to determine if PGE$_2$ also interfered with the ability of NKG2D and CD16 to trigger γδ T cell cytotoxicity. In a redirected lysis assay based on the release of ^{51}Cr by target cells, we analyzed the ability of γδ T cells to kill the P815 cell line coated either with anti-TCR Vδ2, anti-NKG2D or anti-CD16 mAbs (to activate each of

these receptors) in the presence of increasing concentrations of PGE$_2$. We observed that PGE$_2$ significantly inhibited the cytotoxicity of γδ T cells induced either by TCR Vγ9Vδ2, NKG2D or CD16 (Fig. 1c).

3.2. PGE$_2$ inhibits the cytotoxicity of NK cells mediated by NCR, NKG2D and CD16

To determine if PGE$_2$ interfered with the cytotoxicity triggered through the different classes of cytotoxic NKR, we analyzed the level of expression of CD107a after cytolytic degranulation of NK cells co-incubated with P815 cells coated either with anti-NKG2D, anti-CD16 or a mix of anti-NCR mAbs. We observed that the percentage of CD107a$^+$ NK cells was significantly inhibited by increasing doses of PGE$_2$ (Fig. 2a), in a dose-dependent manner (Fig. 2b). To confirm this data, we performed a redirected lysis assay based on the release of ^{51}Cr by target cells in which we analyzed the ability of NK cells to kill the P815 cell line coated either with anti-NKp30, anti-NKp44, anti-NKp46 (alone for Fig. 2c or in combination for Fig. 2d), anti-CD16 or anti-NKG2D mAbs (Fig. 2c) in the presence of increasing concentrations of PGE$_2$ (Fig. 2d). Our results clearly show that PGE$_2$ significantly inhibited cytotoxicity of NK cells induced through all classes of NKR.

3.3. Inhibition of NKR signaling by PGE$_2$ is mediated through EP2 and EP4 receptors

PGE$_2$ is recognized by 4 different subtypes of G protein-coupled E-prostanoid (EP) receptors (EP1–4). Since there is no data available concerning the expression of PGE$_2$ receptors EP1–4 at the surface of NK cells, we decided to analyze the expression of these receptors by NK cells. Using RT-PCR, we show that mRNA for EP2, EP3 and EP4, but not for EP1, was found in NK cells freshly isolated from 3 different donors (Fig. 3a). This was confirmed by flow cytometry analysis of the cell surface expression of the EP2, EP3 and EP4 receptors on freshly isolated NK cells (Fig. 3b). The pattern of expression of the 3 receptors was the same at the surface of NK cells after 3 days in culture with IL-2 (data not shown). EP2 and 4 are coupled to Gs protein, activate adenylate cyclase and increase cyclic adenosine monophosphate (cAMP), whereas the major signaling pathway of EP3 inhibits adenylate cyclase via Gi protein and decreases cAMP [27]. To determine the respective roles of EP2–4 in the inhibition of NCR, CD16 and NKG2D receptors, NK cells were co-incubated with P815 cells coated either with anti-NCR, anti-CD16 or anti-NKG2D mAbs in the presence of PGE$_2$ or
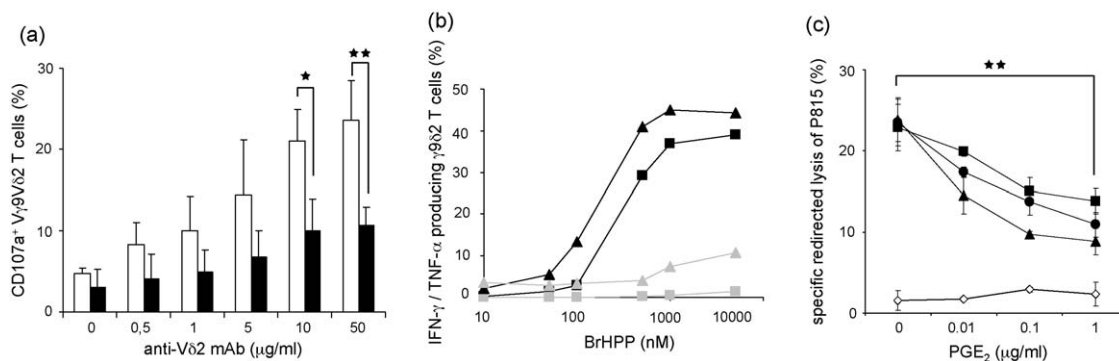

