Published in final edited form as:
*J Immunother*. 2012 ; 35(2): 179–188. doi:10.1097/CJI.0b013e318247a5e9.

# Modulation of host natural killer cell functions in breast cancer via prostaglandin $E_2$ receptors EP2 and EP4

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## Abstract

Breast malignancies often have high levels of COX-2. The COX-2 product prostaglandin $E_2$ ($PGE_2$) contributes to the high metastatic capacity of breast tumors. Our published data indicates that inhibiting either $PGE_2$ production or $PGE_2$-mediated signaling through the $PGE_2$ receptor EP4 (one of four EP expressed on the malignant cell) reduces metastasis by a mechanism that requires Natural Killer (NK) cells. Tumor derived $PGE_2$ and exogenous $PGE_2$ are known to have direct inhibitory effects on NK cell functions, but less is known regarding which EP receptors mediate these effects. We now show that several NK functions (lysis, migration, cytokine production) are compromised in tumor-bearing mice and that tumor produced $PGE_2$ interferes with NK cell functions. $PGE_2$ inhibits the potential of NK cells to migrate, exert cytotoxic effects, and secrete IFNγ. The ability of $PGE_2$ to inhibit NK cells from tumor bearing mice is by acting on EP2 and EP4 receptors. NK cells from tumor-bearing mice were more sensitive to inhibition by EP4 and EP2 agonists compared to endogenous NK cells from healthy mice. $PGE_2$ was inhibitory to most NK functions of either normal or tumor-bearing mice. In contrast, there was a trend for enhanced TNFα production in response to $PGE_2$ by NK cells from tumor-bearing mice. We also report that a recently described EP4 antagonist, frondoside A, inhibits breast tumor metastasis in an NK-dependent manner and protects IFNγ production by NK cells from $PGE_2$ mediated suppression. Taken together these data show that NK functions are depressed in tumor-bearing hosts relative to normal NK cells and that $PGE_2$ suppresses NK functions by acting on EP2 and EP4 receptors.

### Keywords

$PGE_2$; EP4; NK cells; Immunosuppression; Frondoside A

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**Financial Disclosure:** All authors have declared there are no financial conflicts of interest in regards to this work.

## Introduction

NK cells have the ability to recognize and kill tumor cells. Once activated, NK cells have increased cytolytic and proliferative functions to mediate anti-tumor activity. Therefore, it is important for NK cells to mobilize from a resting state. It is well established that the tumor cell releases immunosuppressive agents such as $PGE_2$ that inhibit NK effector function. Other laboratories have found that macrophages from tumor bearing mice produce $PGE_2$ *in vitro* and suppress NK cell activity and this inhibition is relieved with the dual COX-1/COX-2 inhibitor, indomethacin [1]. Monocytes from patients with breast cancer contribute to increased levels of $PGE_2$ produced in culture and this activity correlates with a decrease in NK cell activity [2].

Our lab has demonstrated that murine and human breast tumors secrete high levels of $PGE_2$, which correlate with metastatic disease [3]. $PGE_2$ is the principle COX-2 product in cancer and mediates actions through a family of G-protein coupled receptors, EP1-4. Despite structural and sequence similarities among the four receptors, they are coupled to different intracellular signaling pathways [4]. Ligand binding of EP1 activates phospholipase C (PLC), which generates second messenger release of Ca2+ from intracellular stores and stimulates phosphorylation by protein kinase C (PKC). EP2 and EP4 are coupled to protein kinase A/adenylyl cyclase resulting in increased cyclic AMP (cAMP). The EP4 receptor has also been associated with coupling to phosphatidylinositol 3-kinase activating extracellular signal-regulated kinase (ERK)1 and ERK 2. EP3 inhibits adenylyl cyclase and thus decreases cAMP levels. We have shown that by inhibiting $PGE_2$ production with COX inhibitors or blocking signaling using EP4 receptor antagonists or expressing EP4 siRNA in tumor cells, that mammary tumor cell metastasis to the lung can be reduced [5-7]. This therapeutic mechanism is controlled by NK cells [6]. These studies point to a critical role for EP4 expressed on the tumor cell, but the function of EP4 on the NK cell had not been examined. Since previous work has shown that tumor derived $PGE_2$ suppresses NK activity and NK cells are necessary for therapeutic control of metastasis by $PGE_2$ inhibitors and EP4 antagonists, we wanted to understand which EP receptors participate in regulation of NK cell function during tumor progression. We also investigated the ability of a recently described novel EP4 antagonist to protect NK cells from $PGE_2$-mediated immune suppression.

## Materials and methods

### Cell lines and mice

The Yac-1 (American Type Culture Collection, Manassas, VA, USA) murine T lymphoma cell line that is sensitive to NK mediated cytolysis was used as a tumor-target cell in cytotoxicity assays. Yac-1 cells were cultured at 37°C in 5% $CO_2$ in RPMI 1640 plus media that contained 10mM HEPES, 1mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate and supplemented with 10% FBS. Lines 410.4, 66.1, and 67 were derived from a single, spontaneously occurring mammary tumor in a Balb/cfC3H mouse. Lines 410.4 and 66.1 are highly tumorigenic and metastatic in syngeneic mice; line 67 is poorly tumorigenic and nonmetastatic. Mammary tumor cell lines are maintained in DMEM supplemented with 2mM sodium bicarbonate, 1mM penicillin/streptomycin, 1mM L-glutamine, 1mM minimal essential amino acids, and 10% FBS.

### In vivo studies

For spontaneous metastases assays, $3\times10^5$ of line 410.4 or 66.1 tumor cells were injected subcutaneously proximal to the mammary gland of syngeneic Balb/cByJ female mice (Jackson Laboratory, Bar Harbor, ME, USA). When tumors became palpable, tumor growth

was monitored by caliper measurement of two perpendicular tumor diameters. Preliminary time course studies revealed that NK-EP receptor expression was reduced at day 28 or later, therefore NK functions were examined at day 30-35 (average tumor diameter of 15mm). Mice were euthanized, the spleen removed and immediately placed in phosphate buffered saline (PBS) for spleen cell isolation. Experiments were performed according to IACUC approved protocols.

For experimental metastasis assays, $1\times10^5$ tumor cells were injected into the lateral tail vein of syngeneic immune competent Balb/cByJ or NK-depleted Balb/cByJ female mice and 21 days later, mice were euthanized and surface pulmonary metastases were quantified under a dissecting microscope. To deplete NK cells, mice were injected with rabbit asialoGM1 ganglioside antibody (20μL, Wako Bioproducts, Richmond, VA, USA) or normal rabbit serum 1 day prior to and 3 days after tumor cell injection.

### 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, USA). The entire effluent fraction collected represented the enriched NK cells identified as $DX5^+CD3^-$ cells.

