# Inflammation restraining effects of prostaglandin E2 on natural killer–dendritic cell (NK-DC) interaction are imprinted during DC maturation

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**Among prostaglandins (PGs), PGE2 is abundantly expressed in various malignancies and is probably one of many factors promoting tumor growth by inhibiting tumor immune surveillance. In the current study, we report on a novel mechanism by which PGE2 inhibits in vitro natural killer–dendritic cell (NK-DC) crosstalk and thereby innate and adaptive immune responses via its effect on NK-DC crosstalk. The presence of PGE2 during IFN-γ/membrane fraction of *Klebsiella pneumoniae* DC maturation inhibits the production of chemokines (CCL5, CCL19, and CXCL10) and cytokines (IL-12 and IL-18), which is cAMP-dependent and imprinted during DC maturation. As a consequence, these DCs fail to attract NK cells and show a decreased capacity to trigger NK cell IFN-γ production, which in turn leads to reduced T-helper 1 polarization. In addition, the presence of PGE2 during DC maturation impairs DC-mediated augmentation of NK-cell cytotoxicity. Opposed to their inhibitory effects on peripheral blood–derived NK cells, PGE2 matured DCs induce IL-22 secretion of inflammation constraining NKp44$^+$ NK cells present in mucosa-associated lymphoid tissue. The inhibition of NK-DC interaction is a novel regulatory property of PGE2 that is of possible relevance in dampening immune responses in vivo. (*Blood*. 2011;118(9):2473-2482)**

## Introduction

Prostaglandins (PGs) are potent immune modulators that are produced during inflammation after the conversion of arachidonic acid by cyclooxygenase (COX).[1] Furthermore, PGs are also abundantly produced by various types of tumors.[2] COX2 expression, which is correlated with a poor prognosis, is induced in a variety of human premalignant and malignant tumors, including solid tumors as well as hematologic malignancies.[3-6] Several lines of evidence demonstrate that COX2-derived PGs are involved in the promotion of tumor growth by regulation of cancer cell proliferation, apoptosis, migration, and invasion.[7-11] PGs are also produced by tumor-surrounding cells, creating a tumor-supporting environment by enhancing angiogenesis and inhibiting tumor immune surveillance.[2,11-14]

Of all prostaglandins, PGE2 has a pivotal role in tumor immunosuppression. It has been hypothesized that this effect is caused by induction of a permanent state of inflammation,[2] resulting in phenotypic and functional changes of T-helper ($T_H$) cells, cytotoxic T-lymphocyte (CTL) cells, dendritic cells (DCs), natural killer (NK) cells, and myeloid-derived suppressor cells.[12] PGE2 has been shown to deviate $T_H$ cell skewing from an antitumor $T_H1$ response toward a $T_H2/T_H17$ response by direct binding to these cells.[15-17] In addition, PGE2 is responsible for shifting the balance of IL-12/IL-23 production by DCs toward IL-23, which is a very potent cytokine responsible for $T_H17$ expansion and survival.[18,19] As a consequence, less IL-12 and other proinflammatory cytokines are produced, thereby inhibiting $T_H1$ polarization.[17,20] PGE2 also decreases the cytotoxic capacity of CTLs directly by inducing the expression of inhibitory receptors on CTLs[21] and indirectly by inhibiting DC maturation and antigen presentation.[22,23] Moreover, tumor-associated PGE2 has been reported to be responsible for the preferential attraction and induction of regulatory T cells, creating an immune-regulatory microenvironment.[24,25]

Next to the modulating effects of PGE2 on the effector mechanisms of the adaptive immune response, PGE2 also has an effect on the innate immune response. NK cells, which are implicated in the innate defense against virally infected and malignantly transformed cells, have been reported to be affected by PGE2. It has been shown that PGE2 suppresses proliferation, cytokine secretion, migration, and NK cell–mediated cytotoxicity both in vivo and in vitro.[26-30]

Even though PGE2 has these direct effects on NK cells, it remains to be established whether PGE2 also has an effect on NK-DC crosstalk. We and others have reported previously on the importance of NK-DC crosstalk in modulating adaptive immune responses.[29,31,32] This crosstalk is characterized by DC-dependent recruitment of NK cells and IFN-γ production by NK cells, of which the latter contributes to strong $T_H1$ polarization.[32] In addition, DCs augment NK cell–mediated cytotoxicity of tumor cells. The DC-derived cytokines IL-12, IL-18, and IL-15 have been implicated in NK-cell activation and IL-15 is also responsible for proliferation and survival of NK cells.[32,33]

In the current study, we investigated how PGE2 influences NK-DC interactions. We identified the effects of PGE2 signaling on the production of key chemokines and cytokines responsible for NK-cell interaction with DCs matured with IFN-γ and a membrane

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fraction of *Klebsiella pneumoniae* (FMKp). We show that PGE2 not only has a direct effect on NK cells but also acts indirectly by altering DC-mediated NK-cell effector functions.

## Methods

### Cell isolation

Mononuclear cells from peripheral blood of healthy donors were isolated by density gradient separation using Lymphoprep (Axis-Shield). NK cells and naive $CD4^+$ T cells were negatively selected by immunomagnetic cell separation (Miltenyi Biotec). The purity of isolated populations exceeded 95% as determined by flow cytometry.

Human tonsils were obtained from the Department of Otorhinolaryngology-Head & Neck Surgery, Maastricht University Medical Center. NK cells were isolated to a purity of 70% to 80% as described by Cella et al.[34]

### Generation of DCs

DCs were prepared from peripheral blood–derived monocytes, isolated by density centrifugation and adherence as described previously.[35] In addition, highly purified monocyte fractions obtained by elutriation of leukapheresis products were used (purity, $> 95\%$). Written informed consent was obtained from all leukapheresis donors in accordance with the Declaration of Helsinki. All experiments were approved by the Maastritcht University Medical Center. For every experiment, DCs from both isolation methods were used to avoid the effect of contaminating NK cells ($< 5\%$ in density centrifugation-isolated monocytes) on the results. Differentiation of monocytes was induced during 6 days of culture in AIM V medium (Invitrogen) containing 2000 U/mL IL-4 (Strathmann Biotech) and 400 U/mL GM-CSF (Berlex Laboratories). DCs were matured for 6 hours in AIM V medium containing 500 U/mL IFN-γ (Strathmann Biotech), 1 μg/mL FMKp (Pierre Fabre), and different concentrations of PGE2 (Sigma-Aldrich). Where indicated, 10μM butaprost (E series of prostaglandin [EP]2 agonist), 35μM misoprostol (EP2/EP3/EP4 agonist), 10μM sulprostone (EP1/EP3 agonist; Cayman Chemical), or 100μM dibutyryl-cAMP (Alexis Biochemicals) was added. When selective EP-receptor agonists were compared, 10μM PGE2 was used.