Fig. 1. PGE$_2$ inhibits the responses of γδ T cells mediated by TCR Vγ9Vδ2, NKG2D and CD16. (a) Rates of CD107a$^+$ Vγ9Vδ2 T lymphocytes after co-incubation with the P815 cell line coated with anti-TCR Vδ2 in the presence (black bars) or absence (white bars) of PGE$_2$ at 1 μg/mL (means±SD from 3 independent experiments). (b) Rates of IFN-γ$^+$ (■) and TNF-α$^+$ (▲) Vγ9Vδ2 T lymphocytes stimulated with increasing doses of BrHPP in the presence (black symbols) or absence (grey symbols) of PGE$_2$ at 1 μg/mL (representative of 3 independent experiments). (c) Percentage of specific redirected lysis of ^{51}Cr-loaded P815 cells coated either with anti-TCR Vδ2 (■), anti-NKG2D (▲), anti-CD16 (●) or IgG1 isotype control (◇) by Vγ9Vδ2 lymphocytes in the presence of increasing doses of PGE$_2$ (means ± SD from 3 independent experiments). $^*p < 0.05$, $^{**}p < 0.01$ in a Student's t-test.
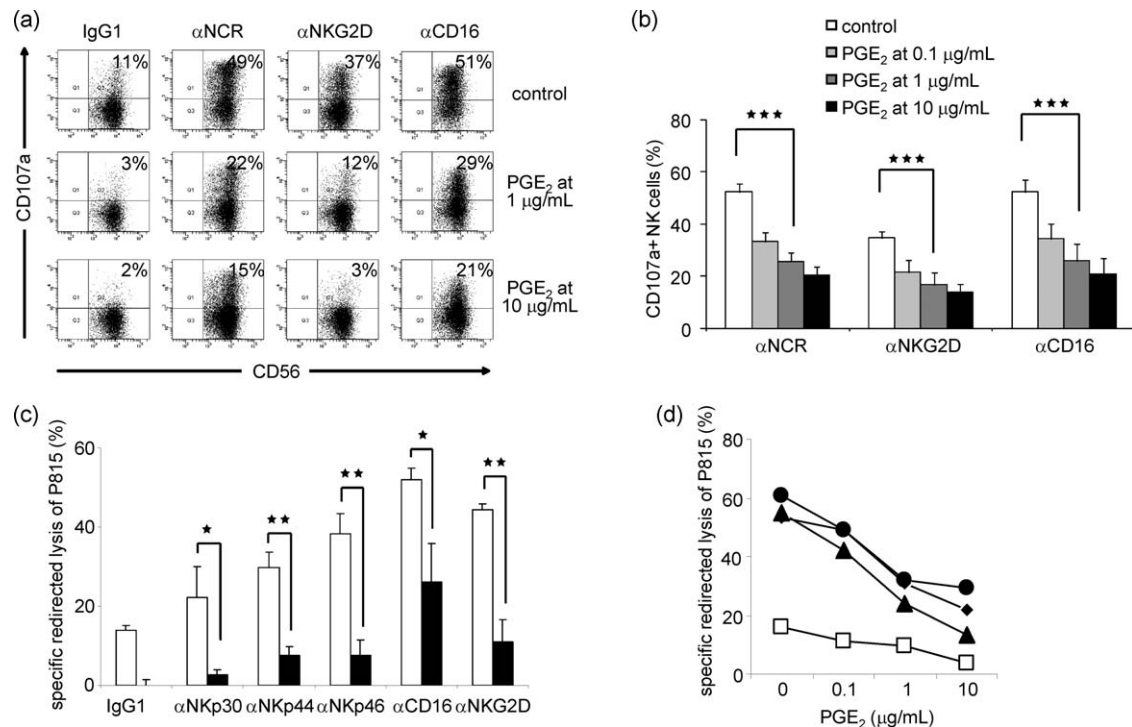