### Flow Cytometry

For EP receptor studies, enriched NK cells were reacted with monoclonal mouse DX5 (CD49b) and CD3ε (BD PharMingen, San Diego, CA, USA) 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, USA) followed by fluorochrome conjugated secondary goat anti-rabbit serum and fixation in 4% paraformaldehyde for 15 minutes with final preparation in PBS. Data expressed as percent of cells positive for the marker minus percent positive in cells treated with IgG as control. 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, USA), 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 (Ann Arbor, MI, USA). 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, GW627368X (GWX), or Frondoside A (FA). To stimulate the receptors, $PGE_2$ (EP1-4), Sulprostone (EP1/EP3), Butaprost (EP2), and Prostaglandin $E_1$ Alcohol ($PGE_1$-OH, EP4) were used as agonists.

### cAMP EIA

NK cells were untreated, treated with vehicle control (DMSO), $PGE_2$ (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 Enzymeimmunoassay System (GE Healthcare, Piscataway, NJ, USA) and intracellular cAMP levels were determined according to manufacturer's instructions.

### Migration Assay

The Chemo Tx® System (Neuro Probe, Gaithersburg, MD, USA) with a 3μm pore size was used to estimate migration. NK cells, at time 0, were treated with or without EP receptor agonists ($PGE_2$, Butaprost, Sulprostone, and $PGE_1$-OH) and the chemotactic response to FBS was assessed. Agonist-treated NK cells were pipetted onto the filter top and incubated at 37°C, 5% $CO_2$ 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, USA), 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 $PGE_2$ concentrations (1.0μM and 10μM), Butaprost (1.0μM and 10μM), Sulprostone (1.0μM and 10μM), or $PGE_1$-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 ($PGE_2$, Butaprost, Sulprostone, $PGE_1$-OH) for 30 minutes. At the end of 30 minutes, cells were stimulated with 1000U/mL of IL2 and incubated at 37°C, 5% $CO_2$. 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, USA) were used to measure cytokines in cell conditioned 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 means of control (DMSO) and experimental groups. Overall test analysis was reported as significant at p-value < 0.05.

## Results

### Natural Killer Cells from Tumor-bearing Mice Have Enhanced $PGE_2$ Production

It is well known that the tumor milieu utilizes many mechanisms to escape NK cell mediated surveillance. One such mechanism is the immunosuppressive action of $PGE_2$. We have previously shown that breast tumor cell lines produce $PGE_2$ and increasing $PGE_2$ correlates with metastatic potential as well as tumor development [3]. To confirm the stability of this property, $PGE_2$ levels were measured in conditioned medium from three mammary tumor cell lines 66.1, 410.4, and 67, as well as the highly NK sensitive lymphoma line, Yac-1. $PGE_2$ levels in the metastatic breast tumor lines 66.1 and 410.4 were 80 and 12 fold greater, respectively, than in conditioned medium from the non-metastatic line 67 (Figure 1a). The mouse lymphoma cell line Yac-1 produced very little $PGE_2$; on a per cell basis, Yac-1 cells produced 107 pg $PGE_2$/mL/$10^6$ cells versus line 67 cells that produced 255 pg $PGE_2$/mL/$10^6$ cells and metastatic cell lines that produced more than 3,000 pg $PGE_2$/mL/$10^6$ cells.

T cells and macrophages produce $PGE_2$, but it has not been reported that NK cells produce $PGE_2$ [8]. Spontaneous release of $PGE_2$ into medium was measured from NK cells isolated from normal mice and treated with vehicle control or with the inhibitor of $PGE_2$ production, indomethacin. Figure 1b shows that an enriched NK cell population produces measurable amounts of $PGE_2$. Normal NK cells produce more $PGE_2$ on a per cell basis, in fact, than non-metastatic cell line 67 and lymphoma cell line Yac-1 but significantly less than metastatic breast cancer cell lines. This $PGE_2$ production was partially inhibited by treatment with indomethacin ($p<0.05$). We also examined $PGE_2$ production from NK cells isolated from tumor bearing mice. Tumor cells were implanted proximal to the abdominal mammary gland in mice and on day 30, splenic NK cells were isolated. NK cells from tumor bearing mice (T-NK) produced 1.5 fold more $PGE_2$ than normal NK cells (N-NK1170 pg $PGE_2$/mL/$1\times10^6$ cells compared to 785 pg $PGE_2$/mL/$1\times10^6$ cells); which could be inhibited by indomethacin (Figure 1c).

### NK Cell EP Receptor Expression is Suppressed in the Tumor Environment

Endogenous NK cells from normal mice express all four EP receptors [9]. To begin to understand the mechanisms by which tumor-derived $PGE_2$ acts on NK cells, we characterized the expression of EP receptors on NK cells from tumor bearing mice and compared them to endogenous NK cells from normal mice. 410.4 or 66.1 tumor cells were injected proximal to the abdominal mammary gland of Balb/cByJ female mice. At 30 days post-injection or when tumors reached 15mm in diameter, the mice were euthanized, lung weights and lung tumor colonies were quantified and splenic NK cells were isolated for further characterization. Mice injected with tumor cells had a 1.3 fold increase in lung weight compared to control mice reflecting the tumor burden in the lungs. Spleens from mice bearing 410.4 tumors were markedly heavier (average weight 0.7088g ± 0.0643) than spleens from normal mice (average weight 0.1039g ± 0.0049); spleens from 66.1 tumor-bearing mice weighed on average 0.1785g ± 0.0093, but were not statistically significantly different than normal spleens. NK cells isolated from spleens from tumor-bearing mice are henceforth referred to as T-NK. We examined the expression of EP on the surface of T-NK by flow cytometry and compared EP expression levels to those on N-NK cells [9]. N-NK cells express all EP receptors (Figure 2a). EP1 is detected on thirty-four percent of N-NK. Thirty-four percent, 20%, and 13% of N-NK are positive for EP2, EP3, and EP4, respectively, confirming our previous report which examined EP function on N-NK cells only [9]. Expression of each EP is reduced on NK cells from mice bearing 410.4 or 66.1 tumors in comparison to N-NK as indicated by the lower percentage of receptor positive cells in T-NK vs. N-NK. Like EP protein levels, EP expression at the mRNA levels is also markedly reduced in T-NK compared to N-NK and to a greater degree compared to protein

expression (Figure 2b) indicating that the EP protein probably has a longer half-life than the mRNA.

### $PGE_2$ Induces Intracellular cAMP Response in NK Cells

EP2 and EP4 receptors are G-protein coupled receptors that upon ligand-mediated activation elevate cAMP levels. We compared the effects of $PGE_2$ on cAMP activity in T-NK to N-NK populations. Figure 3a and 3b confirms our previous findings that $PGE_2$ (1.0 μM, 10.0μM) significantly increased cAMP levels in N-NK cells ranging from 6.7 to 10.6 fold. $PGE_2$ (1.0 μM, 10.0μM) also induced an intracellular cAMP response in T-NK cells obtained from either 410.4 (Figure 3a) or 66.1 (Figure 3b) tumor bearing mice but only by 1.4 to 2 fold. Thus, the ability of $PGE_2$ to induce cAMP in NK cells is significantly blunted in tumor bearing mice.

