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Ludovic Martinet, Christine Jean, Gilles Dietrich, Jean-Jacques Fournié, Rémy Poupot

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

Ludovic Martinet[a], Christine Jean[a], Gilles Dietrich[a], Jean-Jacques Fournié[a], Rémy Poupot[a,*]

[a] INSERM, U.563, Centre de Physiopathologie de Toulouse-Purpan, Toulouse, F-31300 France; Université Paul-Sabatier, Toulouse, F-31400 France

E-mail addresses : ludovic.martinet@ipbs.fr

christine.jean@inserm.fr

gilles.dietrich@inserm.fr

jean-jacques.fournie@inserm.fr

remy.poupot@inserm.fr

* Corresponding author. Pr. Rémy Poupot, INSERM 563 Centre de Physiopathologie de Toulouse-Purpan, CHU Purpan, BP3028, 31024 Toulouse Cedex 3, FRANCE. Tel.: +33 5 6274 8368; fax: +33 5 6274 4558.

E-mail address: remy.poupot@ inserm.fr

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 $Fc\gamma R^+$ 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 cells 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.

Keywords:

NK cells

γδ T cells

PGE2

PKA type I

cytotoxicity receptors

1. Introduction

NK cells (NK) 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 [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. Material 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 (200nM, 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 Vγ9V$\delta 2^+$ 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 lyses 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 4h 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)] x 100.

2.4. Cytokine production

Vγ9Vδ2 T cells were stimulated for 5h with increasing doses of BrHPP in the presence or absence of PGE2 (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 (1mM) for 1h in complete RPMI medium. Cells were pelleted by gentle centrifugation (110 g for 1 min), left in co-incubation for 5h 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 was 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™ reagent and was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp., Paisley, UK). cDNA encoding human EP1, EP2, EP3, EP4 and Hypoxantine 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_3VO_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 1h 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 $\gamma\delta$ T cells mediated by TCR $V\gamma 9V\delta 2$, 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 $Fc\gamma R^+$ 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 1µg/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 four 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, were found in NK cells freshly isolated from three 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 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 30s 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 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 throug the phosphorylation of the latter in Y505 [32]. Previous studies demonstrated that cAMP elevating agents inhibit TCR αβ 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 γδ T cell activation in the presence of PGE_2. Western Blot using specific mAbs against phosphorylated and total Lck in lysates of activated γδ 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 γδ 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 γδ T cells show a great therapeutic interest [1,2]. These cells differ from conventional αβ T cells and B cells in that they use numerous activating and inhibitory receptors which can operate independently one from 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 γδ 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 γδ 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 PGE2 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 γδ 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 γδ 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 γδ T cells induced by activation of TCR Vγ9Vδ2. Secondly, our demonstration is supported by the evidence that PGE_2 inactivates Lck in γδ 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 αβ T cells [33]. The observation that PGE_2 inhibits the cytotoxicity of γδ 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 cells 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 resulting 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.

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Figure captions

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 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.

Fig. 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. (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 γδ 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 +/- SD from 3 independent experiments). ★ $p < 0.05$, ★★ $p < 0.01$ in a Student's *t*-test.

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. (b) Phosphorylated protein *vs* total protein labelling for the protein tyrosine kinase Lck.

Fig. 1.