Fig. 2. PGE$_2$ inhibits the cytotoxicity of NK cells mediated by NCR, NKG2D and CD16. (a and b) Rates of CD107a$^+$ NK cells after co-incubation with the P815 cell line coated either with an IgG1 isotype control (IgG1), anti-NKG2D (αNKG2D), anti-CD16 (αCD16) or a mix of anti-NCR (αNCR) mAbs, in the presence of different concentrations of PGE$_2$ (0, 1 or 10 μg/mL). (a) Representative data from n = 5 independent experiments, (b) means ± SD from 5 independent experiments. (c) Percentage of specific redirected lysis of ^{51}Cr-loaded P815 cells coated with the indicated mAbs in the presence (black bars) or absence (white bars) of PGE$_2$ at 1 μg/mL (means ± SD from 3 independent experiments). (d) Percentage of specific redirected lysis of ^{51}Cr-loaded P815 cells coated either with IgG1 isotype control (□), anti-CD16 (●), anti-NKG2D (◆) or a mix of anti-NCR (▲) mAbs in the presence of increasing doses of PGE$_2$ (representative data from n = 4 independent experiments). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in a Student's t-test.
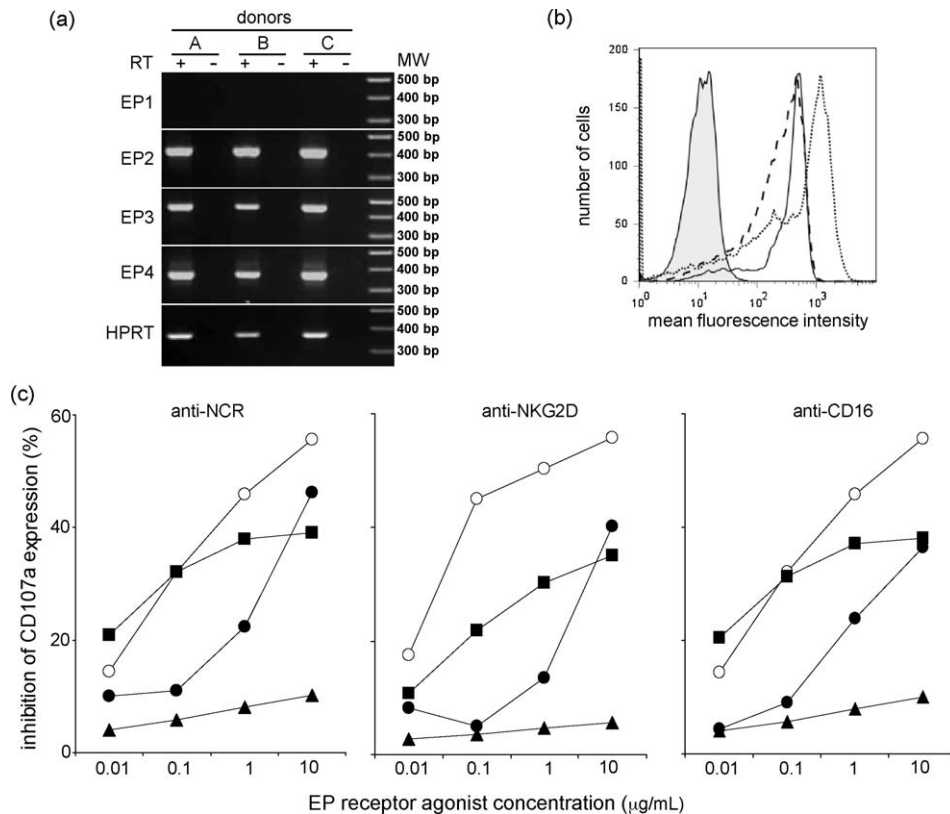