### Flow cytometry

All antibodies for flow cytometry were obtained from BD Biosciences, except for CCR7, 2B4, CXCR3, CCR5, NKp80, NKp44, IL-22 (R&D Systems); NKp30 (BioLegend); and NKG2A, NKG2D, CD158A, CD158B, CD158D, and CD158E (Miltenyi Biotec). Cells were incubated with antibodies at proper dilutions for 30 minutes at room temperature. Analyses were performed on a FACSCanto II flow cytometer (BD Biosciences) and analyzed with BD CellQuest Pro 6.0 software (BD Biosciences), WinMDI 2.1 (J. Trotter, http://facs.scripps.edu/), or FlowJo software (TreeStar).

### Migration assay

Migration of NK cells was analyzed as described previously.[32] To analyze the effect of CCL5 reconstitution on NK-cell recruitment, supernatant of IFN-γ/FMKp DCs matured in the presence of PGE2 was supplemented with 50 ng/mL CCL5. Migration of DCs was analyzed as described previously.[35]

### Cytokine and chemokine secretion by DCs

Quantification of IL-18, IL-12, IL-23, CCL5, CXCL10, and CCL19 in 24-hour maturation supernatants was performed using ELISA (MBL International and R&D Systems) according to manufacturers' instructions.

### Cytokine production by NK cells

NK cells were either cultured in the presence of PGE2 and stimulated with 2.5 μg/mL phorbol 12-myristate 13-acetate (PMA) and 2 μg/mL ionomycin or with supernatant of DCs matured with different concentrations of PGE2. To analyze the effect of CCL5 on NK-cell recruitment, supernatant of IFN-γ/FMKp DCs matured in the presence of PGE2 were supplemented with 2 ng/mL IL-12, 100 pg/mL IL-18, or both. Quantification of IFN-γ production was performed after 16 hours of stimulation using an IFN-γ ELISA kit (R&D Systems).

To evaluate IL-22 production by NK cells obtained from tonsils, NK cells were incubated with DCs in 1:2 ratio. Flow cytometric analysis of IL-22–secreting NK cells was performed as described by Cella et al.[34]

### $T_H$ cell priming

In a 96-well plate, IFN-γ/FMKp–matured DCs ($4 \times 10^4$ cells/well) were coated with 1 ng/mL staphylococcal enterotoxin B (SEB; Sigma-Aldrich) for 1 hour. SEB-coated DCs were washed and placed in culture with $CD45RA^+/RO^-CD4^+$ T cells ($1 \times 10^5$cells/well). Every other day, stimulation medium was replaced with supernatant of NK-DC cultures. In these NK-DC cultures, IFN-γ/FMKp–matured DCs or IFN-γ/FMKp + 1000 ng/mL PGE2–matured DCs ($2 \times 10^5$ cells/well) were cultured with or without NK cells ($1 \times 10^5$ cells/well). At day 10, the expanded $T_H$ cells were washed, plated in 96-well plates ($10^5$ cells/well), and stimulated with PMA/ionomycin (BD Biosciences). After 4 hours, cells were harvested and surface and intracellular staining was performed as described previously.[32]

### NK cell cytotoxicity assay

For NK cell–mediated lysis of K562, unstimulated NK cells were cocultured with K562 cells for 4 hours in the presence of different concentrations of PGE2. For NK cell–mediated lysis of Raji, NK cells were preactivated for 18 hours by either supernatant of IFN-γ/FMKp DCs matured in the presence of different concentrations of PGE2, medium alone, or medium containing 1000 U/mL IL-2 (Proleukin; Chiron Benelux). Target cells were labeled with 3′-dioctadecyloxacarbocyanine according to the manufacturer's instructions (Sigma-Aldrich), and $2 \times 10^4$ target cells were incubated with preactivated NK cells at various effector:target ratios for 12 hours, each ratio in triplicate. Percentages of killed target cells ($PI^+DiO^+$) were determined by flow cytometry. Percentage-specific lysis was calculated as follows[36]: % total target cell death − % spontaneous target cell death ($PI^+$) × 100% and % vital cells (% $PI^-$ cells not incubated with effector cells).

### Statistics

Statistical significance of differences between experimental samples was determined using Student *t* test for paired samples, ANOVA, or Wilcoxon signed rank test. Significance was accepted at the $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***) levels. Data were analyzed using Prism Version 5.00 software (GraphPad Software).

## Results

### PGE2 directly inhibits NK-cell cytokine production and NK cell–mediated cytotoxicity

We investigated whether PGE2 affects the expression of inhibitory and activating receptors on the NK cell surface. To this end, freshly isolated NK cells were cultured for 24 hours in the presence or absence of PGE2. As shown in Figure 1A, only expression of NKG2D (NK cells, mean fluorescence intensity [MFI] = 60.3 ± 16 and NK cells + PGE2, mean MFI = 34.8 ± 9.7) and 2B4 (NK cells, mean MFI = 103 ± 4.5 and NK cells + PGE2, mean MFI = 70.8 ± 2.9) is significantly ($P < 0.05$) reduced in 6 different donors (Wilcoxon signed rank test) because of PGE2 treatment. The expression of the other activating and inhibitory NK cell surface markers, including specific killer immunoglobulin receptors, did not change. In addition, expression of the chemokine receptors CCR5, CXCR3, and CCR7 was evaluated, because these chemokine receptors are implicated in NK-cell migration.[29,31]

