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Prostaglandin E₂ (PGE₂) suppresses Natural Killer cell function primarily through the PGE₂ receptor EP4

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Abstract

The COX-2 product prostaglandin E₂ (PGE₂) contributes to the high metastatic capacity of breast tumors. Our published data indicate that inhibiting either PGE₂ production or PGE₂-mediated signaling through the PGE₂ receptor EP4 reduces metastasis by a mechanism that requires Natural Killer (NK) cells. It is known that NK cell function is compromised by PGE₂, but very little is known about the mechanism by which PGE₂ affects NK effector activity. We now report the direct effects of PGE₂ on the NK cell. Endogenous murine splenic NK cells express all four PGE₂ receptors (EP1-4). We examined the role of EP receptors in three NK cell functions; migration, cytotoxicity, and cytokine release. Like PGE₂, the EP4 agonist PGE₁-OH blocked NK cell migration to FBS and to four chemokines (ITAC, MIP-1α, SDF-1α, and CCL21). The EP2 agonist, Butaprost, inhibited migration to specific chemokines but not in response to FBS. In contrast to the inhibitory actions of PGE₂, the EP1/EP3 agonist Sulprostone increased migration. Unlike the opposing effects of EP4 vs. EP1/EP3 on migration, agonists of each EP receptor were uniformly inhibiting to NK mediated cytotoxicity. The EP4 agonist, PGE₁-OH, inhibited IFNγ production from NK cells. Agonists for EP1, 2, and 3 were not as effective at inhibiting IFNγ. Agonists of EP1, EP2, and EP4 all inhibited TNFα; EP4 agonists were the most potent. Thus, the EP4 receptor consistently contributed to loss of function. These results, taken together, support a mechanism whereby inhibiting PGE₂ production or preventing signaling through the EP4 receptor may prevent suppression of NK functions that are critical to the control of breast cancer metastasis.

Keywords

PGE₂; EP4; NK cells; Immunosuppression

Introduction

Natural Killer (NK) cells are a subset of lymphocytes that participate in innate immunity with diverse functional activities including cytotoxicity and the capacity to produce cytokines and chemokines. NK cells are involved in different effector functions as part of an early defense system. These functions are regulated by the interplay between inhibitory and activating receptor signaling that control the response of NK cells when they encounter a potential target cell. One such target cell is the tumor cell, many of which produce high prostaglandin E₂ (PGE₂) levels due to elevated cyclooxygenase-2 (COX-2) activity. PGE₂ is a small lipid molecule that regulates numerous processes from reproduction to neuronal and

metabolic functions. These effects are mediated through a family of G-protein coupled receptors identified as EP1, 2, 3, and 4. PGE₂ is a potent regulator of inflammation as well as innate and adaptive immune responses and contributes to immune evasion in malignancy. It is known that PGE₂ down regulates IL2 activated LAK cell cytotoxicity through EP2 receptors (1, 2). LAK cells (activated NK cells) are generated from adherent splenocytes cultured in IL2. In a study by Su et al (1), LAK cells were treated with various EP receptor agonists and antagonists to identify which were involved in the immunosuppressive effects of PGE₂. They show that an EP2 agonist significantly inhibits LAK cytotoxicity and that the inhibitory effects of PGE₂ can be blocked using an EP2 antagonist. In contrast, neither an EP1/EP3 agonist nor EP1 or EP4 antagonists modulate LAK cell cytolytic activity. Thus, PGE₂ mediated inhibition of LAK cytotoxic activity is through the EP2, but not the EP1, EP3, or EP4 receptors. The role of individual EP receptors in regulating activities of endogenous, resident splenic NK cells has not been investigated. Therefore, this study has identified the EP receptors that control three NK cell functions in endogenous splenic NK cells: cytotoxicity, cytokine secretion, and migration.