Fig. 3. Inhibition of NKR signaling by PGE$_2$ is mediated through EP2 and EP4 receptors. (a) RT-PCR for EP1, EP2, EP3 and EP4 mRNA from freshly isolated NK cells purified from 3 healthy donors (A, B and C); HPRT: mRNA for hypoxantine phosphoribosyl transferase. (b) Cell surface expression of EP2 (black line), EP3 (dotted line) and EP4 (dashed line) by freshly isolated NK cells. The grey tint histogram on the left is the isotype control. (c) Rates of inhibition of CD107a expression by NK cells after co-incubation with the P815 cell line coated either with anti-NCR, anti-NKG2D or anti-CD16 mAbs in the presence of PGE$_2$ (○), EP1 and EP3 agonist sulprostone (▲), EP2 agonist butaprost-free acid (■) or EP3–4 agonist 1-hydroxy PGE$_1$ (●) (representative from 4 independent experiments).
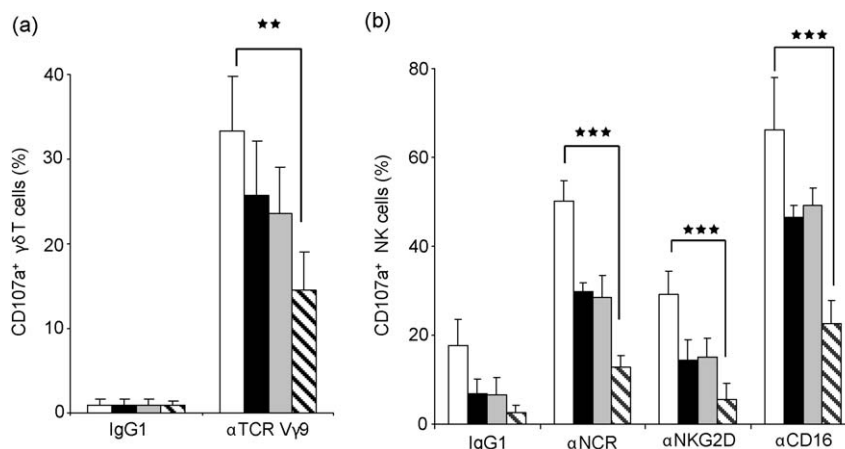
Fig. 4. Activation of PKA type I inhibits NKR- and TCR Vγ9Vδ2-induced degranulation of NK and γδ T cells. (a and b) Rates of CD107a⁺ Vγ9Vδ2 T cells (a) and NK cells (b) after co-incubation with the P815 cell line coated either with an IgG1 isotype control (IgG1), anti-TCR Vγ9 (αTCR Vγ9, for γδ T cells), anti-NCR (αNCR), anti-NKG2D (αNKG2D) or anti-CD16 (αCD16) mAbs in the presence of 6-benz-cAMP (0.5 mM, black bars), 8-HA-cAMP (0.5 mM, grey bars) or both (0.25 mM each, hatched bars) (means ± SD from 3 independent experiments). $^*p < 0.05$, $^{**}p < 0.01$ in a Student's t-test.