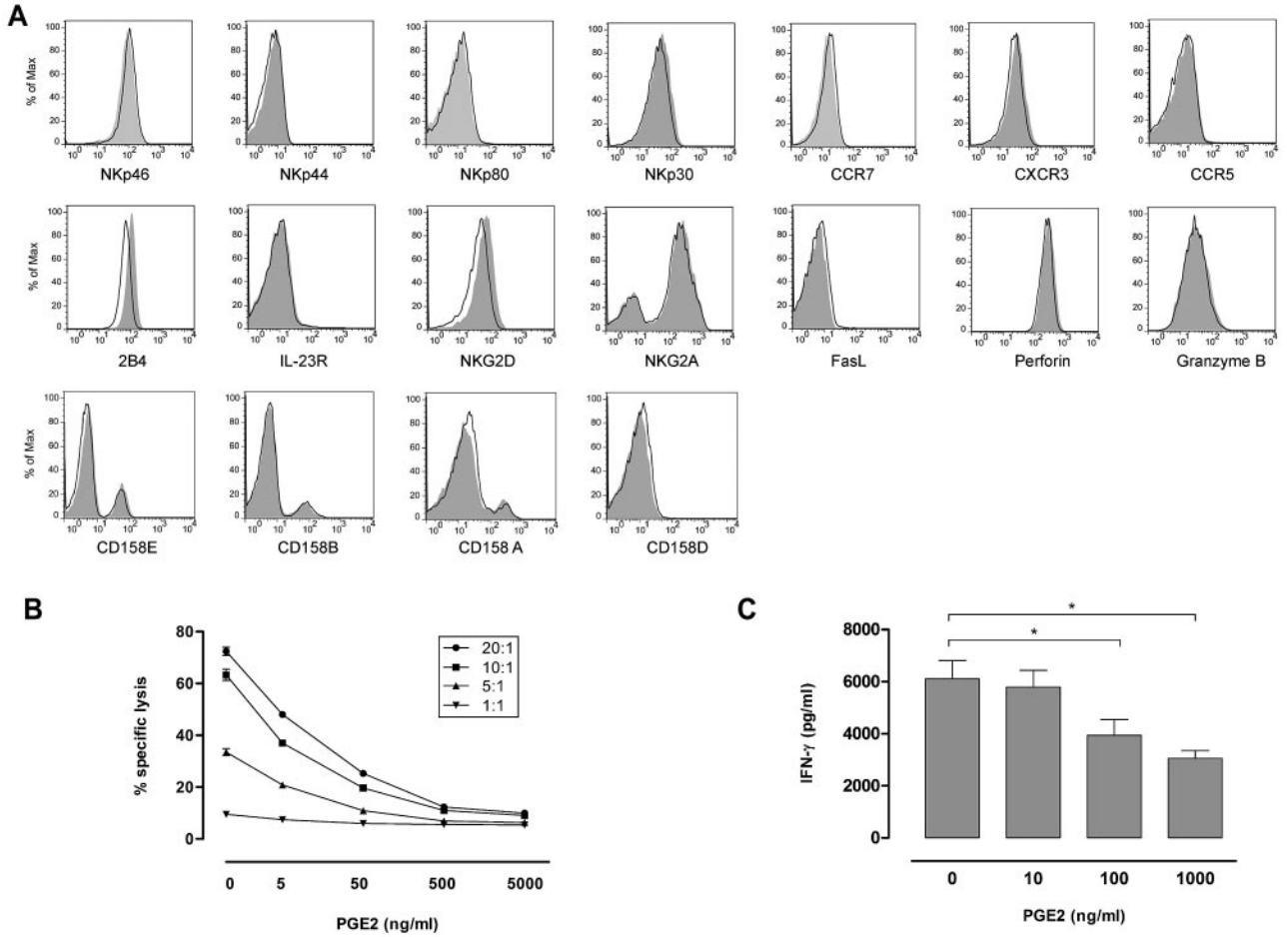


**Figure 1. Direct effect of PGE2 on NK-cell phenotype, cytotoxicity, and cytokine secretion.** Freshly isolated NK cells were cultured for 24 hours in the presence of PGE2. (A) Phenotypic analysis of NK surface receptor expression. Filled histograms, NK cells without PGE2; bold lines, NK cells with 1000 ng/mL PGE2. Representative data of 1 of 6 different donors. (B) Flow cytometry–based NK-cell cytotoxicity assay against K562 by NK cells cultured with different concentrations of PGE2. Data shown are triplicates of 1 representative experiment of 4 different experiments. (C) IFN-γ production by NK cells cultured with different concentrations of PGE2 and stimulated for 16 hours with PMA and ionomycin, as evaluated by ELISA. Results are presented as mean + SEM and are obtained from 3 different donors. $^*P < .05$.

PGE2, however, had no effect on the expression of these receptors. Because PGE2 has been shown to enhance IL-23 receptor expression by $T_H17$ cells,[15] we evaluated whether PGE2 had the same effect on NK cells. Flow cytometric analysis showed that IL-23 receptor expression on NK cells was not altered.

The effect of PGE2 on surface expression of the activating receptors 2B4 and NKG2D suggests that PGE2 has an inhibitory impact on NK-cell effector functions. To evaluate this impact, cytotoxicity assays were performed in either the presence or absence of PGE2. As target cells, K562 cells were used, because these cells fail to express human leukocyte antigen class I and are therefore efficiently killed by NK cells. A dose-dependent decrease in cytolytic activity was observed when PGE2 was present during the cytotoxicity assay (Figure 1B). Similar data have been reported previously.[37] To evaluate the effect of PGE2 on IFN-γ production, a second NK-cell effector function, NK cells were stimulated with PMA and ionomycin in the presence or absence of different concentrations of PGE2, and the 16-hour supernatant was evaluated by ELISA for the presence of IFN-γ production. PGE2 dose-dependently inhibited IFN-γ secretion (Figure 1C).

Taken together, these results show that PGE2 has a direct effect on NK cells, because it down-regulates the expression of the NK cell–activating receptors 2B4 and NKG2A and it dose-dependently inhibits NK cell–mediated cytotoxicity and IFN-γ secretion.

### Effect of PGE2 on DC–derived chemokines is imprinted during maturation and inhibits NK-cell recruitment

NK cells can be recruited by mature DCs, ultimately leading to NK-cell activation.[31,32,38] This recruitment depends on DC-derived chemokines of which CCL5, CXCL10, and CCL19 are the most important. To study whether PGE2 treatment of DCs influences NK-cell recruitment because of effects on the DC chemokine profile, we evaluated CCL19, CXCL10, and CCL5 secretion by DCs after maturation in the presence or absence of PGE2. To this end, monocyte-derived DCs were matured with FMKp and IFN-γ as described previously.[35] DCs were matured for 6 hours with IFN-γ/FMKp and different concentrations of PGE2. After washing, to remove all maturation factors, DCs were incubated for another 18 hours in medium only (total maturation of 24 hours). Effectiveness of DC washing was evaluated by detection of PGE2 by ELISA. None of the samples contained PGE2 (data not shown), indicating that IFN-γ/FMKp–matured DCs do not produce PGE2. Addition of PGE2 during DC maturation did not affect up-regulation of human leukocyte antigen-DR and costimulatory molecules (CD80, CD83, CD86, and CD40), suggesting that addition of PGE2 to the maturation cocktail did not hinder the development of mature DCs (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top