PGE₂ contributes to malignant behavior in breast and other malignancies. Despite the fact that preclinical studies indicate that targeting PGE₂ synthesis through COX-2 inhibition is promising, the safety of COX-2 inhibitors has drawn some concern. Therefore, alternative approaches to target the COX-2 pathway are being sought. Our lab initially showed that COX inhibitors or EP4 antagonists could limit tumor growth and metastasis. Further analysis revealed that COX inhibitors and EP4 receptor antagonism contribute to NK functions critical to the control of metastatic disease (3, 4). In vivo, NK cells were necessary for the therapeutic effects of COX inhibitors. Further, mammary tumor cells treated with COX inhibitors were more sensitive to NK mediated lysis. Moreover, expression of inhibitory ligands for NK cells were decreased and stimulatory ligands were increased by treatment with COX inhibitors or EP4 receptor antagonists. These studies suggest that EP4, expressed on malignant cells, is a potential therapeutic target. Little was known, however, regarding the role of EP4, or any other EP expressed on the NK cell, in regulating anti-tumor responses.

We have now characterized the role of individual EP receptors on endogenous NK functions and show that EP4 expressed in endogenous NK cells and to a lesser extent EP2, contribute to the mechanisms by which PGE₂ suppresses critical NK cell functions.

Materials and methods

Cell lines and mice

The Yac-1 murine T lymphoma cell line that is sensitive to NK mediated cytotoxicity was used. Yac-1 cells were cultured at 37°C in 5% CO₂ in RPMI 1640 plus media that contained 10mM HEPES, 1mM sodium pyruvate, 4500mg/L glucose, 1500mg/L sodium bicarbonate and supplemented with 10% FBS. Immune competent Balb/cByJ female mice were purchased from Jackson Laboratory (Bar Harbor, ME). Experiments were performed according to IACUC approved protocols.

Enriched NK cell isolation

An untouched population of NK cells from spleen cell suspension was isolated following manufacturer's protocol using the NK Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, CA). The entire effluent fraction collected represented the enriched NK cells identified as DX5⁺CD3⁻ cells.

Flow Cytometry

For EP receptor studies, enriched NK cells from normal mice were reacted with monoclonal mouse DX5 (CD49b) and CD3e (BD PharMingen, San Diego, CA) conjugated antibodies. The cells were then stained with an unconjugated primary rabbit polyclonal antibody directed to EP1, EP2, EP3, or EP4 (Cayman Chemical Company, Ann Arbor, MI) followed by fluorochrome conjugated secondary goat anti-rabbit and fixation in 4% paraformaldehyde for 15 minutes with final preparation in PBS. All flow cytometry samples were processed at the Flow Cytometry Shared Services at the University of Maryland Greenebaum Cancer Center.

RT-PCR

RNA was extracted from enriched NK cells using NucleoSpin® RNA II kit (Macherey-Nagel, Bethlehem, PA), and reverse transcribed and amplified using EP-specific or GAPDH control primers. The reaction mixture was then placed in a preheated (94°C) thermal cycler where the initial denaturing step was performed for 2 minutes followed by 36 cycles of denature 94°C for 1 minute, annealing at 56°C for 40 seconds, and extension at 72°C for 50 seconds. Upon completion, a final extension was performed at 72°C for 2 minutes and samples held at 4°C.

EP Receptor Reagents

NK cell EP receptors were pharmacologically targeted using selective agonists and antagonists from Cayman Chemical. SC19220 and SC52089 were used to competitively antagonize the EP1 receptor. The EP1/EP2 receptor was antagonized using AH6809 and EP4 receptor was antagonized with AH23848 and GW627368X (GWX). To stimulate the receptors, PGE₂ (EP1-4), Sulprostone (EP1/EP3), Butaprost (EP2), and Prostaglandin E₁ Alcohol (PGE₁-OH, EP4) were used as agonists.

cAMP EIA

NK cells were either untreated, treated with vehicle control (DMSO), PGE₂ (0.1μM, 1μM, or 10μM), or antagonists to specific EP receptors (AH23848, GWX, AH6809, SC19220, and SC51089). NK cells were treated for 30 minutes and culture media was removed. NK cell pellets were lysed using reagent 1B provided in Amersham cAMP Biotrak Enzyme immunoassay System (GE Healthcare, Piscataway, NJ) and the intracellular cAMP experiment was performed according to manufacturer's instructions.