### IFNγ Secretion is Suppressed in NK Cells through EP2 and EP4 Receptors

NK cells secrete IFNγ to enhance activities of NK and other effector cells. The effect of $PGE_2$ on IFNγ secretion from N-NK and T-NK cells was examined. NK cells from normal and tumor bearing mice were pretreated with $PGE_2$ and then stimulated with 1000U/mL of IL2 to induce cytokine production. T-NK cytokine secretion is reduced compared to N-NK (Figure 4a). N-NK secreted 203 pg/mL IFNγ after IL-2 stimulation whereas only 76 pg/mL IFNγ was secreted from T-NK. Pretreatment with $PGE_2$ reduced the amount of IFNγ produced by N-NK cells (Figure 4b) and eliminated IFNγ production by T-NK cells (Figure 4c). To determine which EP receptor was involved in inhibition, NK cells were targeted with specific EP receptor agonists. The EP4 agonist ($PGE_1$-OH) and EP2 agonist (Butaprost) significantly inhibited IFNγ production by N-NK (Figure 4b, 86 and 66 percent inhibition respectively, $p<0.001$), but the EP1/EP3 agonist Sulprostone only decreased IFNγ by 19 percent; this latter change was not statistically significant. IFNγ secretion in T-NK cells was significantly repressed with addition of 10μM of either an EP2 or EP4 agonist by 99 and 100 percent, respectively (Figure 4c, $p<0.001$). The EP1/EP3 agonist inhibited IFNγ secretion by 14 percent (p = n.s.). Thus in both N-NK and T-NK cells, $PGE_2$ inhibits IFNγ production mainly through EP2 and EP4, but not through the EP1/EP3 receptors.

### $PGE_2$ Suppresses TNFα in N-NK but not T-NK cells

Although TNFα was first identified as an anti-tumor cytokine that induced NK cells, emerging evidence suggests that TNFα acts as a tumor-promoting factor and is linked to migration and invasion of tumor cells [10]. We examined the production of TNFα from T-NK cells. Unstimulated T-NK cells produced TNFα (21.68 ± 2.73 pg/mL/$1\times10^6$cells), which is in contrast to N-NK cells in which no TNFα was detected in the absence of IL2 (Figure 4d). In the presence of IL2, T-NK cells produced higher levels of TNFα than N-NK. We then examined the role of $PGE_2$ and the EP receptors in regulating TNFα secretion by NK cells. $PGE_2$ suppressed TNFα by N-NK (Figure 4e). In contrast, T-NK cells when treated with 1.0μM and 10.0μM $PGE_2$ modestly increased TNFα secretion by 1.4 fold (Figure 4f). Like $PGE_2$, the EP2 agonist Butaprost, EP1/EP3 agonist Sulprostone, and EP4 agonist $PGE_1$-OH were able to inhibit secretion of TNFα by N-NK by 86%, 72%, and 58%, respectively (Figure 4e). In contrast, Butaprost and $PGE_1$-OH modestly increased TNFα secretion by T-NK (Figure 4f). Sulprostone decreased TNFα secretion by 30 percent in T-NK cells. Thus, T-NK cells and N-NK cells are very different concerning cytokine production and in response to $PGE_2$. N-NK cells are superior in terms of IFNγ production whereas T-NK cells produce higher levels of TNFα. Furthermore, $PGE_2$ inhibits TNFα production by N-NK but this cytokine is induced by $PGE_2$ in T-NK.

### NK Cell Migration is Inhibited by $PGE_2$

The ability of NK cells to migrate to tumor targets is important in tumor control. We compared the migratory ability of T-NK to N-NK and assessed the inhibitory effects of $PGE_2$ on migration. $PGE_2$ inhibited migration of N-NK cells by 48 or 70 percent (Figure 5a). T-NK cells migrate poorly in comparison to N-NK even in the absence of exogenous $PGE_2$. T-NK from mice bearing tumor 410.4 could nevertheless be further inhibited by exogenous $PGE_2$ (Figure 5a). To further delineate which EP receptor is involved in $PGE_2$-mediated inhibition of NK cell migration, N-NK and T-NK cells were treated with EP receptor specific agonists and migration was assessed. Like $PGE_2$, the EP4 agonist $PGE_1$-OH significantly blocked N-NK or T-NK cell migration (Figure 5b). The EP2 agonist Butaprost did not significantly impede migration of N-NK, but did inhibit T-NK (Figure 5c); in contrast, the EP1/EP3 agonist Sulprostone modestly increased migration of N-NK cells, but inhibited T-NK cells (Figure 5d). The EP4 agonist $PGE_1$-OH reduced T-NK cell migration by 81-83 percent; the EP2 receptor agonist Butaprost achieved 68-77 percent inhibition and migration was reduced by 46-53 percent by the EP1/EP3 agonist Sulprostone. Like T-NK, the migration of N-NK was also significantly inhibited by EP4 activation, but unlike T-NK, N-NK migration was not inhibited by an EP2 agonist. EP1 and EP3 activation actually promotes migration of N-NK. In contrast, T-NK are inhibited by activation of either EP2 or EP4 and modestly by EP1/EP3 agonists. Thus in N-NK, EP4 is dominant in suppressing migration whereas the broader inhibitory response in T-NK is mediated through all EP receptors.

### NK Cell Cytotoxic Activity is Compromised in the Tumor Environment

Although NK cells have the capacity to infiltrate and kill tumor targets, cytotoxic activity is compromised in the tumor environment. We showed that $PGE_2$ is a major inhibitory factor produced by tumor cells. To determine the effects of the tumor on NK cytolytic actions, NK cell-mediated cytotoxicity from N-NK and T-NK cells was compared. T-NK cells lose the ability to lyse tumor targets compared to N-NK cells (Figure 6). In figure 6, lytic activity was compared at three effector to target ratios. In the absence of exogenous $PGE_2$, T-NK from 410.4-bearing mice were significantly less lytic than N-NK (Figure 6a). Exogenous $PGE_2$ modestly inhibited the cytolytic activity of N-NK cells, T-NK cell lytic activity was at baseline and no further suppression by $PGE_2$ could be observed (Figure 6a). The EP4, EP2, and EP1/EP3 agonists mimicked the ability of $PGE_2$ to inhibit lytic actions of N-NK cells (Figure 6b,c,d), but the minimal activity of T-NK cells was not further suppressed by $PGE_2$ or receptor agonists.