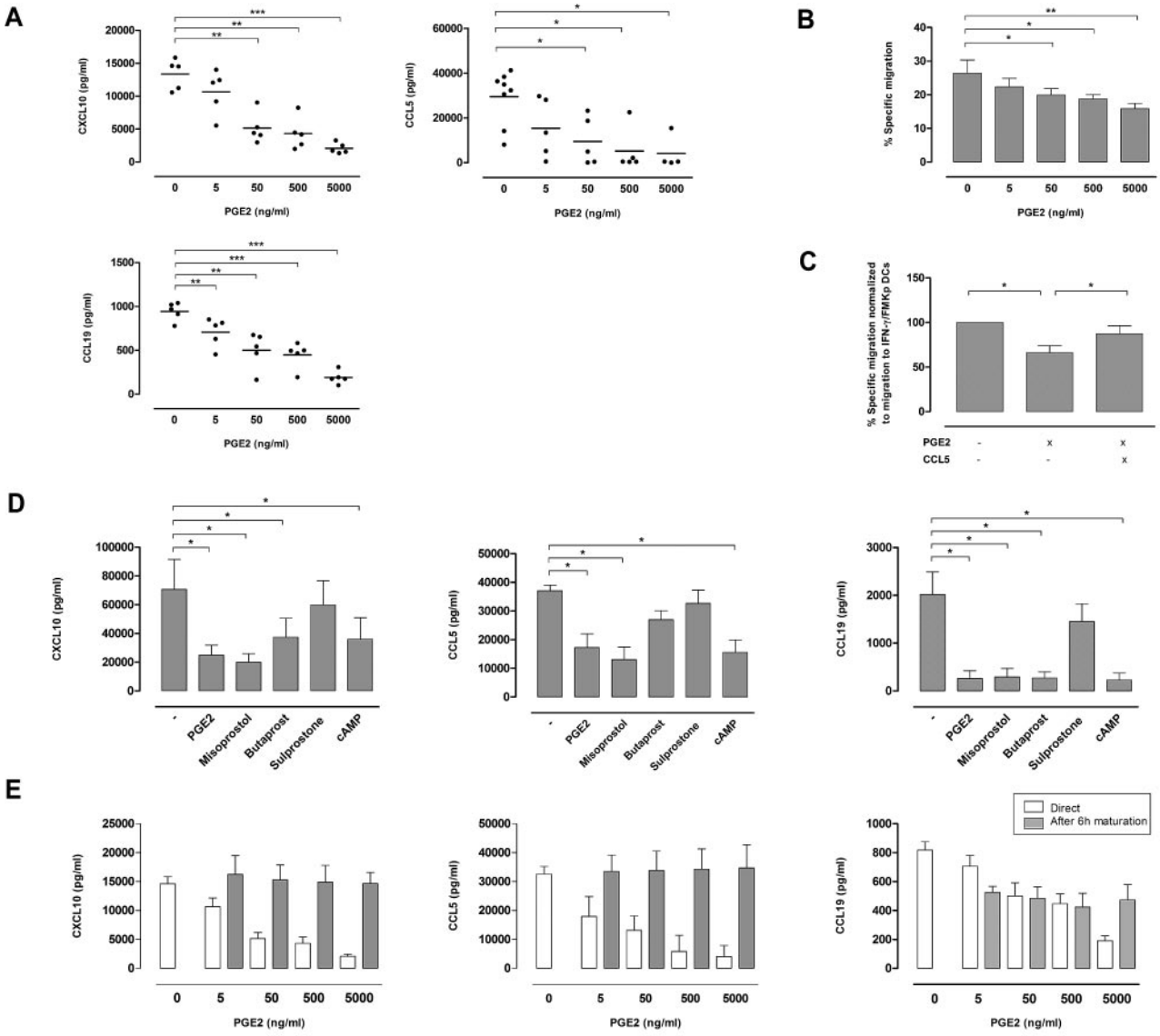


**Figure 2. PGE2 inhibits DC-derived CXCL10, CCL5, and CCL19 secretion.** Monocyte-derived DCs were matured with IFN-γ/FMKp in the presence of different concentrations of PGE2. After 6-hour maturation, DCs were washed to remove the stimulation medium and matured for an additional 18 hours in AIM V medium only. (A) Quantitative comparison of CXCL10, CCL5, and CCL19 production as measured by ELISA. Results shown are the mean + SEM of combined data of at least 5 different donors. (B) Percentage of NK cells migrated in 1.5 hours toward cell-free supernatant of IFN-γ/FMKp DCs matured in the presence of different concentrations of PGE2. Data were calculated as percentage of migrated NK cells. Results presented are the mean + SEM of combined data of 11 different donors. (C) Percentage of NK cells migrated in 1.5 hours toward cell-free supernatant of IFN-γ/FMKp DCs matured in the presence of PGE2 and supplemented with 50 ng/mL CCL5. Data were calculated as percentage of migrated NK cells, normalized to NK-cell migration toward IFN-γ/FMKp DCs matured without PGE2. Results presented are the mean + SEM of combined data of 4 different donors. (D) Quantitative comparison of CXCL10, CCL5, and CCL19 production after stimulation with different EP receptor agonists and cAMP as measured by ELISA. Results shown are the mean + SEM of combined data of at least 4 different donors. (E) Quantitative comparison of CXCL10, CCL5, and CCL19 production after stimulation with PGE2 either during or after DC maturation as measured by ELISA. Results shown are the mean + SEM of combined data of at least 4 different donors. $*P < .05$; $**P < .01$; $***P < .001$.

of the online article). However, PGE2 did induce a significant increase in surface expression of CCR7 (supplemental Figure 1B).

Increased CCR7 expression by TLR-triggered DCs cultured in the presence of PGE2 has been described previously.[39] IFN-γ/FMKp–matured DCs also showed a dose-dependent increase of CCR7 expression that indeed resulted in functionally increased responsiveness to the lymph node–associated chemokine CCL19 (supplemental Figure 1C).

Chemokine (CXCL10, CCL19, and CCL5) production was evaluated by culturing DCs for 14 hours in the presence of different PGE2 concentrations and quantified by ELISA (Figure 2A). Production of these chemokines significantly decreased with increasing concentration of PGE2. Because CCR5 has been identified to be the most important chemokine receptor responsible for NK-cell recruitment by IFN-γ/FMKp–matured DCs,[32] the negative effect of PGE2 on CCL5 production suggested a decrease in the capacity of PGE2-matured DCs to recruit NK cells. To test this hypothesis, migration assays were performed. Freshly isolated NK cells were allowed to migrate during 1.5 hours to supernatant of DCs matured in the presence of different concentrations of PGE2. NK-cell recruitment was significantly decreased when increasing doses of PGE2 were added during DC maturation (Figure 2B). Because DCs were extensively washed after 6 hours of maturation, DC supernatant did not contain any PGE2 anymore; therefore, NK cells were not directly exposed to PGE2. This indicates that the negative effect on NK-cell migration does not

depend on an effect of PGE2 on NK-cell chemokine receptors, which has been described previously.[29] After supplementing the supernatant of PGE2-matured DCs with CCL5, NK-cell recruitment was restored, indicating that indeed the effect of PGE2 on CCL5 production was responsible for decreased NK-cell recruitment (Figure 2C).

DCs have been shown to express all 4 different EP receptors.[40] To evaluate which EP receptors mediate the effect of PGE2 on chemokine production by DCs, they were stimulated with specific agonists that preferentially bind to one or more EP receptors (Figure 2D). Stimulation with butaprost and misoprostol reduced CXCL10, and misoprostol also inhibited CCL5 secretion significantly, whereas sulprostone did not alter secretion of these chemokines. This indicates that mainly EP2 and EP4 signaling are responsible for the effect of PGE2 on the reduced secretion of CXCL10 and CCL5. In contrast, CCL19 production was equally affected by mistoprostol and butaprost, demonstrating that EP2 is the most important EP receptor involved in the inhibition of CCL19 production. The addition of the intracellular cAMP analog dibutyryl-cAMP mimicked the effect of PGE2 for production of all 3 chemokines, suggesting that the effect of PGE2 was dependent on cAMP signaling.

To evaluate whether the effect of PGE2 on chemokine production by DCs is imprinted during maturation or whether PGE2 also can exert its suppressive effect after initiation of maturation, chemokine production of DCs was analyzed when PGE2 was added during or after the first 6 hours of DC maturation. After 24-hour maturation in total, chemokine production was evaluated (Figure 2E). A decrease in CXCL10 and CCL5 secretion could only be detected when PGE2 was present during the first 6 hours of maturation. CCL19 production also was affected by PGE2 after the 6-hour maturation period; however, the effect was less pronounced compared with the effect of immediate addition of PGE2.

These results indicate that the negative effect of PGE2 on production of DC-derived CXCL10, CCL5, and CCL19 is imprinted during maturation and depends on EP2/EP4-mediated cAMP signaling. In addition, we show that the NK-cell recruiting capacity of DCs is reduced because of the influence of PGE2.