different EP-specific agonists. The expression of CD107a at the surface of NK cells was measured by flow cytometry and inhibition of degranulation was calculated (Fig. 3c). We observed that butaprost-free acid, a structural analog of PGE$_2$ with a good selectivity for EP2 and a potent agonist effect, was the most effective inhibitor of NK cell degranulation. Nevertheless, its activity was lower than that of PGE$_2$. The EP3 and EP4 agonist 1-hydroxy PGE$_1$ was also inhibitory although at much higher concentrations. Nevertheless, since sulprostone, an agonist of EP1 and EP3, had not effect on NK cell degranulation, the inhibitory effect of 1-hydroxy PGE$_1$ had to be mediated through EP4. Altogether, these results demonstrate that PGE$_2$ inhibited the ability of NCR, CD16 and NKG2D receptors to activate the degranulation of NK cells through their EP2 and EP4 receptors. We previously found similar results regarding the inhibition of Vγ9Vδ2 T lymphocyte functions by PGE$_2$ [21].

3.4. Activation of PKA type I inhibits NKR- and TCR Vγ9Vδ2-induced degranulation of NK and γδ T cells

As EP2 and 4 activate adenylate cyclase to increase cAMP [27], it was tempting to hypothesize that PGE$_2$ interfered with the cytotoxicity of NK and γδ T cells triggered by NKR and TCR Vγ9Vδ2 through a cAMP-dependent mechanism. Increase of intracellular cAMP is known to activate PKA type I enzyme which has already been implicated in the inhibition of T cell functions and NK cell cytotoxicity against tumor cells [28,29]. The binding of cAMP to the A and B sites on the regulatory subunits (RIA and RIB) of PKA type I results in the dissociation of the enzyme complex releasing two free catalytic subunits which are then able to phosphorylate specific substrate proteins [30]. In order to determine if PKA type I was implicated in the inhibition of the triggering of NK and γδ T cell cytotoxicity by NKR and TCR Vγ9Vδ2, we analyzed the effect of specific analogs of cAMP that selectively bind to RIA and RIB subunits of PKA type I on the degranulation of NK and γδ T cells. NK or γδ T cells were co-incubated with P815 cells coated either with anti-NCR, anti-CD16, anti-NKG2D or anti-TCR Vδ2 mAbs respectively, in the presence of 6-Bz-cAMP, an agonist of the RIA subunit of PKA type I, and/or 8-HA-cAMP, an agonist of the RIB subunit of PKA type I. The rate of CD107a⁺ cells was analyzed by flow cytometry in each condition for γδ T cells (Fig. 4a) and NK cells (Fig. 4b). RIA and RIB both inhibited the cytotoxicity of NK and γδ T cells induced either by TCR Vγ9Vδ2, NCR, CD16 or NKG2D activation.

3.5. Blockade of PKA type I reverses the inhibition by PGE$_2$ of degranulation of NK and γδ T cells induced by NKR and TCR Vγ9Vδ2 triggering

The data presented above indicated that the activation of PKA type I mimicked the inhibitory effects of PGE$_2$ on NKR and TCR Vγ9Vδ2 activation. Rp-8-Br-cAMP is the most potent antagonist of RIA and RIB subunits of PKA type I. Thus, its binding to RIA and RIB prevents the dissociation of PKA type I catalytic units and blocks the ability of the latter to phosphorylate substrate proteins [31]. In order to formally demonstrate that PKA type I is involved in PGE$_2$ inhibitory effects we analyzed the ability of Rp-8-Br-cAMP to prevent the inhibitory effect of PGE$_2$. NK and γδ T cells had been pre-treated with Rp-8-Br-cAMP and then co-incubated with the P815 cell line coated with anti-NCR, anti-CD16, anti-NKG2D or anti-TCR Vδ2 in the presence of PGE$_2$. We observed that the blockade of PKA type I by Rp-8-Br-cAMP significantly prevented the inhibition by PGE$_2$ of the degranulation of Vγ9Vδ2 T cells (Fig. 5a) and NK cells (Fig. 5b) induced by NKR and TCR Vγ9Vδ2 triggering. These results demonstrate that PKA type I was critically involved in PGE$_2$-mediated inhibition of TCR Vγ9Vδ2 and NKR signaling.