### Novel EP4 Antagonist Frondoside A Inhibits Metastasis by an NK-dependent Mechanism

We have shown here that $PGE_2$ exerts inhibitory effects most profoundly through the EP4 receptor in both N-NK and T-NK. This and our previous studies suggest that blocking EP4 activities inhibits metastasis and protects NK cells from $PGE_2$-mediated immune suppression. There is a paucity of EP4 antagonists that can be used clinically. We recently described a novel EP4 antagonist, Frondoside A (FA) that has potent anti-metastatic activity [11]. We compared the ability of FA to control metastatic disease in immune competent Balb/cByJ mice and mice depleted of NK activity. Injection of vehicle-treated tumor cells into competent mice resulted in an average of 50.9 tumor colonies; FA pretreatment of tumor cells before injection reduced the mean number of metastases by 30 percent which was marginally significant ($p=0.064$, Figure 7a). Depletion of NK cells leads to loss of endogenous control of tumor dissemination leading to a 4.25 fold increase in lung metastasis, and in these mice, FA no longer inhibited metastasis. Thus, the ability of an EP antagonist to inhibit metastasis is by an NK-dependent mechanism.

### Frondoside A Reverses PGE2-Mediated Cytokine and Migration Inhibition

To determine how the EP4 antagonist FA and NK cells interact, we determined the effect of FA on NK cell cytokine secretion and migration. N-NK cells were pre-treated with the EP4 antagonist FA (0.1μM or 0.5μM) and EP agonists $PGE_2$ or $PGE_1$-OH. After pre-treatment, N-NK cells were stimulated with 1000U/mL of IL2 to induce cytokine production. As expected, $PGE_2$ and the EP4 agonist $PGE_1$-OH suppressed the secretion of IFNγ from N-NK cells, by 95 and 100 percent respectively (Figure 7b). FA restored the capacity of N-NK cells to secrete IFNγ in the presence of $PGE_2$ or $PGE_1$-OH. We also determined the effect of FA on N-NK cell migration. N-NK cells were treated with FA 0.1μM or 0.5μM with and without the EP4 agonist $PGE_1$-OH and the degree of N-NK cell migration was determined 3h later. $PGE_1$-OH alone markedly inhibited NK cell migration (Figure 7c). FA is able to reverse this $PGE_1$-OH-mediated inhibition.

## Discussion

We examined the effects of the tumor-milieu and specifically $PGE_2$ on NK cell function and identified which EP receptors regulated NK cell functions. T-NK cells have reduced EP1-4 expression compared to N-NK cells. Elkashab and Lala [12] examined whether the presence of tumor caused changes in the affinity of $PGE_2$ binding sites (receptors) on splenic lymphocytes. They completed their study before it was known that multiple EP receptors exist. Their findings revealed that there was no difference in $^3$H-$PGE_2$ binding affinity between normal and tumor bearing lymphocytes, but did reveal possible evidence of lower maximum $^3$H-$PGE_2$ binding in lymphocytes from tumor bearing hosts. Although Elkashab's initial hypothesis was that the findings could be explained by a reduction in the number of $PGE_2$ binding sites on splenic lymphocytes, this theory was ruled out by Scatchard analysis. They concluded that the reduced $^3$H-$PGE_2$ binding was due to partial occupation of receptor sites by tumor-produced $PGE_2$. Our results agree with that study in that we detected less EP receptor expression in T-NK compared to N-NK cells. We expanded these findings to show that expression of each individual EP receptor was reduced at the protein level. G protein coupled receptors are regulated by ligand-induced internalization and degradation. The presence of excess agonist on G-protein coupled receptors is known to cause the apparent decline in receptor number [13]. Gray and Roth report that prolonged exposure to ligand causes GPCRs to be internalized and bound for sorting to be either recycled or down-regulated. The loss of EP receptor expression in T-NK may be due, in part, to chronic exposure to excess tumor derived $PGE_2$ resulting in the internalization of the EP receptors and subsequent degradation. In addition, we also showed that EP mRNA is profoundly reduced in T-NK indicating that the reduced surface expression is not only due to receptor occupancy, turnover, or degradation. Our data suggest that negative regulation of EP transcription and translation also contribute to the loss of EP expression on NK cells from tumor bearing mice.

The reduced EP2 and EP4 receptor expression we observed was accompanied by a dampened cAMP response to EP2 and EP4 activation. $PGE_2$ stimulated cAMP in N-NK cells in a dose dependent manner but this response was blunted in T-NK cells. Since our studies also show that $PGE_2$ is inhibitory to many NK functions, we hypothesized that T-NK down regulate EP in response to either chronic ligand stimulation and/or as a mechanism to escape immune suppression. In spite of EP downregulation however, we show that T-NK cells are still sensitive to further immunosuppressive actions of exogenous $PGE_2$.

It is evident that the tumor releases factors that inhibit NK cell migratory ability. NK cells from tumor bearing mice have partially lost the innate ability to migrate, but this reduced response is still sensitive to further inhibition by $PGE_2$. The treatment of T-NK cells with EP receptor agonists to each receptor revealed that NK cell migration was negatively regulated

through all EP receptors. The EP4 and EP2 receptors appear to inhibit migration of both N-NK and T-NK, but the role of EP1/EP3 appears to diverge in the two populations. In N-NK cells the EP1/EP3 agonist induced migration, but the same agonists inhibited migration of T-NK cells. This differential response may be related to different receptor expression levels in T-NK and N-NK cells.

Although it is evident that NK cells have the potential to recognize and eliminate tumor targets, an imbalance of signals transmitted by inhibitory and activating receptors must be in place before NK cell mediated killing ensues. Tumor-derived $PGE_2$ represents a major barrier to the success of NK cell mediated killing. T-NK cells were 83 percent less cytolytic than N-NK cells at a 10:1 effector to target ratio. $PGE_2$ and the EP2 receptor agonist, Butaprost, inhibited N-NK mediated cytotoxicity, but the further inhibition of cytotoxicity exhibited by T-NK was difficult to detect due to the profound loss of intrinsic killing. In human NK cells from normal donors, $PGE_2$ prevents NK cell activating receptors (NKG2D, NCR, CD16) from inducing NK cell mediated cytotoxicity and this mechanism is through EP2 and EP4 receptors [14]. While our study did not investigate the actions of $PGE_2$ on activated (LAK) cells, it is possible that tumor-derived $PGE_2$ and exogenous $PGE_2$ would decrease activating receptors and increase inhibitory receptors preventing the switch of NK cells from a resting to an activated state. Other investigators have studied the effects of $PGE_2$ on NK cells in the activated state. Su et al [15] showed that LAK cell (activated NK cells generated from adherent splenocytes cultured in IL2) cytotoxic activities were inhibited by $PGE_2$ through the EP2 receptor. This was similar to our findings in endogenous NK cells in that we implicate the EP2 receptor, but we also demonstrate EP4 receptor involvement.