Migration Assay

The Chemo Tx® System (Neuro Probe, Gaithersburg, MD) and a 3μm pore size was used to estimate migration. NK cells, at time 0, were treated with or without EP receptor agonists (PGE₂, Butaprost, Sulprostone, and PGE₁-OH) and the chemotactic response to FBS, ITAC, MIP-1α, SDF-1α, and CCL21 was assessed. Treated NK cells were pipetted onto the filter top and incubated at 37°C, 5% CO₂ for 3 hours. After incubation, cells were gently washed from the top side of the framed filter. Migrated cells were labeled by the addition of Calcein AM. After one-hour incubation, the microplate was placed in a fluorescence reader (485nm) to count migrated cells in the microplate wells. The results were calculated from a generated standard curve and data expressed as number of cells migrated.

NK Cell Mediated Cytotoxicity Assay

NK cell mediated cytotoxicity was measured using the CytoTox 96® Assay (Promega, Madison, WI), which quantitatively measures the release of the stable enzyme lactate dehydrogenase (LDH) upon cell lysis. NK cells were cultured with Yac-1 lymphoma targets at several effector:target (E:T) ratios: 2:1, 5:1, 10:1. NK cells were pre-treated with various

PGE₂ concentrations (1.0μM and 10μM), Butaprost (1.0μM and 10μM), Sulprostone (1.0μM and 10μM), or PGE₁-OH (1μM and 10μM). After pre-treatment with agonists, Yac-1 tumor targets were added and co-incubated for 18 hours. The supernatant was harvested and transferred to the enzymatic assay plate where the Substrate Mix was added, and the reaction halted with Stop Solution and absorbance determined at 490nm. To compute corrected absorbance values the following formula was used to obtain percent cytotoxicity for each effector:target cell ratio:

$$\% \text{Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

ELISA

NK cells were pretreated with either vehicle control (DMSO) or agonists (PGE₂, Butaprost, Sulprostone, PGE₁-OH) for 30 minutes. At the end of 30 minutes, cells were stimulated with 1000U/mL of IL2 and incubated at 37°C, 5% CO₂. At 18 hours, cell culture conditioned media was collected. Mouse IFNγ ELISA Ready-SET-Go!® and Mouse TNFα ELISA Ready-SET-Go!® kits (eBioscience, San Diego, CA) were used to measure cytokines in cell condition media and assays were performed according to the manufacturer's instructions.

Statistical Analysis

The results are expressed as mean ± standard error of at least n=3. A one-way analysis of variance (ANOVA) was used to compare mean control groups (DMSO) and experimental groups. Overall test analysis was reported as significant at p-value < 0.05.

Results

Enriched populations of normal NK cells from Balb/cByJ female mice (6–10 weeks) were isolated from total spleen cells by negative selection using immunomagnetic beads. After selection, the purity of DX5⁺CD3⁻ cells were confirmed by flow cytometry. Seventy-six percent of the isolated cells were identified as DX5⁺CD3⁻ NK cells (Fig. 1a). This enriched population was used in all experiments and referenced as normal endogenous NK cells.

Murine IL2 activated NK (LAK) cells were reported to express all four EP receptors (1). To determine which EP receptors were expressed on normal *endogenous* NK cells, we examined the expression of EP receptors by flow cytometry. All four EP receptors were detected on the surface of endogenous NK cells (Fig. 1b). Fifty-one percent of murine NK cells had detectable EP2. The EP1, EP3, and EP4 receptors were all expressed at similar levels; 29, 33, and 34 percent of cells were positive, respectively. RT-PCR analysis further confirmed the presence of mRNA for all four EP receptors (Fig. 1c).