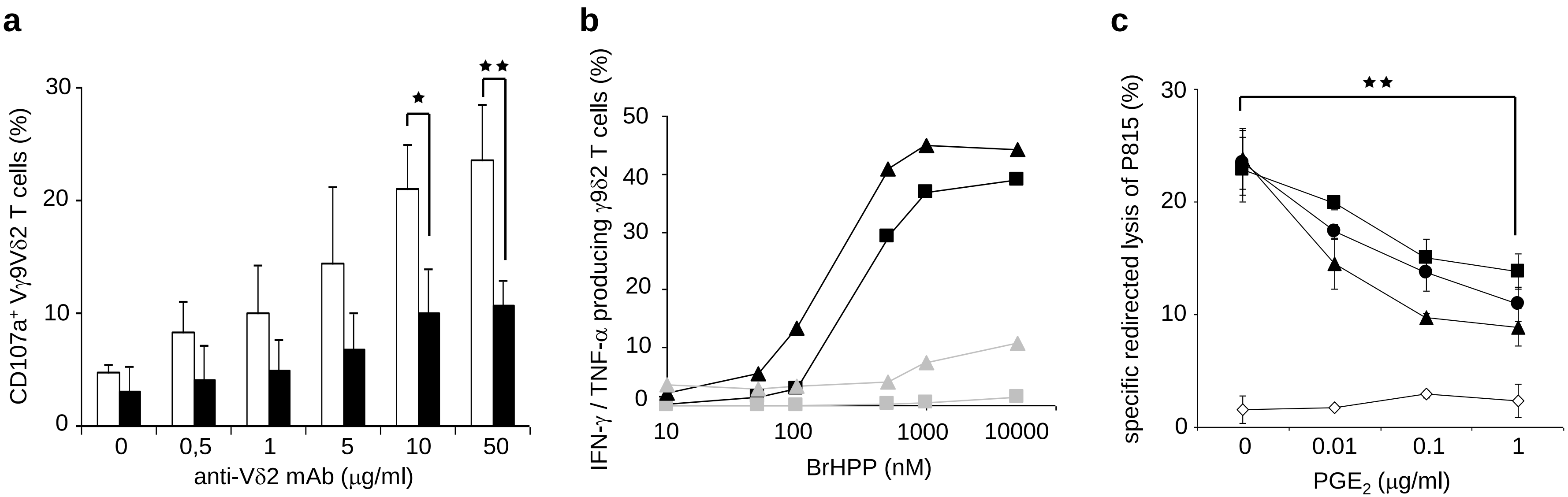

a
b
c
CD107a+ Vγ9Vδ2 T cells (%)
anti-Vδ2 mAb (μg/ml)
0
0,5
1
5
10
50
IFN-γ / TNF-α producing γ9δ2 T cells (%)
BrHPP (nM)
10
100
1000
10000
specific redirected lysis of P815 (%)
PGE2 (μg/ml)
0
0.01
0.1
1

Fig. 2.