In addition to diminished capacity to migrate and to lyse tumor target cells, the intrinsic ability of T-NK cells to secrete IFNγ was compromised and was further inhibited by the addition of exogenous $PGE_2$. In both N-NK and T-NK, IFNγ secretion is negatively regulated through EP2 and EP4. Likewise, EP2 and EP4 activation inhibited TNFα production by N-NK. In contrast, $PGE_2$ induced TNFα secretion from T-NK cells. Consistent with this data, Zeidler et al [16] found that TNFα was induced in co-cultured monocytes and tumor cells (shown to produce $PGE_2$). In data reported here, $PGE_2$, Butaprost (EP2), and $PGE_1$-OH (EP4) increased TNFα secretion by T-NK cells. Thus, in the tumor context regulation of TNFα by $PGE_2$ is very different than in N-NK.

In summary, we have shown T-NK cells have reduced EP receptor expression compared to N-NK cells. Although there is loss of EP expression, the ability to induce adenylyl cyclase activation, while diminished, indicates that EP2 and EP4 are still functionally coupled to adenylyl cyclase in T-NK. Unlike N-NK cells where $PGE_2$ predominately regulated inhibitory functions through the EP4 receptor, $PGE_2$ inhibited T-NK cells through both EP2 and EP4 receptors. Whether this indicates a broadening of the response to include EP2 or whether EP receptors are more sensitive to activation on T-NK remains to be determined. Unlike EP2 and EP4, EP1 and EP3 are coupled to different signaling pathways. This may explain why the later two receptors do not mediate the suppressive effects of $PGE_2$ on NK cells. Taken together, this shows that reduced EP receptor expression during tumorigenesis is not enough to overcome the inhibitory effects of $PGE_2$ and further emphasizes the need to develop antagonists of EP4 and possibly EP2 to prevent $PGE_2$-mediated immune suppression. Our previous work as well as the current study show that EP4 antagonists are dependent on NK cells to inhibit metastasis as well as to enhance NK lytic activity [5-7]. Direct antagonism of EP4 on the malignant cell inhibits metastasis. The current study, showing that T-NK functions are suppressed through EP4 emphasizes the rationale for identifying effective EP4 antagonists that can both protect NK cells and directly reduce tumor metastasis. These studies indicate that Frondoside A may be one potential EP4 antagonist that should be investigated further.

## Acknowledgments

Studies were supported by the National Institutes of Health (U.S.) (AMF), Department of Defense (U.S.) (AMF, DMH), the U.S. Department of Veterans Affairs (AMF) and the Maine Technology Institute, Gardiner, ME (PDC).

**Disclosure of Funding** National Institutes of Health (NIH), Department of Defense, U.S. Department of Veterans Affairs

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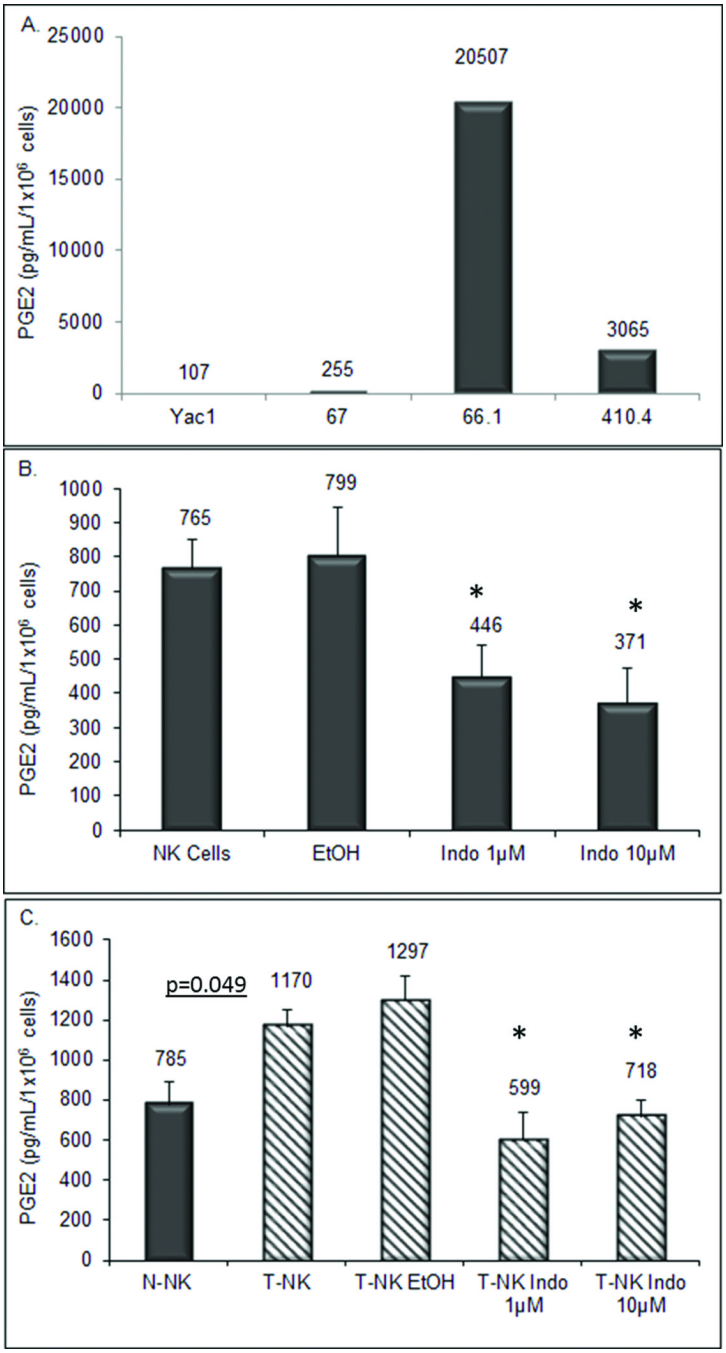


**Fig. 1.**

(a) $PGE_2$ was measured in conditioned media from cultured murine mammary tumor cell lines 66.1, 410.4, 67, and the murine lymphoma line Yac-1. (b) Conditioned media was collected from NK cells, NK cells treated with vehicle control (EtOH) and COX-inhibitor (indomethacin) for 18 hours and $PGE_2$ levels determined by ELISA assay. (c) Conditioned media was isolated from NK cells and T-NK cells, T-NK cells treated with vehicle control (EtOH) and COX-inhibitor (indomethacin). Results are from triplicate wells. Data reported as mean pg $PGE_2$/mL/1×$10^6$ cells ± standard error.