EP2 and EP4 receptors are G-protein coupled receptors that upon activation elevate intracellular cAMP levels. If the reported inhibitory effects of PGE₂ on NK cells are mediated through EP2/EP4 signaling, PGE₂ treatment of NK cells should increase the intracellular cAMP levels in these cells. To test this, the intracellular cAMP levels of NK cells treated with vehicle control were compared to PGE₂ treated NK cells. PGE₂ (0.01μM-10.0μM) increased cAMP levels by 1.3–3.4 fold in NK cells in a dose dependent manner (Fig. 2a). To identify which EP receptor mediated cAMP activation, specific EP receptor antagonists were added to block PGE₂ stimulation of cAMP. The EP1 antagonists SC19220 and SC51089, EP1/EP2 antagonist AH6809, and EP4 antagonists AH23848 and GW627368X (GWX) were all utilized (Fig. 2b, c). In the experiment shown in Fig. 2b, PGE₂ (1.0μM) stimulated cAMP production by approximately 2.5 fold. The EP2 and EP4

antagonists alone did not significantly affect the basal cAMP levels. In the presence of PGE₂, the EP2 antagonist (AH6809) or the EP4 antagonists (AH23848 or GWX) were able to significantly block the PGE₂-mediated increase in cAMP (Fig. 2b). Neither EP1 antagonist (SCI9220 or SC51089) was capable of reversing the elevation of cAMP in PGE₂-treated NK cells (Fig. 2c). These results indicate that PGE₂ induces adenylate cyclase in NK cells through the EP2 and EP4 receptors as reported in other cells.

Whether PGE₂ affects the migration of NK cells was assessed. Migration of NK cells was determined by estimating the number of cells migrating through a porous membrane for 3 hours. NK cells migrated across the membrane in the presence of four percent fetal bovine serum or the chemokines; ITAC, MIP1 α , SIP1, or CCL21 all at 100nM. These chemokines were chosen because of their known ability to induce migration of resting NK cells (5). Treatment with PGE₂ (1.0 μ M, or 10 μ M) blocked migration of NK cells in response to each chemokine as well as to FBS (Fig. 3a). PGE₂ inhibited migration by 35 to 71 percent. To identify which EP receptor is involved in PGE₂-mediated inhibition of NK cell migration, NK cells were treated with EP receptor specific agonists. Like PGE₂, the EP4 agonist PGE₁-OH blocked NK cell migration to each stimulant (Fig. 3b). The EP2 agonist Butaprost did not significantly blunt migration to FBS, but significantly reduced the response to all chemokines tested (Fig. 3c). In contrast to the inhibitory actions of PGE₂, the EP1/EP3 agonist Sulprostone increased migration of NK cells regardless of the stimulant (Fig. 3c). These data show that the ability of PGE₂ to inhibit migration is relevant to physiological chemotactic factors and always mimicked by activation of the EP4 receptor, and in the case of specific chemokines, by EP2. Conversely, activation of the EP1 and/or EP3 receptors promotes migration.

Cytotoxicity is a major function of NK cells; therefore, effects of PGE₂ on the cytotoxic capacity of NK cells were investigated. NK cells were pre-treated with different concentrations of PGE₂ (0.01–10.0 μ M) for 30 minutes before testing their capacity to lyse Yac-1 tumor targets. Cytolytic activity of endogenous NK cells ranges from 10–20 percent (Fig. 4a). Pre-incubation with PGE₂ modestly inhibited NK mediated cytotoxicity (Fig. 4a). To determine which EP receptor mediated the inhibitory effect, NK cells were pretreated with EP receptor agonists and cytolytic activity was measured. Unlike migration where only the EP4 and EP2 agonists mimicked PGE₂ inhibition, agonists of each of the four receptors were able to inhibit NK mediated cytotoxicity (Fig. 4b, c, d).