### Effect of PGE2 on DC-derived IL-12/IL-18 production is imprinted during maturation and inhibits NK-cell IFN-γ secretion

We showed previously that production of IL-12 and IL-18 by IFN-γ/FMKp matured DCs is needed to induce NK-cell activation.[32] To evaluate the effect of PGE2 on NK-cell activation, production of these cytokines by IFN-γ/FMKp DCs stimulated with PGE2 was determined in 24-hour DC supernatant. As described previously, TLR-triggered DCs produce less IL-12 when matured in the presence of PGE2.[17,39] We show here that PGE2 also has this effect on IL-12 production by IFN-γ/FMKp–matured DCs and that in addition PGE2 triggering results in less IL-18 secretion (Figure 3A). Moreover, PGE2 exerted its effect on the secretion of these cytokines only when it was present during DC maturation, as evidenced by lack of inhibition of IL-12 and IL-18 production when PGE2 was added after the 6-hour DC maturation period (Figure 3B).

NK-cell activation, evaluated by IFN-γ production of NK cells cultured for 16 hours in DC supernatant, was significantly reduced when DCs were matured in the presence of PGE2 (Figure 3C). NK-cell activation was completely restored after addition of IL-12 to the supernatant of IFN-γ/FMKp DCs matured in the presence of PGE2, indicating that the effect of PGE2 on DC-mediated NK-cell activation mainly depends on the inhibition of IL-12 secretion (Figure 3D). Although the decrease in DC-mediated NK-cell activation is caused by the decrease in IL-12 secretion, these data do not exclude that other factors might contribute. One candidate cytokine produced by TLR-triggered DCs in the presence of PGE2 is IL-23, a cytokine implicated in the induction of $T_H17$ responses.[19] Also, IFN-γ/FMKp–matured DCs produce IL-23 that is enhanced when DCs are matured in the presence of PGE2 (Figure 3E), albeit with an apparent optimum at 50 ng/mL PGE2. Addition of IL-23 to DC supernatant during NK-cell activation did not affect IFN-γ production by NK cells (data not shown).

These data indicate that PGE2 added to the maturation of DCs is not only responsible for decreased production of the NK-activating cytokines IL-12 and IL-18 but also enhances IL-23 secretion.

### Presence of PGE2 during DC maturation stimulates IL-22 production by NKp44$^+$ NK cells isolated from human tonsils

Recent studies showed that in mucosa-associated lymphoid tissue, an NK-cell subset with inflammation-restraining properties resides. This NKp44$^+$ subset produces IL-22, IL-26, and leukemia inhibitory factor in response to IL-23 or after coculture with TLR-matured DCs and are not proficient at typical NK-cell functions such as cytotoxicity and IFN-γ secretion.[34] In contrast to conventional NK cells that secrete cytokines in response to IL-12, this NK-cell subpopulation produces IL-22 after IL-23 triggering and, to a lesser extent, after triggering with IL-6 and IL-15.[34] Furthermore, these NKp44$^+$ NK cells express the transcription factor RORγT and therefore show functional and phenotypic similarity to $T_H17$ cells.[41,42] Given the positive effect of PGE2 matured DCs on the induction of $T_H17$ cells,[15] we hypothesized that the addition of PGE2 to IFN-γ/FMKp DC maturation may enhance activation of NKp44$^+$ cells.

To test this hypothesis, NK cells were isolated from human tonsils and cocultured for 16 hours with DCs that were matured in the presence or absence of 50 ng/mL PGE2. Production of IL-22 by NKp44$^+$ NK cells was evaluated by flow cytometry. There was a significant increase in percentage of NK cells accumulating IL-22 when NK cells were cocultured with PGE2-stimulated DCs compared with cocultures with DCs matured in the absence of PGE2 (Figure 4A-B). Notably, the IL-22–producing NK-cell subset expressed NKp44$^+$ and did not accumulate IFN-γ.

Taken together, these data indicate that PGE2 is responsible for the induction of DCs that activate inflammation-restraining NK cells.

### Decreased activation of NK cells by PGE2-matured DC inhibits NK cell–dependent $T_H1$ polarization and augmentation of cytotoxicity

NK-derived IFN-γ has been implicated in the induction of $T_H1$ polarization.[31,43] Consequently, it can be anticipated that the decreased activation of NK cells by DCs matured in the presence of PGE2 results in decreased $T_H1$ polarization. To evaluate whether this hypothesis is true, DCs (washed after 6-hour maturation) were matured with or without PGE2, and after 24 hours, NK cells were added. In this experiment we were only interested in the effect of NK cell–derived cytokines on $T_H1$ polarization and not in the previously reported negative effect of PGE2-matured DCs on $T_H1$ skewing. Therefore, DCs without PGE2 stimulation were coated with SEB and used in a T-cell stimulation assay. To study the effect of NK cell-secreted cytokines, T-cell stimulation medium was exchanged every other day by the supernatant of the NK-DC