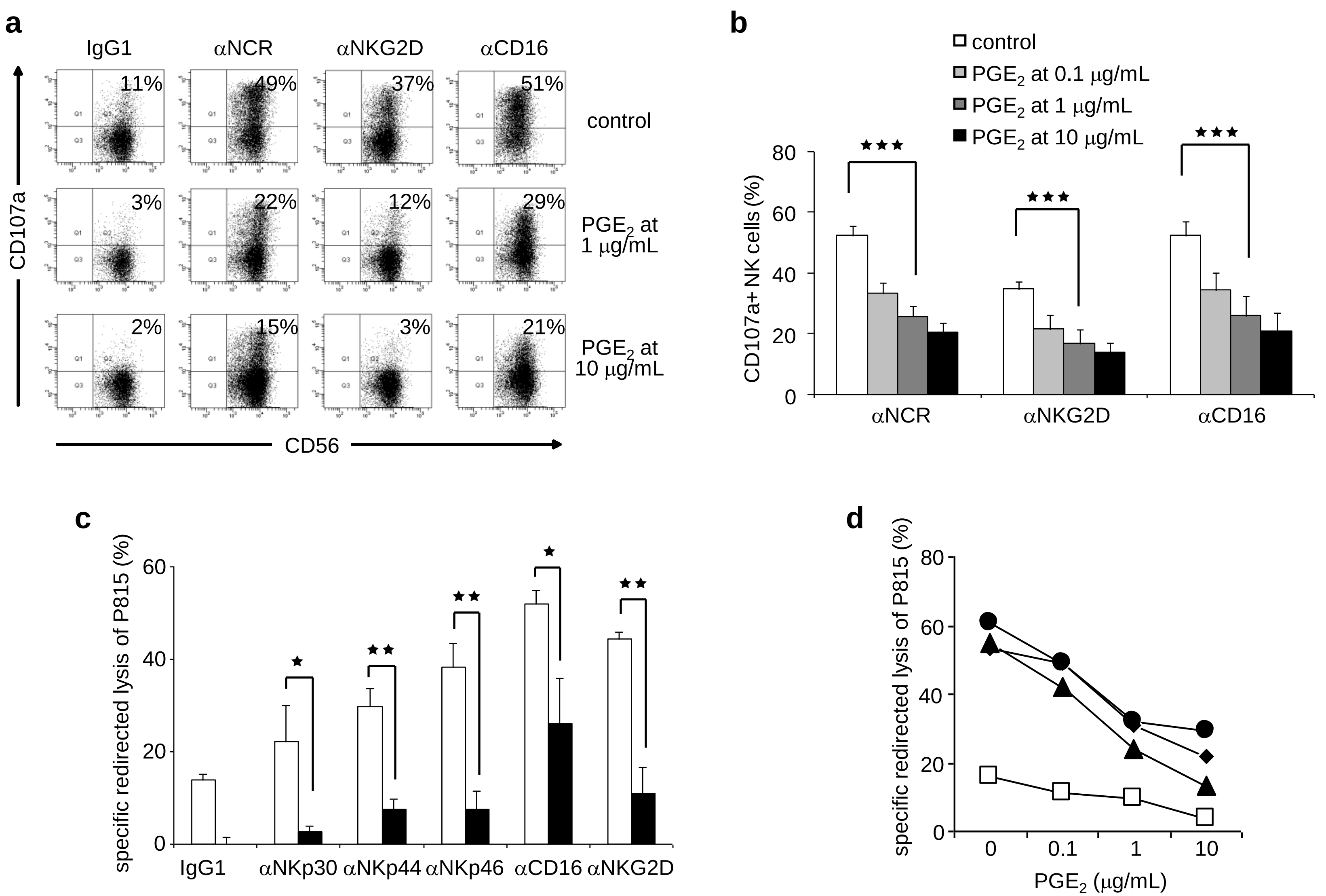

a
IgG1
αNCR
αNKG2D
αCD16
11%
49%
37%
51%
control
3%
22%
12%
29%
PGE2 at 1 μg/mL
2%
15%
3%
21%
PGE2 at 10 μg/mL
CD107a
CD56
b
control
PGE2 at 0.1 μg/mL
PGE2 at 1 μg/mL
PGE2 at 10 μg/mL
CD107a+ NK cells (%)
80
60
40
20
0
αNCR
αNKG2D
αCD16
c
specific redirected lysis of P815 (%)
60
40
20
0
IgG1
αNKp30
αNKp44
αNKp46
αCD16
αNKG2D
d
specific redirected lysis of P815 (%)
80
60
40
20
0
0
0.1
1
10
PGE2 (μg/mL)

Fig. 3.