To examine the effects of PGE₂ on the ability of NK cells to secrete cytokines, the effects of PGE₂ on the production of two type I cytokines (IFN γ and TNF α) were examined. NK cells were pretreated with PGE₂ for 30 minutes, then stimulated with 1000U/mL of IL2 to induce cytokine production. Pretreatment with PGE₂ profoundly reduced the amount of IFN γ produced by NK cells compared to control (Fig. 5a). At 1.0 μ M and 10.0 μ M, PGE₂ inhibited IFN γ production by 92 and 94 percent, respectively. To determine which EP receptor is involved in inhibition, NK cells were targeted with specific agonists. Inhibition of IFN γ production by NK cells was observed when EP4 agonist PGE₁-OH pretreated NK cells were stimulated with IL2 (Fig. 5a). Eighty-eight and 85 percent inhibition of IFN γ production at 1.0 μ M and 10.0 μ M concentrations of PGE₁-OH, respectively, were observed compared to DMSO treated cells. The agonists for the EP1, EP2, and EP3 receptors were not as efficient as either PGE₂ or an EP4 agonist at inhibiting IFN γ production (Fig. 5b). The EP2 agonist Butaprost at 1.0 μ M inhibited IFN γ production by 15%; eighty percent inhibition was achieved by Butaprost at 10.0 μ M concentration. The EP1/EP3 agonist Sulprostone inhibited IFN γ by only 10 and 28 percent at the 1.0 μ M and 10.0 μ M concentrations, respectively.

TNF α is secreted by NK cells, especially during tumorigenesis. Pretreatment with PGE₂ led to a 51–100 percent inhibition of TNF α secretion by NK cells in a dose dependent manner

(Fig. 5c). Inhibition of TNF α secretion was achieved by using all three EP receptor agonists. Stimulating the EP4 receptor with PGE₁-OH yielded results that most resembled PGE₂ (Fig. 5c) with complete inhibition of TNF α secretion at both concentrations employed. The EP2 agonist Butaprost and the EP1/EP3 agonist Sulprostone also inhibited TNF α secretion but were less effective than PGE₂ or an EP4 agonist (Fig. 5d).

Discussion

The ability of PGE₂ to regulate immune responses of T-lymphocytes has long been recognized but much less is known regarding the role of PGE₂ in mediating NK cell functions. In this study, we examined the effects of PGE₂ on normal murine NK cells and identified which EP receptors regulate NK cell functions. We showed that endogenous murine NK cells expressed all four EP receptors. An interesting question is whether the same NK cell expresses multiple EP receptors. This question has been technically challenging because all EP receptors are identified with rabbit polyclonal antibodies. Our preliminary results indicate that EP4 positive NK cells rarely express EP2. One recent report contradicted our findings reporting that NK cells from three human donors expressed simultaneously, the EP2, EP3, and EP4, but not EP1 receptors (6). In addition to possible species differences, they cultured NK cells in IL2; our unpublished data shows that IL2 activation of murine NK cells downregulates EP receptor expression.

Elevations in intracellular cAMP suppress innate immune functions of macrophages, monocytes, and neutrophils (7). Early work in NK cells suggests an association between elevated cAMP and suppression of NK cell activity (8). We also examined the effects of PGE₂ on cAMP activity. PGE₂ stimulated increased intracellular cAMP in a dose dependent manner in NK cells. Based on the ability of EP2 or EP4 agonists to mimic the activities of PGE₂, we concluded that the PGE₂ mediated increase in cAMP was regulated through both EP2 and EP4 receptors. This was anticipated, since in other cells, the EP2 and EP4 receptors are both coupled to the G α_s subunit known to be involved in stimulating adenylyl cyclase (9). The cAMP response was not linked to the EP1 receptor because an EP1 agonist did not affect cAMP levels. On other cells, the EP1 receptor is coupled to the G α_q subunit (9). Upon PGE₂ stimulation, antagonists to the EP1 receptor will most likely block calcium mobilization. There were limitations with determining whether the EP3 receptor was involved in PGE₂ stimulated adenylyl cyclase activity because there are no commercially available specific EP3 antagonists. However, based on our results and current knowledge that the EP3 receptor is coupled to the G α_i subunit, it is postulated that PGE₂ would not stimulate adenylyl cyclase through EP3 on NK cells.