3.6. Lck Y505 phosphorylation induced by PGE$_2$ inhibits TCR Vγ9Vδ2 signaling pathway

In order to determine how PGE$_2$ interfered with the signaling pathway of TCR Vγ9Vδ2, cell lysates were prepared from γδ T cells activated by the cross-linking of their TCR with an anti-CD3 mAb, in the presence or the absence of PGE$_2$. The activation status of key proteins (the protein tyrosine kinase ZAP70, PLCγ and ERK 1/2 MAP kinases) of the TCR signaling pathway was then evaluated by Western Blot using specific mAbs against phosphorylated and total proteins. We observed that 30 s after the activation of γδ T cells, proximal signaling proteins ZAP70 and PLCγ became both strongly phosphorylated and then their phosphorylation level decreased (Fig. 6a). Following ZAP70 and PLCγ phosphorylation, the maximal phosphorylation of the downstream MAP kinases ERK1/2 occurred 15 min after activation of γδ T cells (Fig. 6a). In sharp contrast, in the presence of PGE$_2$, ZAP70 and PLCγ phosphorylation was notably decreased and ERK 1/2 phosphorylation was nearly abolished (Fig. 6a). It is noteworthy that the inhibition of ZAP70 phosphorylation by PGE$_2$ strongly suggests that the blockade of TCR Vγ9Vδ2 signaling by PGE$_2$ takes place during the very first
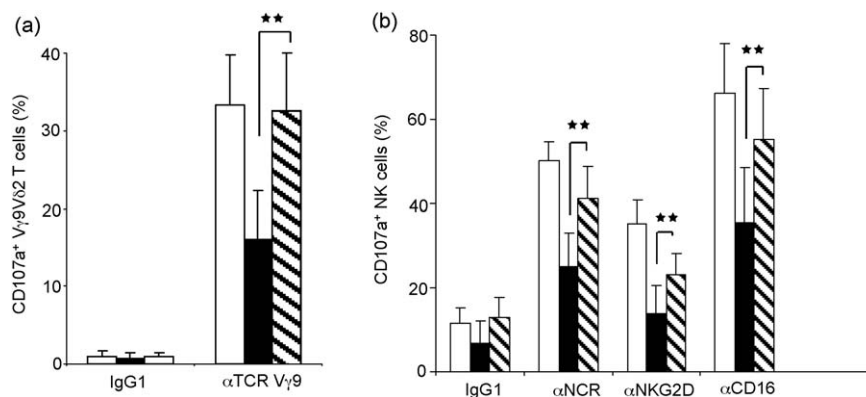
Fig. 5. Blockade of PKA type I reverses the inhibition by PGE$_2$ of degranulation of NK and $\gamma\delta$ T cells induced by NKR and TCR Vγ9Vδ2 triggering. (a and b) Rates of CD107a$^+$ Vγ9Vδ2 T cells (a) and NK cells (b) pre-treated with 1 mM of Rp-8Br-cAMP (white and hatched bars) and then co-incubated with the P815 cell line coated either with an IgG1 isotype control (IgG1), anti-TCR Vγ9 (αTCR Vγ9, for $\gamma\delta$ T cells), anti-NCR (αNCR), anti-NKG2D (αNKG2D) or anti-CD16 (αCD16) mAbs in the presence (black and hatched bars) or in the absence (white bars) of PGE$_2$ at 1 μg/mL (means $\pm$ SD from 3 independent experiments). *$p < 0.05$, **$p < 0.01$ in a Student's t-test.

events of the TCR signaling cascade. Following T cell activation, tyrosine phosphorylation of the TCR-associated CD3ζ chain mediated by Lck (a protein kinase of the Src family) is a key step leading to the recruitment and the phosphorylation of ZAP70 which in its turn propagates the activating signal downstream the TCR. The COOH-terminal Src kinase Csk plays an important regulatory role in T cell activation by its ability to inhibit the activity of Lck through the phosphorylation of the latter in Y505 [32]. Previous studies demonstrated that cAMP elevating agents inhibit TCR $\alpha\beta$ signaling through the phosphorylation of Csk at S364 by PKA type I. Phosphorylation of Csk at S364 increases its stability and activity (phosphorylation of Lck at Y505) and consequently inactivates Lck [29,33,34]. Since the PKA type I was involved in the inhibition of TCR Vγ9Vδ2 by PGE$_2$, we analyzed the Y505 phosphorylation status of Lck after $\gamma\delta$ T cell activation in

Fig. 6. Lck Y505 phosphorylation induced by PGE$_2$ inhibits TCR Vγ9Vδ2 signaling pathway. (a and b) Lysates of Vγ9Vδ2 T cells were prepared after 0, 0.5, 1, 5 or 15 min of activation by cross-linking of the TCR with an anti-CD3 mAb. Experiments were conducted in the presence or the absence of PGE$_2$ at 1 μg/mL. Data shown are representative of 3 independent experiments. (a) Phosphorylated protein vs total protein labelling for the protein tyrosine kinase ZAP70, PLCγ and ERK 1/2 MAP kinases and (b) phosphorylated protein vs total protein labelling for the protein tyrosine kinase Lck.

the presence of PGE$_2$. Western blot using specific mAbs against phosphorylated and total Lck in lysates of activated $\gamma\delta$ T cells in the presence or absence of PGE$_2$ indicates that Lck phosphorylation was strongly increased in the presence of PGE$_2$ during the activation of $\gamma\delta$ T cells (Fig. 6b). Altogether, these results establish that the blockade of TCR Vγ9Vδ2 signaling cascade by PGE$_2$ is due to the phosphorylation of Lck at Y505 by the cAMP-activated PKA type I.