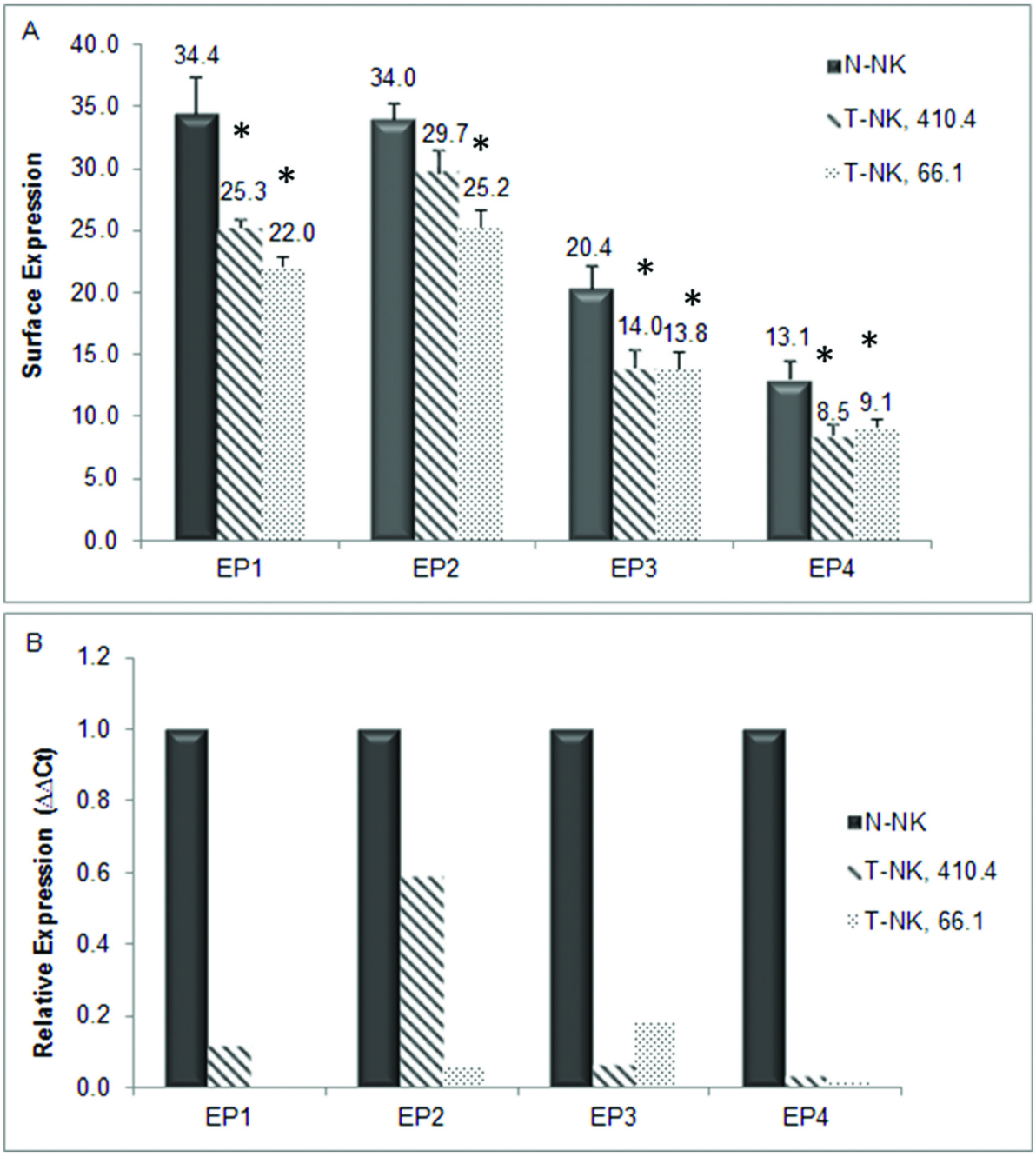


**Fig. 2.**

N-NK cells and T-NK cells from 410.4 and 66.1 bearing mice were assessed by (a) Flow cytometry analysis and (b) quantitative PCR for EP receptor expression. Relative to N-NK, T-NK EP receptor expression *p<0.05.

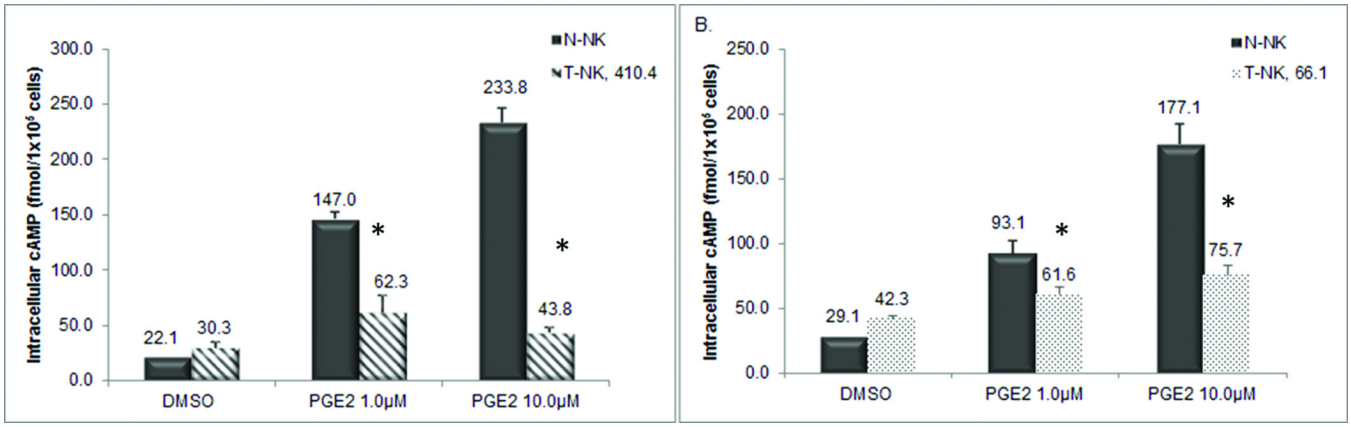


**Fig. 3.**

N-NK cAMP was compared to (a) T-NK 410.4 and (b) T-NK 66.1. Cells were stimulated in the presence of $PGE_2$ for 30 minutes. Intracellular cAMP was assessed from cell lysates. Data represented as mean ± standard error fmol cAMP/$1\times10^5$ cells in triplicate wells. Relative to N-NK, T-NK with $PGE_2$ treatment *$p<0.05$.