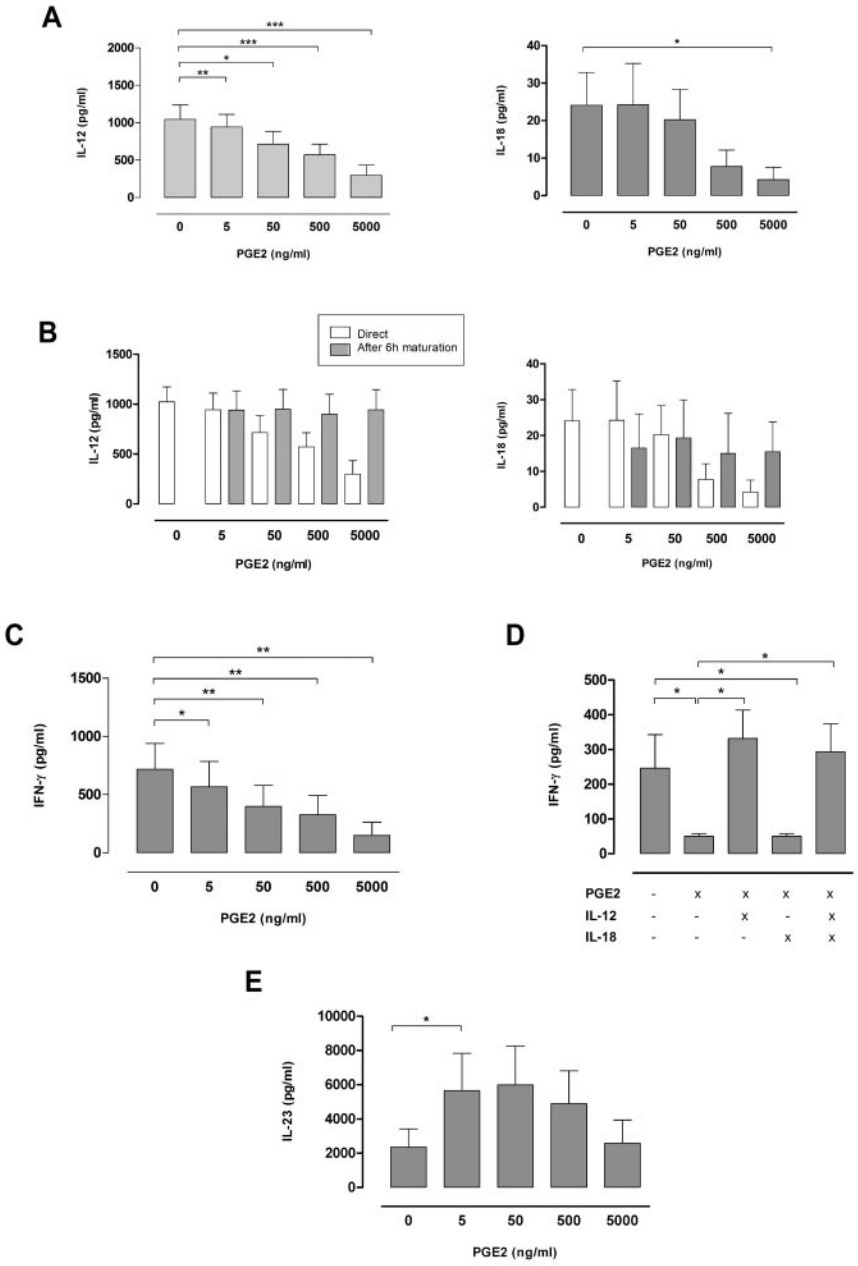


**Figure 3. PGE2 inhibits DC-derived NK-cell activation.** Monocyte-derived DCs were matured with IFN-γ/FMKp in the presence of different concentrations of PGE2. After 6-hour maturation, DCs were washed to remove the stimulation medium and matured for an additional 18 hours in AIM V medium only. (A) Quantitative comparison of IL-12 and IL-18 production as measured by ELISA. Results shown are the mean of combined data of at least 4 different donors + SEM (IL-12, n = 16; IL-18, n = 4). (B) Quantitative comparison of IL-12 and IL-18 production after stimulation with PGE2 either during or after 6-hour DC maturation as measured by ELISA. Results shown are the mean of combined data of at least 3 different donors + SEM (C) NK-cell IFN-γ production, as measured by ELISA, after incubation for 16 hours with DC supernatant. Results presented are the mean + SEM of combined data of 11 different donors. (D) NK-cell IFN-γ production, as measured by ELISA, after incubation for 16 hours with DC supernatant, or DC supernatant supplemented with 2 ng/mL IL-12, 100 pg/mL IL-18, or both. Results presented are the mean + SEM of combined data of 6 different donors. (E) Quantitative comparison of IL-23 production as measured by ELISA. Results shown are the mean + SEM of combined data of 4 different donors. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

cocultures (Figure 5A). $T_H1$ polarization was detected by accumulation of the $T_H1$ cytokine IFN-γ (Figure 5B). Only T cells stimulated with medium of NK-DC cocultures, in which the DCs were not triggered with PGE2 during maturation, benefited from the $T_H1$-polarizing effect of NK cells, because more IFN-γ–producing T cells were detected (Figure 5C).

Previously, we showed that IFN-γ/FMKp matured DCs are able to augment NK-cell cytotoxicity, which is mediated by DC-derived IL-12.[32] These observations opened the possibility that PGE2 triggering of DCs could abrogate the beneficial effect on NK-cell cytotoxicity. To evaluate this, killing assays against the NK-cell resistant Raji cells were performed. Raji cells are insensitive for lysis by naive NK cells; however, if NK cells are preactivated, they become able to kill Raji cells. When DCs were matured with increasing concentrations of PGE2, NK cell–mediated lysis of target cells decreased (Figure 5D). These data indicate that PGE2 not only inhibits NK-cell cytotoxicity via direct action on NK cells (Figure 1B) but also indirectly affects augmentation of NK-cell cytotoxicity via its effect on DC maturation. Taken together, these data show that PGE2 inhibits NK cell–mediated cytotoxicity via two independent mechanisms.

To determine whether activation of NK cells by NK-DC crosstalk could overcome the negative effect of PGE2 on NK cell–mediated cytotoxicity, killing of Raji cells was tested with either NK cells preincubated with PGE2 (preincubation) before stimulation with DC supernatant or NK cells triggered with PGE2 during stimulation with DC supernatant (early incubation). Flow cytometric analysis showed that NK cells preincubated with PGE2 are able to kill Raji cells when activated by DCs (Figure 5E). When PGE2 was present during the NK-cell activation, kill of Raji cells was decreased. However, when NK cells are preactivated by DCs, PGE2 had a minor inhibitory effect on NK-cell cytotoxicity, indicating that the negative effect of PGE2 on NK cells is partly overcome.

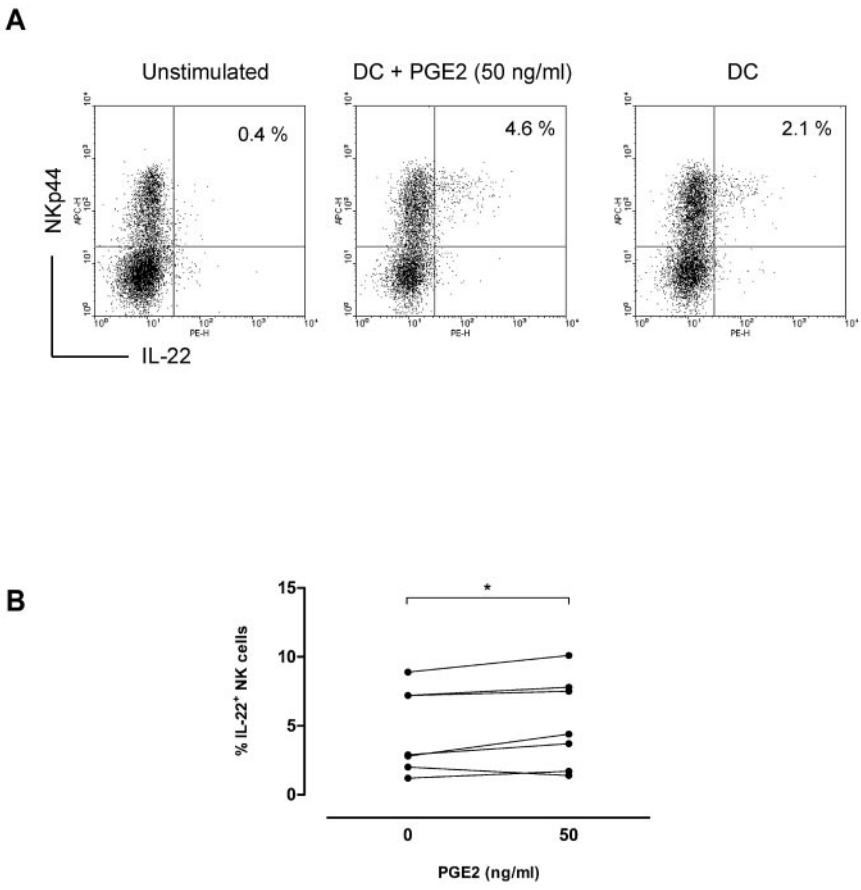


**Figure 4. Effect of PGE2 matured DCs on IL-22 production by NK cells residing in the tonsil.** Monocyte-derived DCs were matured with IFN-γ/FMKp in the presence or absence of 50 ng/mL PGE2. After 6-hour maturation, DCs were washed to remove the stimulation medium and matured for an additional 18 hours in AIM V medium only. (A) Percentage of NKp44$^+$ NK cells isolated from tonsils, accumulating IL-22 after 16 hours of coculture as analyzed by flow cytometry, gated on CD56$^+$CD3$^-$ cells. Representative data of 1 of 6 independent experiments are shown. (B) NKp44$^+$ NK cells accumulating intracellular IL-22 of 7 different donors. In 6 of 7 donors, there is an increase in percentage of IL-22–positive NK cells. Wilcoxon signed rank test significance, $^*P < .05$.