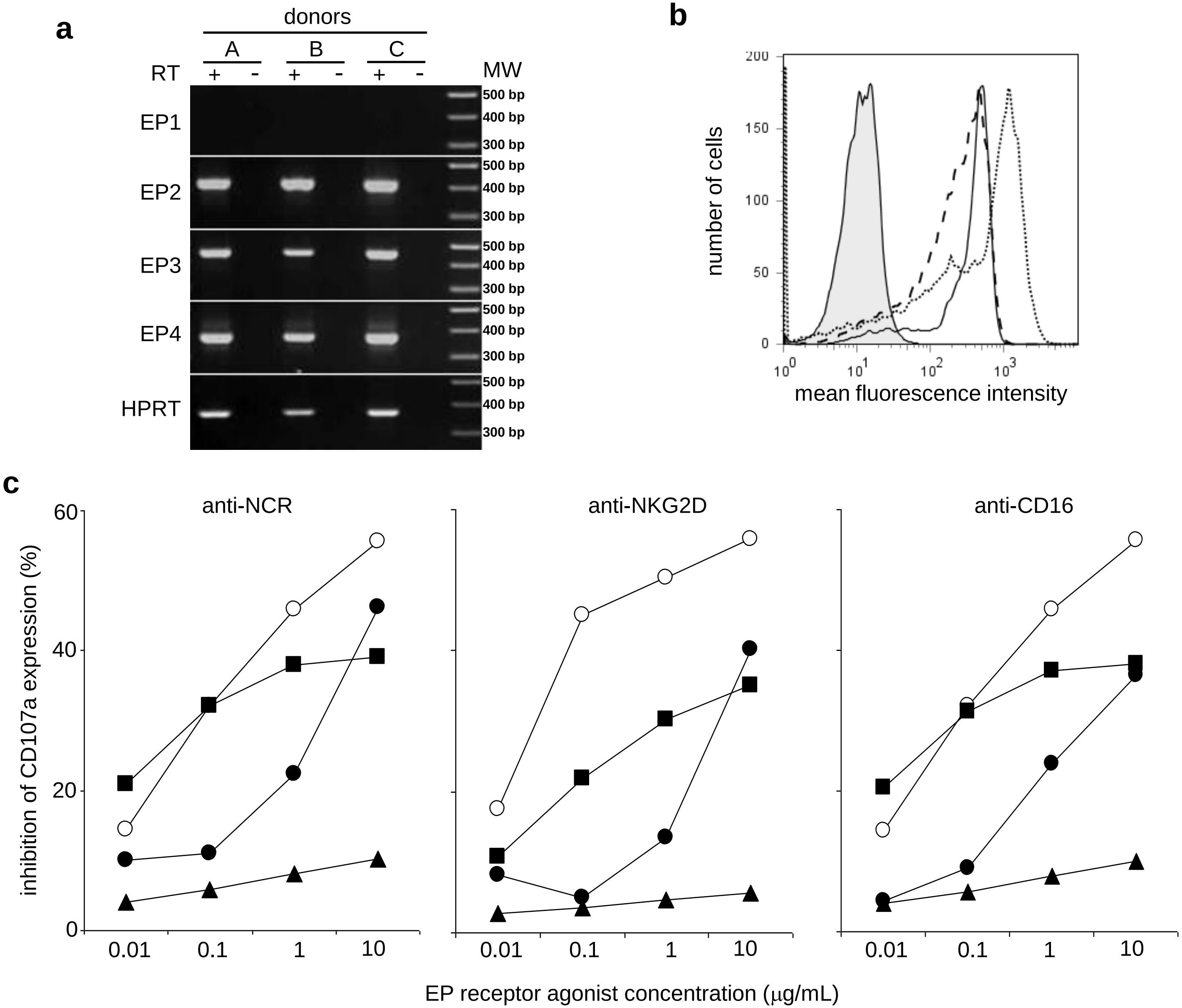

a
donors
A
B
C
RT
+
-
+
-
+
-
MW
EP1
EP2
EP3
EP4
HPRT
500 bp
400 bp
300 bp
b
number of cells
mean fluorescence intensity
c
anti-NCR
anti-NKG2D
anti-CD16
inhibition of CD107a expression (%)
0.01
0.1
1
10
EP receptor agonist concentration (μg/mL)

Fig. 4.