4. Discussion and conclusions

The inhibition of immune cell responses by PGE$_2$ derived from tumor stroma represents a major barrier to the success of immunotherapies [15]. By their ability to sense ligands up-regulated by oncogenic stress on cell surface, to mediate ADCC and to kill tumor cells without MHC-dependent presentation of tumor antigens, NK and $\gamma\delta$ T cells show a great therapeutic interest [1,2]. These cells differ from conventional $\alpha\beta$ T cells and B cells in that they use numerous activating and inhibitory receptors which can operate independently from one another to trigger or inhibit destruction of target cells [3]. Even if previous reports have demonstrated that PGE$_2$ is able to suppress the destruction of some tumor cell lines by NK and $\gamma\delta$ T cells [21,23,24], it remains undetermined which receptors are subjected to inhibition by PGE$_2$. In this report, using mAbs for redirecting the specific lysis of the mouse FcγR$^+$ cell line P815, we clearly demonstrate that the major NKR (NKp30, NKp44, NKp46, NKG2D and CD16) and the major $\gamma\delta$ T cell receptors (TCR Vγ9Vδ2, NKG2D and CD16) are all inhibited by PGE$_2$ through a cAMP-mediated PKA type I-dependent mechanism following the binding of PGE$_2$ on EP2 and EP4.

The activation of PKA type I by agents increasing cAMP – such as PGE$_2$ and adenosine – has already been shown to suppress global responses of NK and $\gamma\delta$ T cells [28,29]. To our knowledge, this is the first report demonstrating that the activation of PKA type I mediated by PGE$_2$ suppresses $\gamma\delta$ T cell cytolytic responses triggered by TCR Vγ9Vδ2, NKG2D or CD16. A first argument lies in the fact that Rp-8-Br-cAMP – a selective antagonist of PKA type I – prevents the inhibition by PGE$_2$ of the cytolytic degranulation of $\gamma\delta$ T cells induced by activation of TCR Vγ9Vδ2. Secondly, our demonstration is supported by the evidence that PGE$_2$ inactivates Lck in $\gamma\delta$ T cells through its phosphorylation at Y505. This inactivation prevents in turn the phosphorylation of ZAP70, a key enzyme in TCR signaling. It is reasonable to speculate that the phosphorylation of Csk at S364 mediated by PKA type I is involved in the phosphorylation of Lck at Y505 by PGE$_2$. This has already been demonstrated in $\alpha\beta$ T cells [33]. The observation that PGE$_2$ inhibits the cytotoxicity of $\gamma\delta$ T cells triggered by any of their major
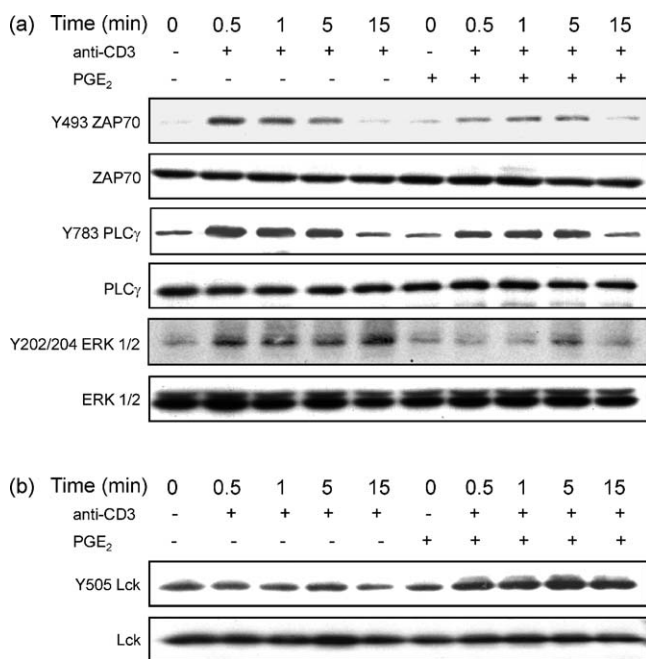
cytolytic receptors is of importance in regard with the prospect of developing γδ T cell-based therapy of cancer to restore strong anti-tumor immunity in humans [1].