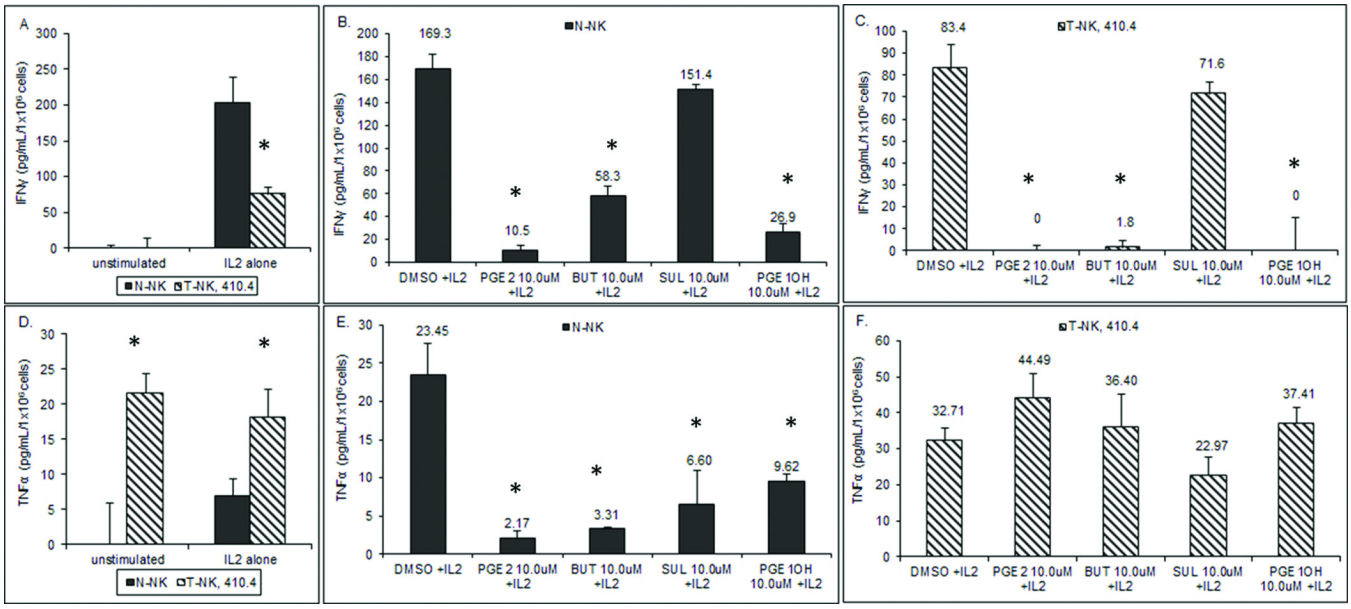


**Fig. 4.**

(a,d) N-NK and T-NK were analyzed for IFNγ or TNFα in the presence or absence of IL2 stimulation. (b,e) N-NK cells and (c,f) T-NK cells were pretreated with 10μM of $PGE_2$, $PGE_1$-OH, Butaprost (BUT), or Sulprostone (SUL). Cell culture supernatants were assayed for IFNγ or TNFα. Results are reported as mean pg/mL/$1\times10^6$ cells ± standard error, n=3. Relative to DMSO, treatment *$p<0.05$.

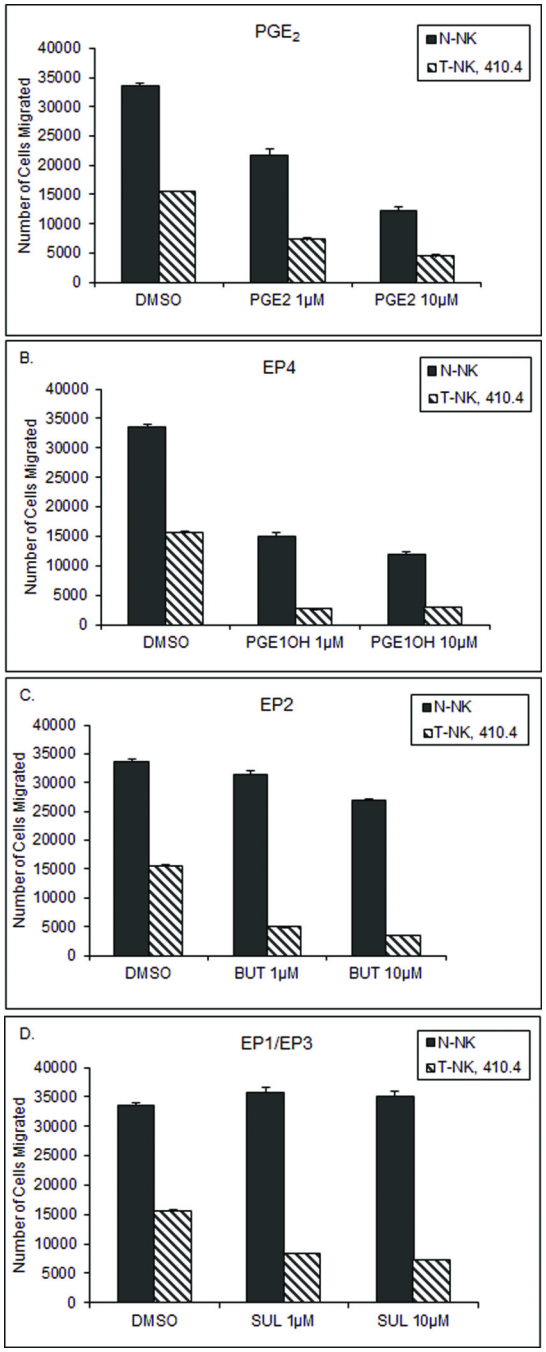


**Fig. 5.**

N-NK or T-NK cells were pretreated with 1μM or 10μM of (a) $PGE_2$, (b) $PGE_1$-OH, (c) Butaprost (BUT), or (d) Sulprostone (SUL) and migration capacity was determined. The number of cells that crossed a 3μm pore membrane was counted based on fluorescence. Data reported as mean number of cells migrated ± standard error, n=3. Relative to DMSO, treatment $p<0.05$ and N-NK relative to T-NK, $p<0.05$.