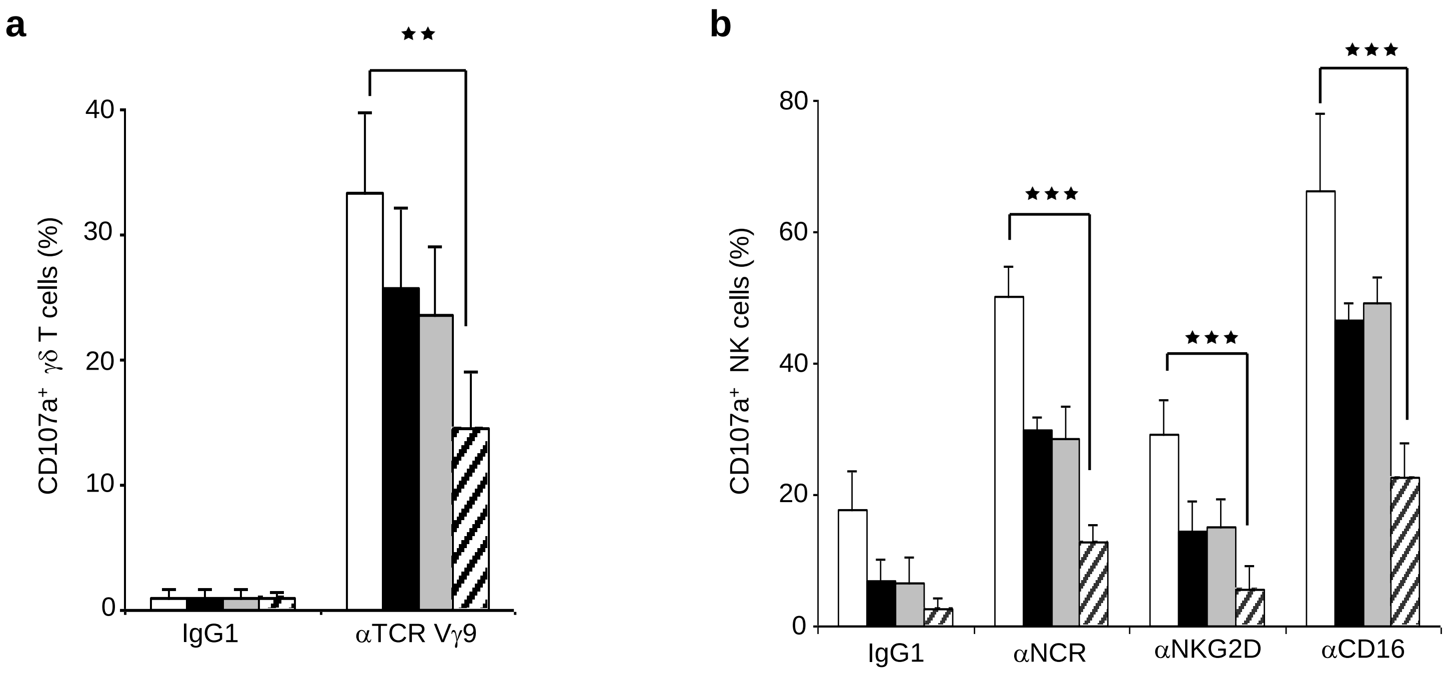
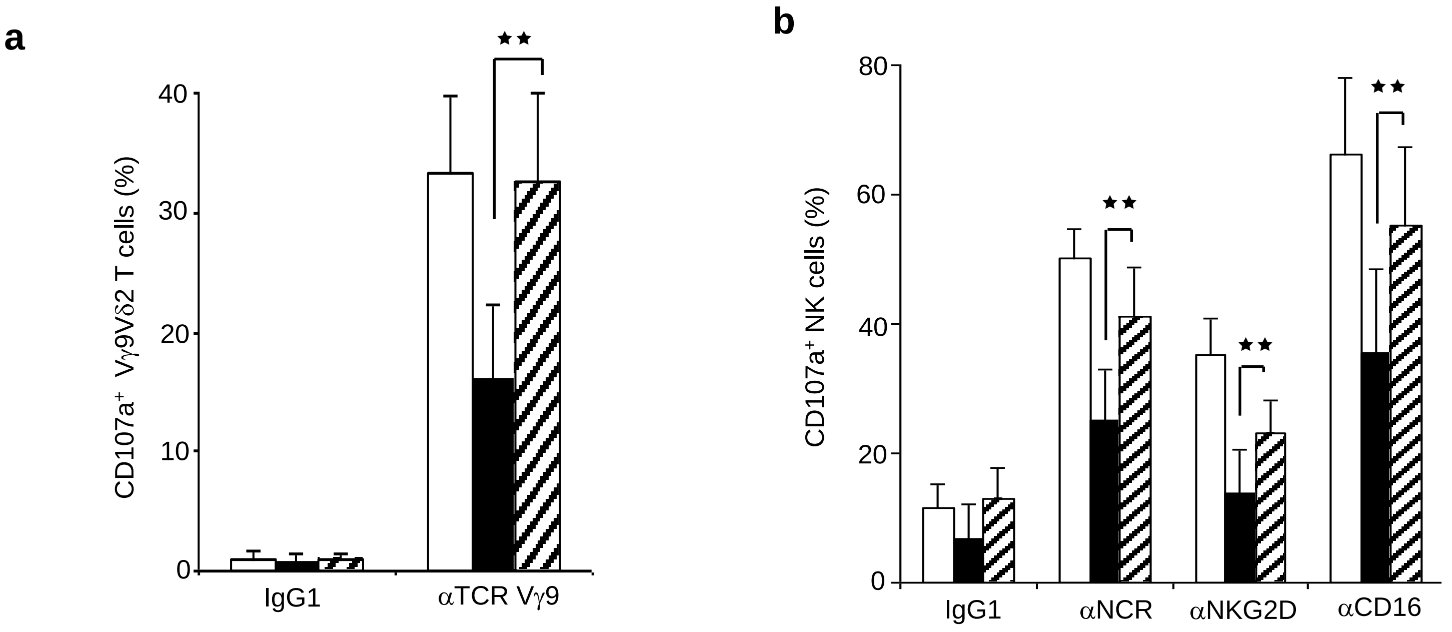
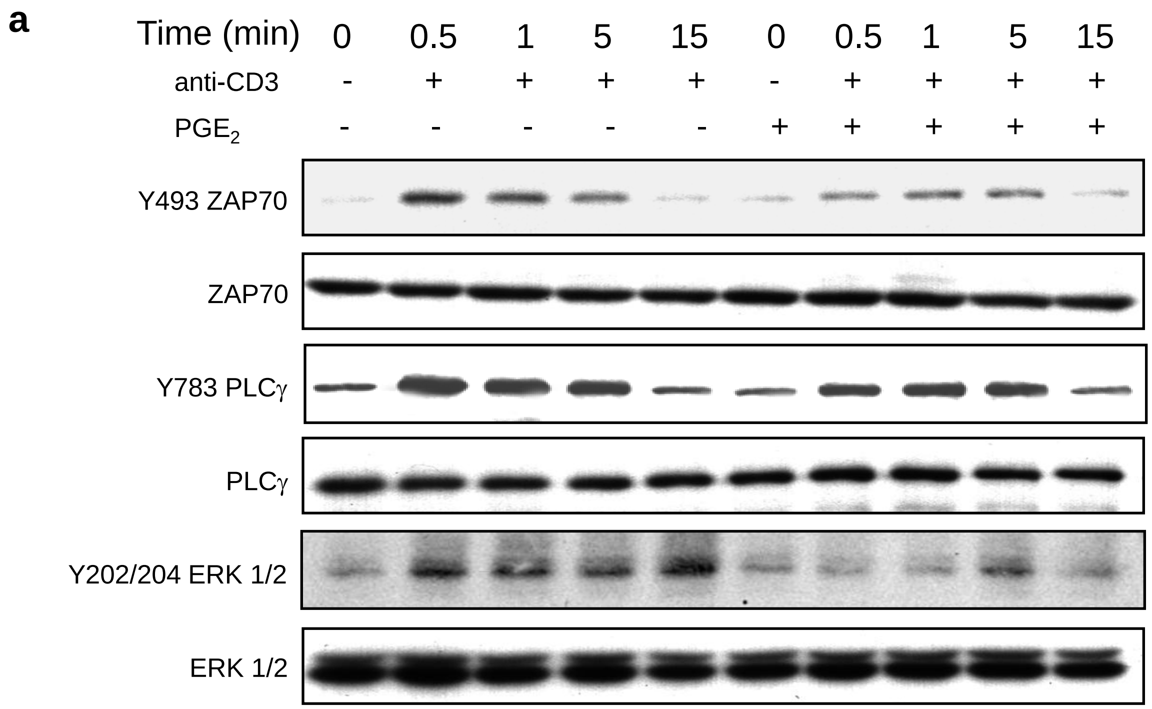