In summary, these results show that PGE2 matured DCs inhibit NK cell–mediated $T_H1$ polarization and also suppress augmentation of NK-cell killing capacity.

## Discussion

COX2-derived PGE2 is abundantly expressed in various malignancies and is one of many factors that directly promote tumor growth by regulation of cancer cell proliferation, apoptosis, migration, and invasion.[7-11] Moreover, PGE2 plays a pivotal role in immunosuppression, creating a tumor-supportive environment by inhibiting tumor immune-surveillance. Increasing the knowledge about the mechanisms by which PGE2 mediates immunosuppression is crucial in understanding tumor formation and might lead to the development of new immunotherapeutic strategies for cancer.

Here, we report on a novel mechanism by which PGE2 inhibits NK-cell effector functions. In addition to its inhibition of NK-cell activation by direct binding to NK cells, PGE also inhibits crosstalk with IFN-γ/FMKp–matured DCs. This is mediated via modulation of chemokine and cytokine secretion of IFN-γ/FMKp–matured DCs, which is responsible for inhibition of NK-cell recruitment and activation.

We show that PGE2 has an inhibitory effect on the secretion of CCL5, CXCL10, and CCL19, which are implicated in NK-cell migration.[31,38] Decreased production of DC-derived CCL19 (implicated in naive T cell recruitment) and CCL5 because of PGE2 triggering has been described recently.[22,44] Interestingly, the effect of PGE2 on chemokine secretion is imprinted during DC maturation because we show that PGE2 stimulation after maturation does not influence chemokine secretion.

In addition, our data evidence that NK-cell activation is altered because of the effect of PGE2 on IFN-γ/FMKp DC–derived cytokine secretion. PGE2-induced changes in the IL-12/IL-23 balance of DCs have been described to account for skewing of $T_H1$ responses toward $T_H2/T_H17$ responses.[15,16,19] Here, we show that it is also responsible for decreased IFN-γ secretion by NK cells. In addition, PGE2 is responsible for decreased production of IL-18 that in addition to IL-12 is an important cytokine for NK-cell activation. This is in apparent paradox to the reported IL-18–mediated induction of PGE2 secretion in the synovial fluid of osteoarthritis patients.[45] Possibly, this represents a feedback loop by which PGE2 constrains inflammation during a normal immune response. Mainly the decreased production of IL-12 by IFN-γ/FMKp–matured DCs because of PGE2 accounts for the inhibition on IFN-γ secretion, because addition of IL-12 restores IFN-γ secretion by NK cells. However, our data do not preclude that other cytokines that have an inhibitory effect on NK-cell activation are produced by DCs in response to PGE2 triggering. As a functional consequence of the DC-mediated effect of PGE2 on NK-cell activation, NK cell–dependent $T_H1$ polarization and tumor cell lysis are inhibited. Interestingly, the reported effect of PGE2 on NK-DC interaction was not only demonstrated for monocyte-derived DCs but also for blood-derived BDCA1$^+$ DCs that were matured with IFN-γ/FMKp in the presence of PGE2 (supplemental Figure 2). In addition, the effect of PGE2 could also be induced by tumor cell–derived PGE2 (supplemental Figure 3A-B). Under different cell culture conditions, the same tumor cells did not produce PGE2, but they were still able, although less efficiently, to inhibit NK-DC crosstalk (supplemental Figure 3A-B). A possible candidate for this inhibition is TGF-β, which is possibly one of