NK cells are one of the first immunological cells arriving at sites of injury or inflammation. To do so, the NK cell must respond to either soluble or surface bound signals that stimulate cell migration. Our studies show that PGE₂ inhibited NK cell migration to a broad spectrum of chemotactic signals through the EP4 and EP2 receptors. While we know that NK cell migration is negatively regulated through the EP4 receptor, the downstream events are not fully understood. One mechanism may be through cAMP as a second messenger. Although we found PGE₂ to inhibit migration through the EP4 receptor, in Langerhans cells, a type of dendritic cell in the epidermis, the EP4 receptor plays an important role in promoting, rather than inhibiting, migration (10). This was confirmed using human monocyte-derived dendritic cells. Legler and colleagues (11) showed that PGE₂ induced migration of dendritic cells through the EP4 receptor. Since dendritic cells play a role in T cell activation, we postulate that PGE₂ may play a pro-migratory function in this setting allowing dendritic cells to come into proximity with T lymphocytes. Thus, the same EP4 receptor can promote or inhibit migration of different immune effector cells.

An interesting finding was that Sulprostone, an EP1/EP3 receptor agonist, actually increased migration to chemokines by 1.3–1.6 fold. The EP1/EP3 mediated increase in migration may be driven by EP1 stimulation of phospholipase C (PLC) which leads to downstream release of inositol 1,4,5-triphosphate (IP₃) and activation of protein kinase C (PKC). In T cells, migration across the vasculature is controlled by PKC (12). Thus, all EP receptors are not uniformly suppressive to all NK cell functions. An opposing role for EP1 versus EP4 has also been described in tumor metastasis. We showed that EP4 promotes mammary tumor cell metastasis, but EP1 is a metastasis suppressor (3, 13, 14). Because EP1 is a lower affinity receptor compared to EP2 and EP4, it is possible that pro-migratory responses are only elicited when tissue PGE₂ levels are very high.

Natural Killer cells have been named for their ability to mediate cytotoxicity against transformed and infected cells. In the presence of PGE₂, NK mediated cytotoxicity against Yac-1 tumor targets is inhibited. Our lab has previously shown that the COX inhibitor indomethacin, which suppresses PGE₂ production, decreases expression of the inhibitory ligand MHC class I and increases activating ligands H60 and Rae-1 on mammary tumor cells (4), unpublished data). Therefore, PGE₂ may inhibit NK-mediated cytotoxicity by allowing engagement of the inhibitory receptor Ly49 and preventing activation of the NKG2D receptor as a result of diminished H60 and Rae-1 levels. Consistent with this hypothesis, Zeddou and colleagues (15) show that PGE₂ increases the percentage of cells expressing CD94/NKG2A, an inhibitory receptor, resulting in diminished cytolytic potential. Although that study examined T cells, it can be presumed that this mechanism could be relevant to NK cells.

Specific agonists of each EP receptor modestly inhibited cytotoxicity. In contrast, PGE₂ inhibited cytotoxicity in activated NK cells (LAK cells) only through the EP2 receptor (1). In that study, LAK cells were treated with EP2 agonist (Butaprost), EP1/EP3 agonist (Sulprostone), and antagonists to the EP1 (SC19220), EP2 (AH6809), and EP4 (AH23848) receptors. Only Butaprost could inhibit cytotoxicity and the antagonist AH6809 blocked PGE₂-mediated inhibition of cytotoxicity. Therefore, the authors concluded that the EP2 receptor, but not EP1, EP3, or EP4, was involved in the inhibitory signaling on LAK cells. While our data also implicates the EP2 receptor, we also show the EP1, EP3, and EP4 receptors are also involved in the regulation of cytotoxicity mediated by endogenous NK cells. We have observed that activated NK cells have a different EP receptor profile than endogenous NK cells (data not shown) which may lead to different signaling patterns in these two populations.