a
★★
40
30
20
10
0
CD107a⁺ γδ T cells (%)
IgG1
αTCR Vγ9
b
★★★
★★★
★★★
80
60
40
20
0
CD107a⁺ NK cells (%)
IgG1
αNCR
αNKG2D
αCD16

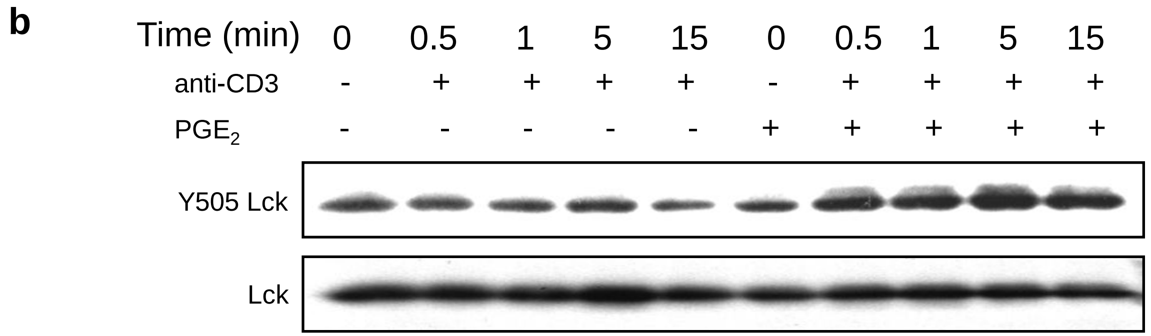
Fig. 5.



a
★★
40
30
20
10
0
CD107a+ Vγ9Vδ2 T cells (%)
IgG1
αTCR Vγ9
b
80
60
40
20
0
CD107a+ NK cells (%)
★★
★★
★★
IgG1
αNCR
αNKG2D
αCD16

Fig. 6.


a
Time (min) 0 0.5 1 5 15 0 0.5 1 5 15
anti-CD3 - + + + + - + + + +
PGE2 - - - - - + + + + +
Y493 ZAP70
ZAP70
Y783 PLCγ
PLCγ
Y202/204 ERK 1/2
ERK 1/2


b
Time (min) 0 0.5 1 5 15 0 0.5 1 5 15
anti-CD3 - + + + + - + + + +
PGE2 - - - - - + + + + +
Y505 Lck
Lck

Graphical Abstract

binding of PGE_2 on EP2 and EP4 receptors (b versus a) expressed by NK and Vγ9Vδ2 T cells activates cAMP-dependent PKA type I resulting in the inhibition of cytotoxicity