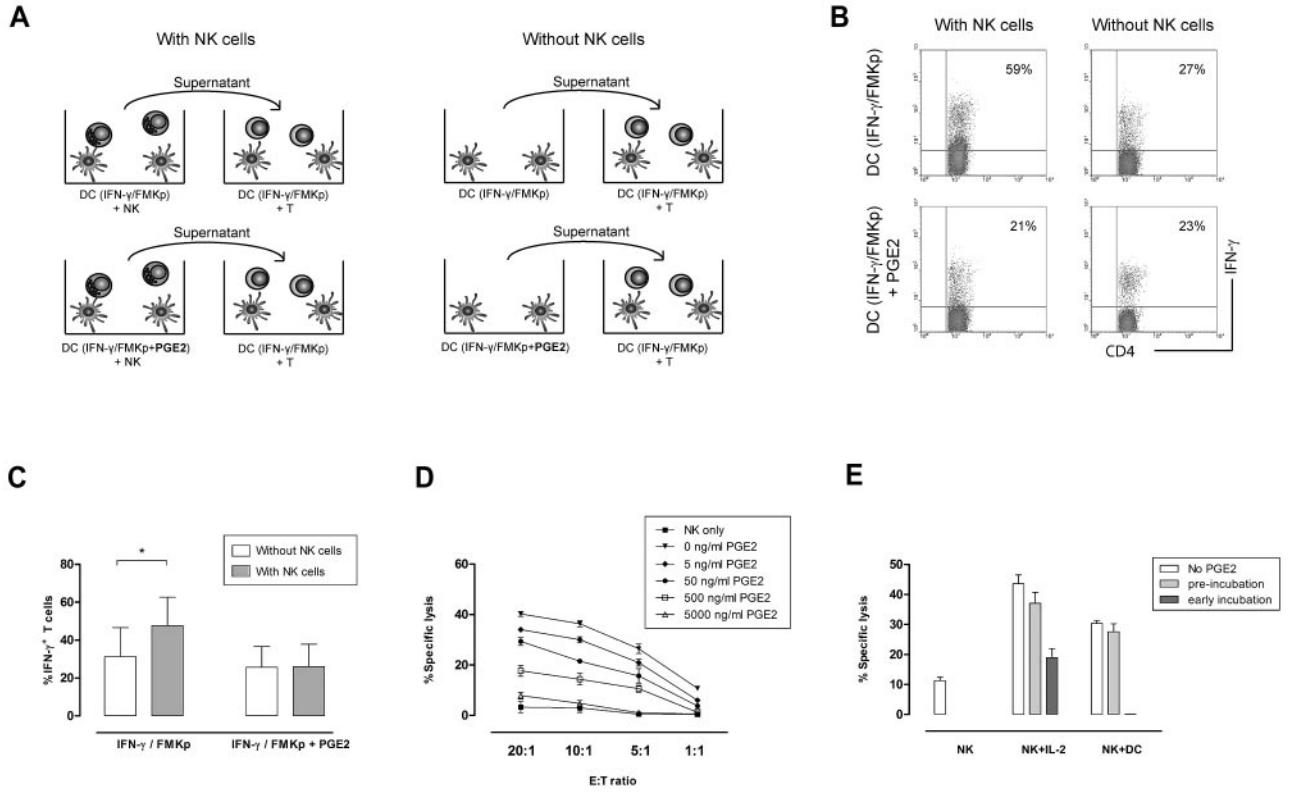


**Figure 5. Effect of PGE2 matured DC on NK-cell function.** (A) IFN-γ/FMKp–matured DCs with or without 5 μg/mL PGE2 were cultured for 24 hours in the presence or absence of freshly isolated NK cells. IFN-γ/FMKp matured DCs were coated with SEB and cocultured with freshly isolated $CD45RA^+/RO^-CD4^+$ $T_H$ cells; every other day, stimulation medium was exchanged with supernatant from IFN-γ/FMKp–matured DCs (with or without 5 μg/mL PGE2) cultured with or without NK cells. (B) Percentage of naive $CD4^+$ T cells accumulating IFN-γ after 10 days of coculture and stimulation with PMA/ionomycin for 4 hours as analyzed by flow cytometry, gated on $CD4^+CD3^+$ T cells. Representative data of 1 of 3 independent experiments are shown. (C) $CD45RA^+/RO^-CD4^+$ $T_H$ cells accumulating intracellular IFN-γ of 5 different donors. Results presented are the mean of combined data of 5 different donors + SEM. (D) Freshly isolated human NK cells and undiluted, filtered supernatants of IFN-γ/FMKp DCs matured in the presence of different concentrations of PGE2 were used for induction of NK-cell cytotoxicity. NK-cell cytotoxicity toward Raji cells was assessed by a flow cytometry–based kill assay. Data represent means ± SEM of triplicate wells. Data shown are representative of 3 independent experiments. (E) Cytotoxicity toward Raji without and after preincubation and early incubation of NK cells with PGE2 and activation in DC supernatant. Data represent means + SEM of triplicate wells of effector:target (E:T) ratio of 10:1. Data are representative of 3 independent experiments. $*P < .05$.

many immunosuppressive factors produced by this cell line (supplemental Figure 3C). Thus, these data illustrate the redundancy of different tumor-derived factors on NK-DC crosstalk, during different culture conditions. We anticipate that among the many mechanisms tumors have to escape immune surveillance, PGE2 is very potent to prevent NK-DC interactions because the application of COX inhibitors in tumor bearing mice completely restores the reduced CCL5 and IL-12 production of myeloid cells.[14,44]

In relation to the enhanced production of IL-23 by PGE2-stimulated DCs, it is relevant to note that Cella et al[34] reported on an NK-cell subset (NK-22 cells) that resides in mucosa-associated lymphoid tissue that expresses NKp44 and produces IL-22 rather than IFN-γ on IL-23, IL-6, and IL-15 stimulation. In addition, they showed that these NK cells produce IL-22, IL-26, and leukemia inhibitory factor after contact with IL-23–producing monocytes that were stimulated with lipopolysaccharide. This NK-cell subset was suggested to contribute to mucosal homeostasis, because IL-22 induces production of the anti-inflammatory cytokine IL-10 by epithelial cells.[34,41,46,47] We show here that PGE2 is responsible for the maturation of DCs that are capable of inducing mucosal $NKp44^+$, IL-22–secreting NK cells, and not IFN-γ–secreting peripheral NK cells. Given the fact that $NKp44^+$ NK cells produce IL-22 in response to IL-23, the increased IL-23 secretion of PGE2 matured IFN-γ/FMKp DCs could represent one of several mechanisms by which DCs induce IL-22 secretion of these NK cells.[34] In this light, it is interesting to note that IL-22 as well as tumor-derived PGE2 have the same immune-protective effects on epithelial cells, which is characterized by increased proliferation and decreased apoptosis.[7] It can be hypothesized that the IL-22, secreted by NK cells, also has this effect on tumor cells (supplemental Figure 4). Future studies addressing this question are of significance to elucidate the function of these $NKp44^+$ NK cells in cancer.

As demonstrated by us and others, PGE2 induces expression of CCR7 on DCs and functionally increases their migratory responsiveness to lymph node-homing chemokines in vitro.[22,35,39] Based on these observations, PGE2 is often incorporated in DC maturation cocktails used in clinical vaccination studies. Our data on the inhibitory effect of PGE2 on NK-DC interaction in combination with previous reports on enhanced $T_H17$ and $T_H2$ induction by PGE2-matured DCs and the decreased ability to induce antigen-specific CTLs[35] argue against the use of PGE2-matured DCs in DC-based vaccines, because it does not contribute to in vivo DC migration and has detrimental effects on immune effector mechanisms. In addition, Muthuswamy et al[48] showed that increased CCR7 expression of PGE2-matured DCs, compared with TLR-matured DCs, correlates with their lack of CCL19 production. If TLR-matured DCs that express low levels of CCR7 are cultured in

an environment with low concentrations of CCL19, CCR7 expression is increased. In addition, they showed in a clinical trial that PGE2-matured DCs demonstrated no advantage over TLR-triggered DCs in lymph node homing. In the current study, we show that the effect of PGE2 is imprinted during IFN-γ/FMKp DC maturation, because cytokine and chemokine secretion is only affected by PGE2 in the first 6 hours of maturation, and this effect is mainly mediated via EP2 and EP4 and depends on cAMP signaling. In terms of vaccination protocols, these data support the application of ex vivo–matured DCs in patients with tumors secreting PGE2. Moreover, our data indicate that PGE2 does not induce permanent changes in NK-cell cytotoxic capacity, because the suppressive effect of PGE2 on NK cells can be easily overcome by DC-induced NK-cell activation. However, to effectively activate NK cells, NK-DC interaction should take place in an environment with low concentrations of PGE2, emphasizing the need of NK-cell recruitment by DCs. Our results also open the possibility to combine DC vaccination with COX2-inhibitory therapy, which has been shown previously in mouse models to enhance the efficacy of cancer vaccines.[49,50]

In conclusion, we report on a novel immunosuppressive effect of PGE2, mediated by IFN-γ/FMKp–matured DCs, on NK-cell function. These current data help to understand the complex role of PGE2 in the regulation of immune responses during inflammation. The extent of effect of PGE2 on the immune system in relation to other tumor-derived factors and thereby the redundancy of this mechanism is yet not completely elucidated and in vivo studies should be performed to answer this question. However, we show in vitro that the immunosuppressive effect of PGE2 on NK-DC interaction possibly represents one of many tumor-mediated mechanisms to hamper acute immune responses.

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## Authorship

Contribution: C.H.M.J.V.E. designed and performed experiments and designed and wrote the paper; J.V. designed and performed experiments and wrote the paper; T.O. and B.L.M.G.S.-G. performed experiments; and W.T.V.G. and G.M.J.B. helped write the paper and designed and supervised the project.

Conflict-of-interest disclosure: C.H.M.J.V.E. is employed by PharmaCell. The remaining authors declare no competing financial interests.

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