The ability of the EP1/EP3 receptor agonist Sulprostone to inhibit NK mediated cytotoxicity was not predicted. Based on migration data, we predicted that Sulprostone treatment would increase NK mediated cytotoxicity or have no effect on this function. FasL is a member of the tumor necrosis factor (TNF) superfamily that can trigger apoptotic cell death following receptor-ligand binding. O'Callaghan and colleagues (16) observed that PGE₂ increased FasL expression on colon tumor cells increasing cytotoxicity against Fas-sensitive Jurkat T cells. We hypothesize that Sulprostone is stimulating the EP1 receptor, which may play a role in increased Fas ligand (FasL) expression on tumor cells triggering apoptosis of the NK cell.

In addition to mediating cytotoxicity, NK cells play an important regulatory role in the immune response through production of cytokines. IFN γ exerts anti-proliferative, immunoregulatory and proinflammatory activities and is thus important in host defense mechanisms. We show that pretreatment with PGE₂ reduced the amount of IFN γ and TNF α secretion by NK cells. Likewise, in human NK cells, IFN γ secretion was suppressed by PGE₂ in a dose dependent manner (17). Our data revealed that PGE₂ mediated suppression

of IFN γ was primarily through the EP4 receptor. In CD4⁺ T cells, PGE₂ also inhibited IFN γ production via EP4 receptor (18). The mechanism controlling inhibition was through the cAMP-PKA pathway. Thus, EP4 is a common mechanism to negatively regulate IFN γ in both CD4⁺ T cells and NK cells.

PGE₂ inhibited NK cell production of TNF α through all three EP receptors. At 10 μ M, the EP agonists Butaprost, Sulprostone, and PGE₁-OH inhibited TNF α production by 51, 54, and 100 percent, respectively. In macrophages, evidence for inhibition of TNF α secretion through either the EP4 or EP2 receptors had been presented (19, 20), but the inhibitory effects produced by the EP1/EP3 agonist that were observed were unanticipated. While we examined the effects of EP1 on TNF α , Spaziani and colleagues (21) examined changes in EP1 protein, EP1 mRNA, and PGE₂ concentrations in response to TNF α treatment in amnion WISH cell culture. They reported that treatment of WISH cells with TNF α increased EP1 expression and PGE₂ concentrations. In dental pulp cells, which are made of defense cells such as macrophages, granulocytes, and mast cells, TNF α stimulated PGE₂ production that in turn stimulated Ca²⁺ signaling. Therefore, it may be that inhibitory effects of EP1/EP3 agonist Sulprostone are through the EP1 receptor regulated by downstream Ca²⁺ signaling.

In summary, we have shown NK cells express all four EP receptors (Table 1). The EP2 and EP4 receptors on NK cells, which are coupled to adenylyl cyclase activation, were found to be functional. PGE₂ predominantly inhibits NK cell migration and cytokine secretion through the EP4 receptor and, to a lesser extent, the EP2 receptor. NK cell mediated cytotoxicity is negatively regulated through all four receptors. The results of several laboratories indicate that targeting the EP4 receptor may prevent metastatic disease and the current study shows that targeting the EP4 receptor may prevent NK inhibition and have immunotherapeutic potential. Therefore, these findings support the further investigation of using specific antagonists of EP4 receptors to prevent disease progression via NK cell mechanisms.

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Fig. 1. NK cells express all four EP Receptors

(a) Enriched populations of NK cells from spleen cells by negative selection using immunomagnetic beads. (b) DX5⁺ NK cells freshly isolated from normal murine spleen by magnetic cell sorting were analyzed for EP1-4 receptor expression by flow cytometry using antibodies specific for DX5, EP1, EP2, EP3 or EP4 or isotype control. Upper right quadrant shows the percent of DX5⁺ NK cells expressing the indicated EP receptor. (c) mRNA was isolated from enriched NK cells, reverse transcribed, and analyzed using the primer pairs specific for EP1, EP2, EP3, EP4, or GAPDH. RT-PCR analysis confirms the presence of all four EP receptors on murine NK cells at the correct size; EP1, 168bp, EP2, 233bp, EP3 198bp, and EP4 213bp.