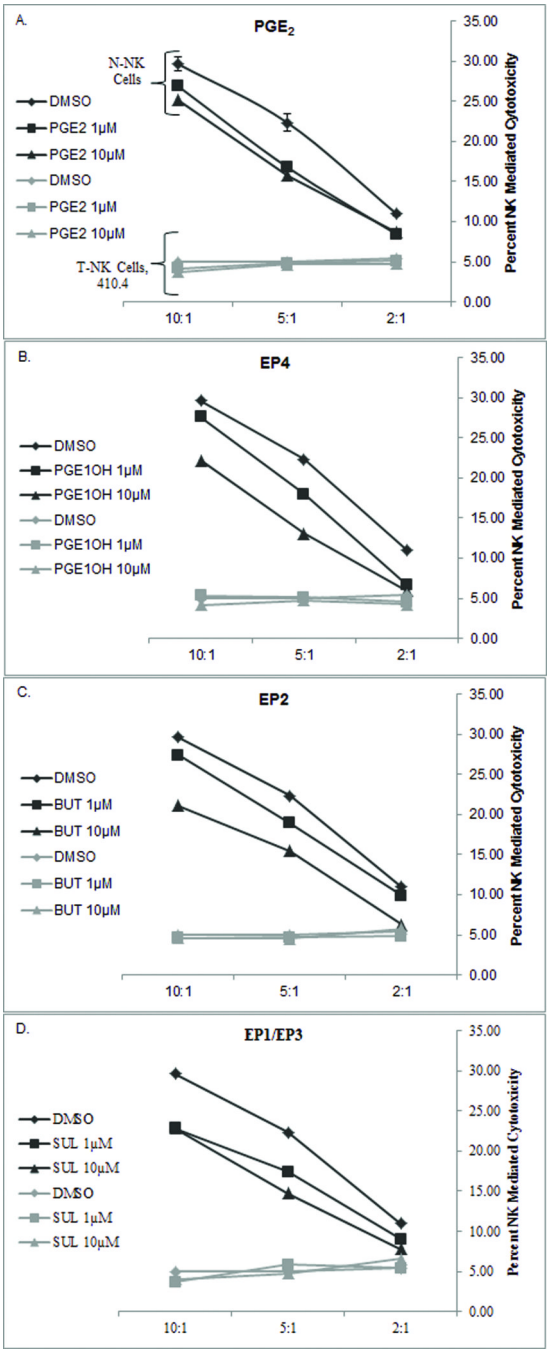


**Fig. 6.**

N-NK cells or T-NK cells were assessed for the ability to lyse Yac-1 cell targets. N-NK and T-NK cells were pre-incubated with either $PGE_2$, $PGE_1$-OH (EP4), Butaprost (EP2), or Sulprostone (EP1/EP3). Three effector to target ratios were used: 2:1, 5:1, and 10:1. Data presented as percent NK mediated cytotoxicity ± standard error, n = 3. Key: Black = N-NK, Gray = T-NK

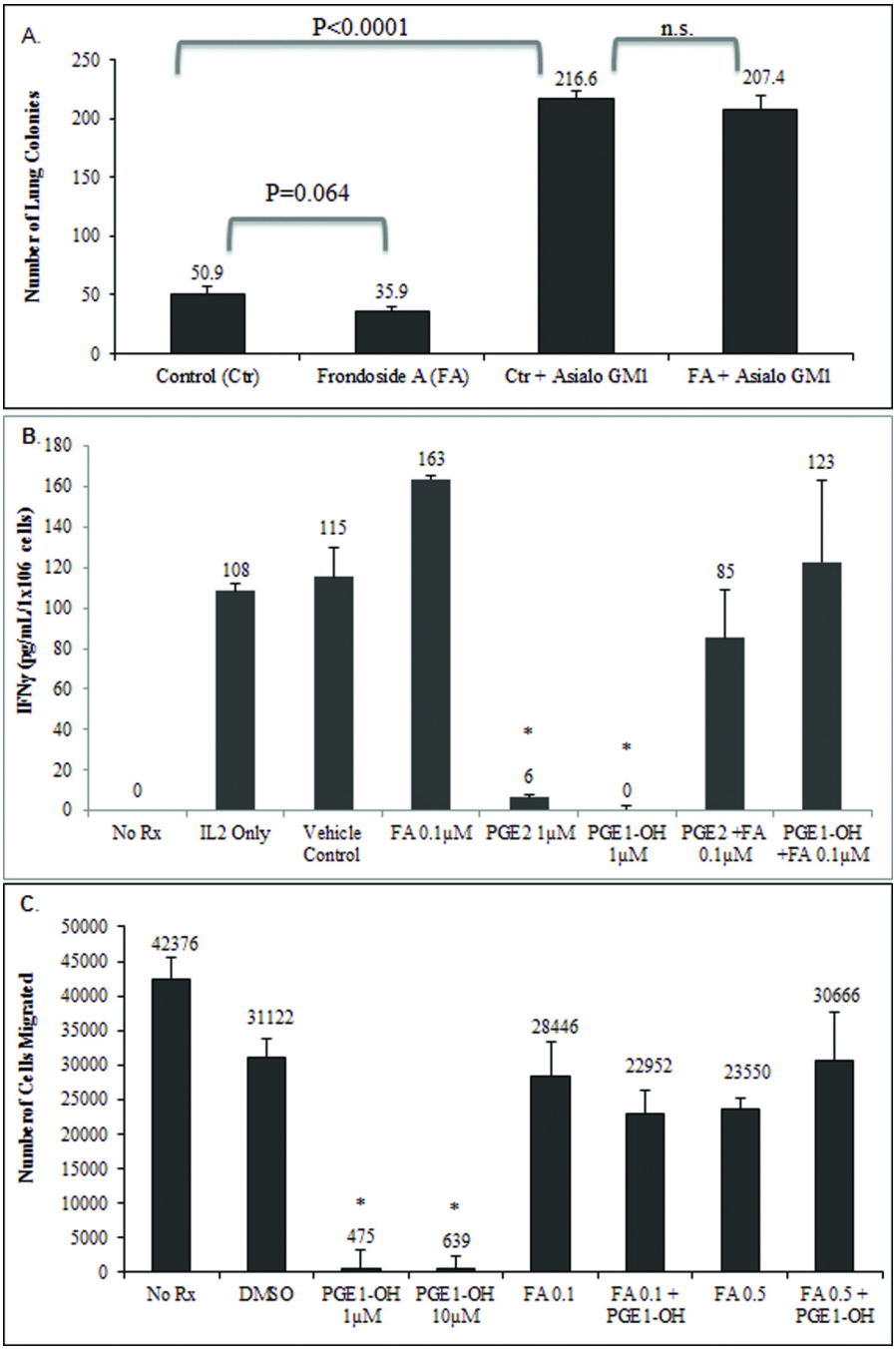


**Fig. 7.**

(a) Line 66.1 tumor cells were treated with FA (1μM) or PBS control for 24h, washed and $1\times10^5$ viable cells injected intravenously into Balb/cByJ mice or Balb/cByJ mice depleted of NK cells with asialoGM1 antibody. Twenty-one days later, mice were euthanized and surface lung tumor colonies were enumerated. Mean ± SE of lung metastases in 10 mice/group. (b) N-NK cells were pretreated with $PGE_2$, $PGE_1$-OH, and FA (0.1μM) with and without EP receptor agonists and cell culture supernatants were assayed for IFNγ. Results are reported as mean pg/mL/$1\times10^6$ cells ± standard error. (c) N-NK cells were pretreated with FA (0.1μM and 0.5μM) in the presence and absence of $PGE_2$ and $PGE_1$-OH and migration capacity was determine by the number of cells that crossed a 3μm pore

membrane. Data reported as mean number of cells migrated ± standard error. Relative to DMSO, treatment *p<0.05.