Like TCR, NCR and CD16 are associated with adapter proteins (such as CD3ζ, DAP12 and FcεRI-γ) which bear immunoreceptor tyrosine-based activating motifs (ITAM) and recruit the tyrosine kinases ZAP70 and Syk [35]. In contrast, NKG2D is associated with the adapter protein DAP10 which contains an YINM motif and recruits the adapter Grb2 and the kinase PI3K [36,37]. All these adapter proteins need to be phosphorylated by kinases of the Src family (such as Lck) to transmit their activating signal [35]. We show that cAMP and PKA type I are both implicated in the inhibition of NCR, CD16 and NKG2D by PGE$_2$ suggesting that, like αβ and Vγ9Vδ2 TCR, blockade by PGE$_2$ is dependent on the inactivation of Lck mediated by Csk. Other kinases of the Src family such as Fyn, Src, Yes and Lyn are also expressed in NK cells and might have redundant functions with Lck [35]. It remained to be determined if these Src kinases are also targeted by the activation of PKA type I mediated by PGE$_2$.

Studies on the mechanism of action of therapeutic mAbs (such as Trastuzumab and Rituximab) used in breast cancer and lymphomas suggest an important role for ADCC mediated by CD16 [38,39]. NK and γδ T cells have been shown to actively participate in the ADCC of cancer cells coated with therapeutic mAbs [8–10]. Since many cancers over-express COX2 and produce high amounts of PGE$_2$ [16,17], the ability of PGE$_2$ to down regulate NK and γδ T cell cytotoxicity triggered by CD16 might be involved in the numerous cases of tumor resistance to treatment with mAbs depicted until now [39]. Neutralizing the biosynthesis of PGE$_2$ with COX2 inhibitors is already aimed at improving αβ T cell-based cancer immunotherapy [40]. Our results suggest that such an approach could also benefit to mAbs-based therapies.

We also demonstrate that PGE$_2$ suppresses the cytotoxicity of NK cells mediated by NCR (NKp30, NKp44 and NKp46) when they are triggered separately or in combination. NCR have been studied extensively, demonstrating the importance of these receptors in the destruction of tumor cells by NK cells. Blocking one or more of these receptors often inhibits killing of tumor cells by human NK cells in vitro [11]. Mice with targeted mutation in the gene encoding NKp46 are impaired in their ability to eradicate transferred lymphoma [41]. It has been suggested that a functional cross-talk between the different members of NCR occurs in NK cells possibly results in the amplification of these activating signals [42].

From its part, NKG2D has received considerable attention owing to evidence of its important role in immunosurveillance of tumors [6,7]. Indeed, genotoxic stress that activates DNA damage responses has been shown to induce the expression of NKG2D ligands (MICA, MICB, and ULBPs) and most of established tumor cell lines constitutively express one or more of these NKG2D ligands [43]. NK and γδ T cells express high levels of NKG2D which has been shown to directly promote their cytotoxic responses against target cells [5,44]. Therefore, the recognition of NKG2D ligands on tumor cells must play an important role in tumor immunosurveillance or eradication by NK and γδ T cells [6,7,45]. Several inhibitory factors produced by tumor stroma such as TGF-β have already been shown to interfere with the activation of NK cells by NKG2D triggering [46]. Here we report that PGE$_2$ inhibits through a cAMP-dependent mechanism the cytotoxicity activated by NKG2D triggering. This might be another way for tumor cells to avoid eradication by NK and γδ T cells. NKG2D has also been shown to act as a co-receptor allowing the destruction of tumor cells by cytotoxic T cells (CTL) [47]. The present study did not investigate the ability of PGE$_2$ to inhibit NKG2D co-activation of CTL. Nevertheless, the ability of PKA type I to inhibit NKG2D-mediated activation of NK and γδ T cells, strongly suggests that PGE$_2$ will inhibit NKG2D signaling in CTL since previous studies

demonstrated that the increase of cAMP mediated by PGE$_2$ leads to PKA type I activation in these cells [29,48].

Taken together, our results outline the broad inhibitory properties of PGE$_2$ towards the major cytotoxic receptors of NK and γδ T cells. Considered from its deleterious side, the problem raised by PGE$_2$ should be taken into account when designing anti-cancer immunotherapies (based either on molecular or cellular tools) in which the cytotoxic properties of NK and γδ T cells are targeted.

Acknowledgements

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