Fig. 2. PGE₂ stimulates intracellular cAMP in NK cells

(a) NK cells were stimulated in the presence of PGE₂ for 30 minutes. Intracellular cAMP was assessed from cell lysates. Triplicate measurements taken for each treatment group. Data represented as mean fmol/1x10⁵ cells intracellular cAMP. (b–c) NK cells were incubated with various EP antagonists for 15 minutes prior to addition of 1 μM PGE₂ agonist. (b) 10 μM AH6809 with and without agonists and 10 μM AH23848 with and without agonist. (c) 10 μM SC19220 with and without agonist and 10 μM SC51089 with and without agonist. Triplicate measurements taken for each treatment group. Data represented as mean fmol/1x10⁵ cells intracellular cAMP. Relative to DMSO, PGE₂ $p < 0.01$ and SC19220 and SC51089 with PGE₂ treatment $p < 0.05$. Overall test for main effect of treatment p -value = 0.0003.

Fig. 3. PGE₂ inhibits NK cell migration through EP4 and EP2 receptors

(a) NK cells were treated with various concentrations of PGE₂ and migration of NK cells in response to FBS or four chemokines (ITAC, MIP-1α, SDF-1α, and CCL21) was assessed. The number of cells that crossed a 3μm pore membrane were counted based on their fluorescence. Data reported as mean number of cells migrated ± standard error, n=3. NK cells were incubated with either 1μM or 10μM of (b) PGE₁-OH (EP4), (c) Butaprost, BUT (EP2), or (c) Sulprostone, SUL (EP1/EP3). Cells were allowed to migrate across a 3μm pore membrane to enriched buffer.

Results reported as mean number of cells migrated ± standard error, n=3. Relative to DMSO, * p<0.05.

Fig. 4. PGE₂ inhibits NK mediated cytotoxicity through all EP receptors

NK cells were pre- incubated with either 1 μ M or 10 μ M of (a) PGE₂, (b) Butaprost (BUT), (c) Sulprostone (SUL), or (d) PGE₁-OH and ability to kill Yac-1 cells was determined.

Three effector to target ratios were used; 2:1, 5:1, and 10:1. Data presented as percent NK mediated cytotoxicity $\pm$ standard error and represents n of 3. Overall testing for main effect of PGE₂ inhibition p-value < 0.0001.

Fig. 5. PGE₂ inhibits IFN γ and TNF α production by NK cells predominantly through EP4 receptor

NK cells were pretreated with (a,c) 1 μ M or 10 μ M of PGE₂ or PGE₁-OH; and (b,d) 1 μ M or 10 μ M Butaprost (BUT), or Sulprostone (SUL). Cell culture supernatants were assayed for IFN γ in a and b and for TNF α in c and d. Results are reported as mean pg/mL/1x10⁶ cells $\pm$ standard error, n=3. Relative to DMSO, EP receptor agonists (PGE₂, PGE₁- OH, Butaprost, and Sulprostone) *p<0.05, **p<0.01, ***p<0.001.

EP receptors present on NK cells and their role in NK functions

In summary, the EP4 and EP2 receptors are involved in all NK cell functions analyzed, with the EP4 receptor consistently contributing to loss of function. Targeting the EP4 receptor may have immunotherapeutic potential. ☑ EP receptor is present on cells; ↑ and ↓ PGE₂ exerts functions through specified receptor.

Table 1

	PGE ₂	EP1	EP2	EP3	EP4
Receptor Expression	☑	☑	☑	☑	☑
CAMP	↑		↑		↑
Migration	↓	↑	↓	↑	↓
Cytotoxicity	↓	↓	↓	↓	↓
Cytokine Production					
-IFN γ	↓	→	↓	→	↓
-TNF α	↓	↓	↓	↓	↓